PHEROID^{TM} TECHNOLOGY FOR THE TOPICAL APPLICATION OF SELECTED COSMECEUTICAL ACTIVES

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ABSTRACT

Aging can be described as an extremely complex occurrence from which no organism can be excluded. Intrinsic and extrinsic aging make out the two components of skin aging and they differ on the macromolecular level while sharing specific molecular characteristics which include elevated levels of reactive oxygen species (ROS) and matrix metalloproteinase (MMP) while collagen synthesis decreases.

The skin functions as a protective barrier against the harsh environment and is essential for regulating body temperature. The stratum corneum (SC) is responsible for the main resistance to the penetration of most compounds; nevertheless the skin represents as an appropriate target for delivery. The target site for anti-aging treatment includes the epidermal and dermal layers of the skin.

Calendula oil and L-carnitine L-tartrate was utilised as the cosmeceutical actives as they can be classified as a mixed category of compounds/products that lie between cosmetics and drugs. Both show excellent properties which can prove valuable during anti-aging treatment, whether it is due to the scavenging of ROS (calendula oil), moisturising effects (calendula oil and L-carnitine L-tartrate) or the improvement of the skin turnover rate (L-carnitine L-tartrate).

The Pheroid™ delivery system can enhance the absorption of a selection of active ingredients. The aim of this study was to determine whether the Pheroid™ delivery system will enhance the flux and/or delivery of the named actives to the target site by performing Franz cell diffusion studies over an 8 h period, followed by tape stripping experiments. The Pheroid™ results of the actives were compared to the results obtained when 100 % calendula oil was applied and the L-carnitine L-tartrate was dissolved in phosphate buffer solution (PBS), respectively.

In the case of calendula oil only a qualitative gas chromatography mass spectrometry (GC/MS) method could be employed. No calendula oil was observed to permeate through the skin, but linoleic acid (marker compound) was present in the epidermis and dermis layers. Components in the Pheroid™ delivery system hampered the results as the marker compound identified is a fundamental component of the Pheroid™, making it difficult to determine whether or not the Pheroid™ delivery system enhanced calendula oil's penetration.

The aqueous solubility and log D partition coefficient of L-carnitine L-tartrate was determined. Inspection of the log D value of -1.35 indicated that the compound is unfavourable to penetrate the skin, whereas the aqueous solubility of 16.63 mg/ml in PBS at a temperature of 32 °C indicated favourable penetration.

During the Franz cell diffusion and tape stripping studies it was determined by liquid chromatography mass spectrometry (LC/MS) that carnitine may be inherent to human skin. Pheroid™ enhanced the flux (average of 0.0361 μg/cm^2.h, median of 0.0393 μg/cm^2.h) of the L-carnitine L-tartrate when compared to PBS (average of 0.0180 μg/cm^2.h, median of 0.0142 μg/cm^2.h) for the time interval of 2 - 8 h. The PBS was more effective in delivering the active to the target site (0.270 μg/ml in the epidermis and 2.403 μg/ml in the dermis) than Pheroid™ (0.111 μg/ml and 1.641 μg/ml in the epidermis and dermis respectively).

Confocal laser scanning microscopy (CLSM) confirmed the entrapment of L-carnitine L-tartrate in the Pheroid™ vesicle, while in the case of calendula oil it was impossible to differentiate between the oil and the Pheroid™ components.

Keywords: Calendula oil, L-carnitine L-tartrate, Pheroid™, Skin aging, Topical delivery

OPSOMMING

Veroudering kan beskryf word as 'n uiters komplekse gebeurtenis wat geen organisme kan vryspring nie. Die twee komponente waaruit vel veroudering bestaan is intrinsieke en ekstrinsieke veroudering wat verskil op die makromolekulêre vlak, terwyl hulle spesifieke molekulêre karakteristieke deel, wat verhoogde vlakke van reaktiewe suurstof spesies (RSS) en matriks metalloproteinase (MMP) insluit, onderwyl kollageen sintese ook afneem.

Die vel funksioneer as 'n beskermende versperring teen die ru omgewing en is essensieel vir die regulering van liggaamstemperatuur. Die stratum corneum (SC) is verantwoordelik vir die hoof weerstand wat die vel bied teen die penetrasie van meeste verbindings; nieteenstaande verteenwoordig die vel 'n toepaslike teiken vir aflewering. Die teikengebied vir anti-verouderingbehandeling sluit die epidermale en dermale lae van die vel in.

Calendula olie en L-karnitien L-tartraat was gebruik as die kosmeseutiese aktiewe, omdat hulle geklassifiseer kan word as 'n hibried kategorie van verbindings/produkte wat lê tussen kosmetika en geneesmiddels. Beide toon uitmuntende eienskappe wat waardevol kan wees tydens die anti-verouderingsbehandeling; hetsy of dit is as gevolg van die opruiming van RSS (*calendula olie*), bevogtigingseffekte (calendula olie en L-karnitien L-tartraat) of die vel se vernuwingstempo verbeter (L-karnitien L-tartraat).

Die Pheroid™ afleweringsisteem kan die absorpsie van 'n verskeidenheid aktiewe bestanddele verhoog. Die doelwit van hierdie studie was om te bepaal of die Pheroid™ afleweringsisteem die vloed en/of aflewering van die genoemde aktiewe geneesmiddels na die teikengebied bevorder, deur gebruik te maak van Franz sel diffusiestudies oor 'n 8 h periode gevolg deur kleefbandafstropingseksperimente. Die Pheroid™ resultate van die aktiewe geneesmiddels was onderskeidelik met 100 % calendula olie toegedien en L-karnitien L-tartraat opgelos was in fosfaat buffer oplossing (PBS) vergelyk.

In die geval van calendula olie, kon slegs 'n kwalitatiewe gaschromatografie-massaspektrometrie (GC/MS) metode gebruik word. Geen calendula olie het deur die vel gedring nie, maar linoliensuur (merker-verbinding) was teenwoordig in die epidermis en dermis lae. Komponente in die Pheroid™ afleweringsisteem het die resultate belemmer, aangesien die geïdentifiseerde merker-verbinding ook 'n fundamentele komponent van die Pheroid™ uitmaak, wat dit moeilik gemaak het om te bepaal of die Pheroid™ afleweringsiseem die calendula olie se penetrasie bevorder al dan nie.

L-karnitien L-tartraat se wateroplosbaarheid en log D verdelingskoëffisiënt was bepaal. Die log D waarde van -1.35 toon dat die verbinding nie gunstig is om die vel te penetreer nie, terwyl die wateroplosbaarheid waarde van 16.63 mg/ml in PBS by 'n temperatuur van 32 °C gunstige penetrasie aandui.

Tydens die Franz sel diffusie- en kleefbandafstropingstudies is dit bepaal met behulp van vloeistofchromatografie-massaspektrometrie (LC/MS) dat karnitien natuurlik mag voorkom in mensvel. Pheroid™ het die vloed (gemiddeld van 0.0361 $\mu g/cm^2.h$, mediaan van 0.0393 $\mu g/cm^2.h$) van L-karnitien L-tartraat bevorder wanneer dit vergelyk word met PBS (gemiddeld van 0.018 $\mu g/cm^2.h$, mediaan van 0.0142 $\mu g/cm^2.h$) vir die tyd interval vir 2 - 8 h. Die PBS was meer effektief om die aktief af te lewer by die teikengebied (0.270 µg/ml in die epidermis en 2.403 µg/ml in die dermis) as Pheroid™ (0.111 µg/ml en 1.641 µg/ml in die epidermis en dermis respektiewelik).

Konfokale laserskanderingsmikroskopie (KLSM) het bevestig dat L-karnitien L-tartraat in die Pheroid™ vesikel vasgevang is, alhoewel, in die geval van calendula olie was dit onmoontlik om te onderskei tussen die olie en die Pheroid™ komponente.

Sleutelwoorde: Calendula olie, L-karnitien L-tartraat, Pheroid™, Vel veroudering, Topikale aflewering

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ABBREVIATIONS

AP-1	-	Activator protein 1 complex
ATP	-	Adenosine triphosphate
BF_3	-	Boron trifluoride
CACT	-	Acylcarnitine carnitine translocase
CH_3CN	-	Acetonitrile
CH_2O_2	-	Formic acid
CLSM	-	Confocal laser scanning microscopy
CoA	-	Coenzyme A
CPT-I	-	Carnitine palmitoyltransferase I
CPT-II	-	Carnitine palmitoyltransferase II
DNA	-	Deoxyribonucleic acid
DPPH	-	Diphenylpicrylhydrazyl
ECM	-	Extracellular matrix
GC/MS	-	Gas chromatography mass spectrometry
He	-	Helium gas
HIV	-	Human immunodeficiency virus
HPLC	-	High pressure liquid chromatography
IL-1	-	Interleukin-1
IL-6	-	Interleukin-6
IL-8	-	Interleukin 8
KH_2PO_4	-	Potassium dihydrogen phosphate
LCAS	-	Long-chain acyl-CoA syntetase

LC/MS	-	Liquid chromatography mass spectrometry
Log D	-	Octanol-PBS partition coefficient
Log P	-	Octanol-water partition coefficient
LOOHs	-	Lipid hydroperoxides
LOX	-	Lysyl oxidase
MAP	-	Mitogen-activated protein kinases
MeOH	-	Methanol
MMP	-	Matrix metalloproteinase
MMP-1	-	Collagenase-1
MMP-2	-	Gelatinase-A
MMP-3	-	Stromelysin-1
MMP-9	-	Gelatinase-B
MRM	-	Multiple reaction monitoring
N_2	-	Nitrogen gas
NaCl	-	Sodium chloride
NaOH	-	Sodium hydroxide
Na_2SO_4	-	Sodium sulphate anhydrous
NF-κB	-	Nuclear factor kappa B
PBS	-	Phosphate buffer solution
ROS	-	Reactive oxygen species
SC	-	Stratum corneum
TEWL	-	Transepidermal water loss
TGF-β	-	Transforming growth factor beta

TIMP-1 - Tissue inhibitor of metalloproteinase-1

TSP-1 - Thrombospondin-1

TSP-2 - Thrombospondin-2

UV - Ultraviolet

UV-VIS - Ultraviolet/visible spectra

VEGF - Vascular endothelial growth factor

CHAPTER 1: INTRODUCTION AND STATEMENT OF THE PROBLEM

From the ancient to the medieval ages there has always been a fascination on combating aging and conserving eternal youth (Makrantonaki & Zouboulis, 2008:e153). A considerable increase in the average life span of citizens in the industrialised world (North America, Western Europe and Japan) has been observed with the mean life span of women reaching to about 80 years and 76 years for men (Cauwenbergh, 2002:468). Developing countries also show an increasing trend, although the percentages are lower (Tinker, 2002:279). On the African continent the HIV (human immunodeficiency virus) epidemic has somewhat reduced this trend (Cauwenbergh, 2002:468). This has led to the development of rejuvenation procedures which people will utilise no matter the cost (Makrantonaki & Zouboulis, 2008:e153); thereby they have created a multibillion-dollar cosmetic industry (Cauwenbergh, 2002:469).

The mechanisms which underlie skin aging should be understood, as it is vital to use safe and proper intervention modalities (Makrantonaki & Zouboulis, 2008:e153). Intrinsic and extrinsic aging are the two components of aging and both have as basis increased production of reactive oxygen species (ROS) and matrix metalloproteinase (MMP) expression along with decreased pro-collagen synthesis (Jenkins, 2002:801; Rittié & Fisher, 2002:706; Varani *et al.*, 2000:480). Cosmeceuticals provide a new therapeutic frontier for aging in the human skin and can be seen as a hybrid category of products that lie on the spectrum between drugs and cosmetics (Draelos, 2007:2; Choi & Berson, 2006:163). The cosmeceutical actives investigated in this study were calendula oil and L-carnitine L-tartrate.

Calendula oil is responsible for scavenging the ROS (Ćetković *et al.*, 2004:648) and also shows re-epithelising and moisturising properties (Centerchem, 2006:7). L-carnitine L-tartrate accelerates the epidermal turnover rate in the skin which leads to younger, softer and more radiant-looking skin (Held, 2004:41). These properties make them excellent actives for treating and combating skin aging.

According to Brain *et al.* (1998:161) the topical and transdermal routes have become accepted above the more conventional methods of drug delivery. The target site of delivery for treating/preventing skin aging includes the epidermal and dermal layers of the skin. The transport of a drug to the viable epidermal and/or dermal tissues of skin in order to have a local therapeutic effect, with minimal systemic blood circulation, is known as topical delivery (Roy, 1997:139).

The novel Pheroid™ delivery system employed during this study consists mainly of modified essential fatty acids (Grobler, 2008:283) which are oriented in the *cis*-formation making it similar

to fatty acids found in humans, leading to a skin-friendly carrier for cosmetic compounds (Grobler *et al.*, 2008:285). Pheroid™ is capable to enhance the absorption of various categories of drugs (Grobler, 2004:3) including lipophilic, hydrophilic and even insoluble compounds (Grobler, 2004:7).

The aims and objectives set for this study included the following:

- Experimentally determine the aqueous solubility and partition coefficient of L-carnitine L-tartrate.
- Confirming whether L-carnitine L-tartrate was entrapped within the Pheroid™ by use of confocal laser scanning microscopy (CLSM).
- Developing and validating a liquid chromatography mass spectrometry (LC/MS) method to quantitatively determine L-carnitine L-tartrate.
- Experimentally determine whether the boron trifluoride (BF_3) catalised methylation is effective for converting fatty acids of the calendula oil into their simplest convenient volatile derivatives.
- Developing a qualitative gas chromatography mass spectrometry (GC/MS) method for determining the presence of calendula oil.
- Experimentally determine the transdermal flux of L-carnitine L-tartrate in both phosphate buffer solution (PBS) (pH 7.4) and Pheroid™.
- Experimentally determine whether the PBS (pH 7.4) or Pheroid™ delivered the L-carnitine L-tartrate to the target site, i.e. the epidermis and dermis, *via* tape stripping.
- Experimentally determine whether calendula oil diffuses through the skin or into the target site of delivery (tape stripping) when applied as is and when encapsulated within the Pheroid™.

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CHAPTER 2: TOPICAL DELIVERY OF CALENDULA OIL AND L-CARNITINE L-TARTRATE

2.1 INTRODUCTION

The aging process is the progressive accumulation of changes associated with time. This universal phenomenon is responsible for the ever-increasing susceptibility to disease and death with advancing age (Harman, 1981:7124). The cosmetic industry is attempting to develop treatments in order to maintain the skin's youthful appearance. This can prove to be a huge business opportunity for this fast pathway growing industry (Rhein *et al.*, 2000:114). This chapter presents an overview of the changes that take place during skin aging, and describes skin structures affected by aging, as well as factors that influence transdermal penetration. The cosmeceutical actives calendula oil and L-carnitine L-tartrate will also be discussed in accordance with the role they play in prevention and/or treatment of skin aging.

2.2 SKIN AGING

Aging is commonly associated with increased wrinkling, sagging and laxity (Jenkins, 2002:801). As a very complex biological phenomenon, cutaneous aging can be divided into two components: intrinsic and extrinsic aging (Jenkins, 2002:801) as can be seen in **Figure 2.1** and **Figure 2.2**. Intrinsic aging is largely due to a person's genetics; whereas aging caused by environmental exposure, predominantly ultraviolet (UV) light, can be classified as extrinsic or photoaging (Jenkins, 2002:801). Several factors that influence the aging of the skin are hormonal changes, genetics, environmental exposure (mechanical stress, xenobiotics, UV irradiation) and metabolic processes (formation of reactive chemical compounds such as activated oxygen species, aldehydes and sugars) (Rittié & Fisher, 2002:705).

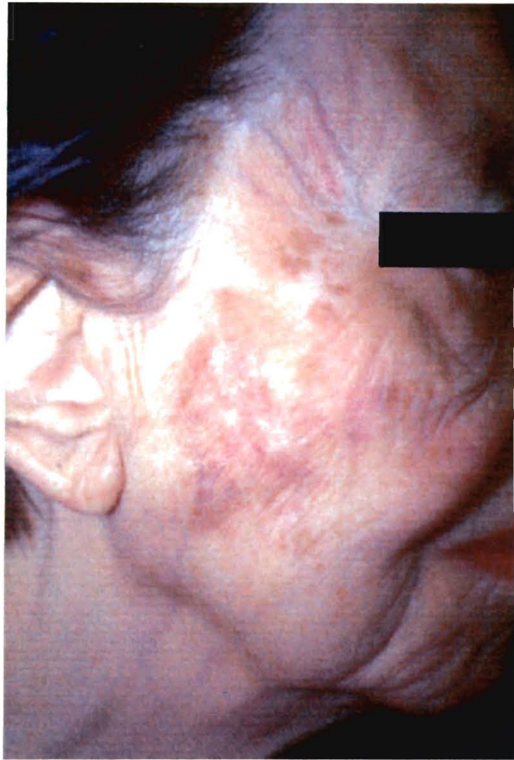

Figure 2.1: The difference between photoaging (face) and chronologic aging (neck) in a 92-year-old woman (Ramos-e-Silva & Coelho Da Silva Carneiro, 2001:414).

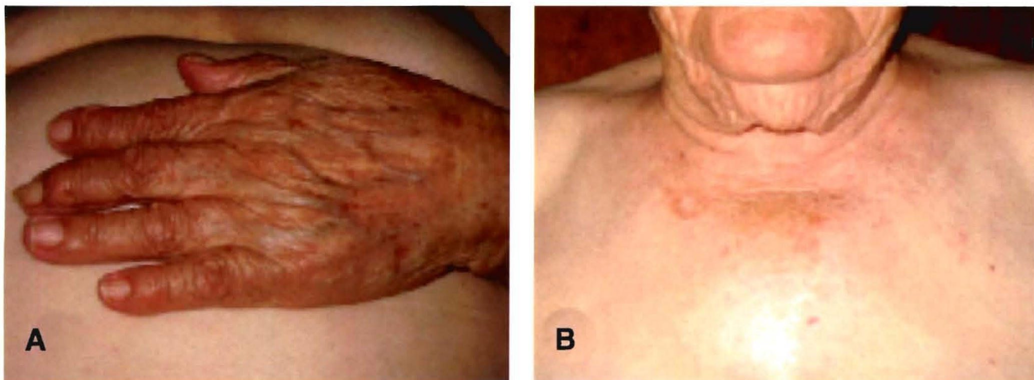

Figure 2.2: Skin aging. Photoaged skin appears coarse, irregularly pigmented and dark while chronologically aged skin appears to be pale and dry with laxity and fine wrinkles. **A** depicts the contrast between the dorsal hand and sun-protected abdomen of a 91-year old woman. **B** shows the skin above and below the customary neckline of the same woman (Kosmadaki & Gilchrest, 2004:156).

2.2.1 OVERLAPPING CHARACTERISTICS BETWEEN INTRINSIC AND EXTRINSIC AGING

As stated by Billek (2002:111) both intrinsic and extrinsic aging are accountable for the dysfunction of the skin's natural repair and self-protection and both coincide during a person's lifetime. There are marked differences between intrinsically and photoaged skin on the macromolecular level, while recent evidence suggests that they share notable molecular characteristics (Rittié & Fisher, 2002:706; Jenkins, 2002:807). This includes increased levels of the MMP enzymes due to altered signal transduction pathways, connective tissue impairment and a decline in pro-collagen synthesis (Rittié & Fisher, 2002:706; Varani *et al.*, 2000:480) as well as reduced response to growth factors and a decreased cellular lifespan (Jenkins, 2002:807). It is suggested that many aspects of the chronological aging process is accelerated by UV irradiation and skin aging due to UV exposure being superimposed on chronological skin aging (Rittié & Fisher, 2002:706).

Makrantonaki & Zouboulis (2008:e156) suggested the following diagram (**Figure 2.3**) to give a schematic overview of the major biochemical changes and signalling pathways involved in the generation of intrinsically and extrinsically aged skin (NF-κB: nuclear factor kappa B, VEGF: vascular endothelial growth factor, TSP-1: thrombospondin-1, TSP-2: thrombospondin-2, IL-1: interleukin-1, IL-6: interleukin-6, IL-8: interleukin 8). The typical histological characteristics of the sun-exposed skin **(b)** show an accumulation of disoriented elastic tissue (blue arrows) in the dermis after elastic staining. This is in contrast to the moderate histological changes found in the sun-protected skin **(a)**. A variety of cellular functions are regulated by mitogen-activated protein (MAP) kinase signal transduction pathways in aged skin. The c-Jun and c-Fos transcription factors are some of the downstream effectors of the MAP kinases which heterodimerise in order to form the activator protein 1 (AP-1) complex (Makrantonaki & Zouboulis, 2008:e156). AP-1 is a vital regulator of skin aging, as it induces the expression of the MMP-family and inhibits the gene expression of Type I pro-collagen *via* its interference with the transforming growth factor beta (TGF-β) signalling pathway (Makrantonaki & Zouboulis, 2008:e156). It was postulated that the excess production of ROS activates MAP kinases. The ROS production may be superimposed by extrinsic factors such as UV/IR irradiation. ROS production leads to the accumulation of cellular damage; this includes the oxidation of deoxyribonucleic acid (DNA), which results in mutations, oxidation of membrane lipids which leads to altered transmembrane-signalling and reduced transport efficiency; and oxidation of proteins which leads to reduced function (Makrantonaki & Zouboulis, 2008:e156).

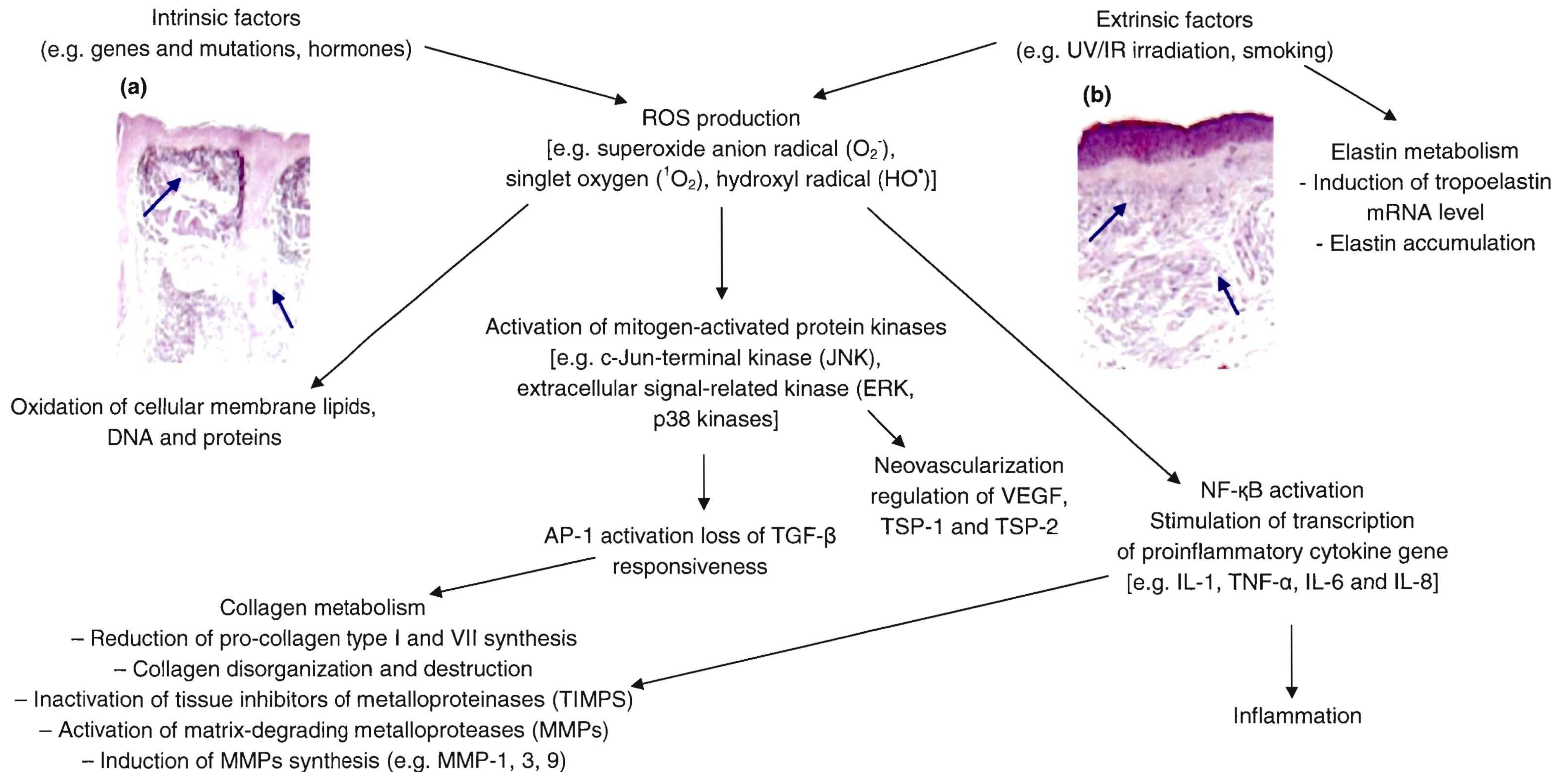

Figure 2.3: A schematic overview of the major biochemical changes and signalling pathways involved in the generation of **(a)** intrinsically aged skin obtained from the inner side of the upper arm of a 83-year-old woman and **(b)** extrinsically aged skin obtained from the face of a 75-year-old woman (Makrantonaki & Zouboulis, 2008:e156).

2.2.1.1 PRO-COLLAGEN BREAKDOWN

This section describes the role that collagen degradation plays during intrinsic as well as extrinsic skin aging. In order to better understand it, the synthesis of collagen is described as background.

2.2.1.1.1 COLLAGEN SYNTHESIS

Collagen fibres comprise approximately 75 % of the dry weight of the dermis (Oishi *et al.*, 2002:859). Collagen can be found in connective tissues and it exists as large bundles of regularly oriented fibres which are composed of fibrils and micro-fibrils (Uitto & Eisen, 1979:164). The basic collagen molecule has an approximate molecular weight of 290 000 and exists of three polypeptide chains, each with a molecular weight of 94 000 dalton (Uitto & Eisen, 1979:164). This ensures that this super polymer has an enormous tensile strength (Dafforn *et al.*, 2001:49310). These three polypeptides (α-chains) are coiled on each other like the strands of a rope to give the collagen molecule a triple-helical structure. Collagen's unique properties can be attributed to the helical conformation and in the absence of this triple helix no collagen fibres would be formed and the connective tissues would appear seriously defective (Uitto & Eisen, 1979:165).

Collagen is initially synthesised as a larger precursor molecule, named pro-collagen which is soluble under physiologic conditions; this is in contrast with collagen which is insoluble (Dafforn *et al.*, 2001:49310; Uitto & Eisen, 1979:166). Pro-collagens are larger than collagens because they contain additional peptide sequences at both ends of the molecule. The precursor polypeptides of pro-collagen, pro-α-chains, are synthesised on the membrane-bound ribosomes of fibroblasts and related cells (Uitto & Eisen, 1979:166). After the amino acids are assembled into pro-α-chains on the ribosomes, the polypeptides undergo several modifications before the completed collagen molecules are deposited into extracellular fibres (Uitto & Eisen, 1979:168). Pro-collagen is converted to collagen by several successive steps and possibly several enzymes (Uitto & Eisen, 1979:172).

According to Uitto & Eisen (1979:174) collagenases, which belong to the family of MMPs (Oishi *et al.,* 2002:860), are enzymes responsible for cleaving the native collagen triple helix under non-denaturing conditions at physiologic levels of pH, temperature and salt concentration. Collagenases catalyse the initial cleavage of the collagen polypeptide chains (Uitto & Eisen, 1979:174). The precise regulation of a specific collagenase will help with the maintenance of the normal architecture of the human skin. The dermal cellular components are responsible to react to acute requirements for degradation (i.e. in the wound healing process) as well as to oversee the fairly slow collagen breakdown that occurs during normal turnover (Uitto & Eisen, 1979:174).

According to Epstein & Munderloh (1975:9304) collagen is not a unique molecule, but makes out a family of molecules. Type I collagen is the most extensively characterised and most widely distributed form of collagen and can be found primarily in bone and tendon (Uitto & Eisen, 1979:165). Furthermore, Type I collagen is the most abundant protein in skin connective tissue (Rittié & Fisher, 2002:706; Uitto & Eisen, 1979:165). The skin also contains other types of collagen (III, V and VII), fibronectin, proteoglycans, elastin and other extracellular matrix (ECM) proteins (Rittié & Fisher, 2002:706). Collagen III makes out approximately 10 % of the total collagen in the adult human dermis; whereas it predominates in early foetal skin (Epstein, 1974:3225).

2.2.1.1.2 COLLAGEN DEGRADATION

The coarse, rough, wrinkled appearance of aged skin is thought to be underlined by damage to the collagenous matrix (Varani *et al.*, 2001:940). Fligiel *et al.* (2003:846) suggested that collagen fragmentation *in vivo* could underlie the loss of collagen synthesis in photodamaged skin and to a lesser extent in aged skin. The capacity of fibroblasts in old individuals to synthesise collagen is less than in young individuals (Fligiel *et al.*, 2003:842). They also found less extensive collagen damage in sun-protected naturally aged skin of individuals aged 80 years or older (Fligiel *et al.*, 2003:842). Their findings were consistent with other studies that suggested that the loss of collagen synthetic activity that characterised aged/photodamaged skin was contributed by MMP which fragments collagen *in vivo* (Fligiel *et al.*, 2003:846-847) which will be described in Section 2.2.1.3. High molecular weight fragments of Type I collagen are produced by collagenolytic enzymes (primarily MMP-1) which in turn inhibits the synthesis of Type I pro-collagen (Varani *et al.*, 2002:123). The inhibitory capacity decreases when these high molecular weight fragments are processed further (Varani *et al.*, 2002:123).

In an *in vitro* study, collagen, partially degraded by exposure to collagenolytic enzymes (from either human skin or bacteria), undergoes contraction in the presence of fibroblasts which are found in the matrix, within close opposition of collagen fibres (Varani *et al.*, 2001:931). These fibroblasts showed reduced proliferative capacity and synthesised less Type I pro-collagen (Varani *et al.,* 2001:931). The method by which it takes place is not fully understood (Fligiel *et al.*, 2003:847). One possibility could be the change in cell shape which occurs when the collagen contracts. Fibroblasts express the typical elongated spindle-cell morphology when they attach to collagen fibres (Varani *et al.*, 2001:940). If enough breaks are introduced to the three-dimensional scaffold, it is unable to resist the contractile force of the cells, and it consequently collapses (Varani *et al.*, 2001:940). This is followed by the disassembling of the cytoskeleton and the changing of the cell shape from elongated to round, which ultimately underlies the reduced growth and collagen production on partially degraded collagen (Varani *et al.*, 2001:940-941). Another possibility may be abnormal signalling which causes abnormal cell

function, brought about by cellular interactions with degraded collagen, rather than with the intact triple helical molecule (Varani *et al.*, 2001:941).

2.2.1.2 GENERATION OF REACTIVE OXYGEN SPECIES (ROS)

Both chronological and photoaging leads to increased production of ROS (Rittié & Fisher, 2002:709). This changes protein and gene function and structure, which leads to the deregulation of extracellular and intracellular homeostasis that can alter the cellular behaviour as well as the cell-matrix interactions. This eventually leads to the diminished function of the skin (Rittié & Fisher, 2002:709).

According to Nicolaÿ & Paillet (2002:268) keratinocyte membranes as well as the deep-sited fibroblasts are targets for oxidants. These oxidants can come from the atmosphere, such as ozone, free radicals or peroxides, or it can accumulate directly on the surface of the skin, for example heavy metals and chemicals (Nicolaÿ & Paillet, 2002:268). When these ROS species reach the biological membranes it oxidizes its constituents, especially the phospholipids which are the basic components of cell membranes (Nicolaÿ & Paillet, 2002:268). **Figure 2.4** illustrates a schematic representation of the oxidation of phospholipids in the membrane by ROS.

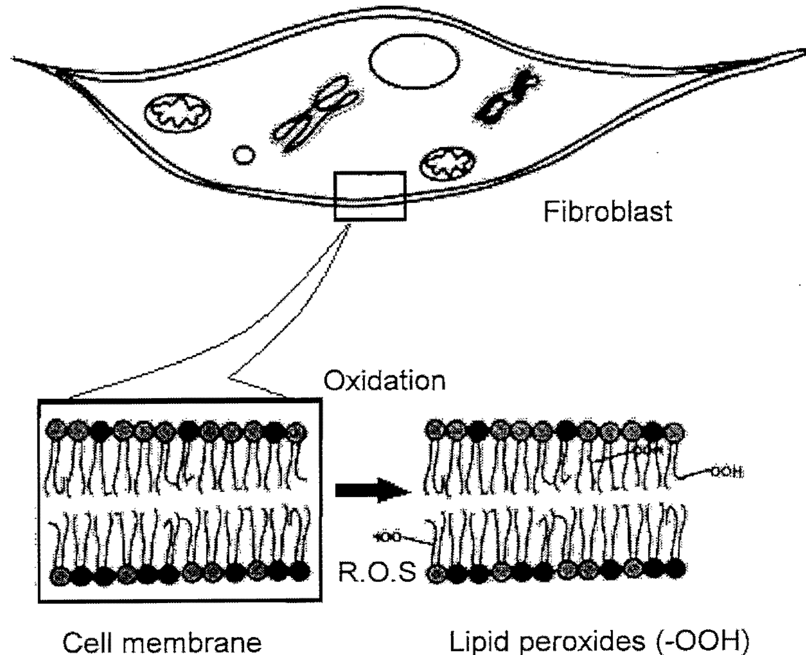

Figure 2.4: Schematic representation of the oxidation of phospholipids in the membrane by ROS (Nicolaÿ & Paillet, 2002:269).

Lipid hydroperoxides (LOOHs) are derived from the unsaturated phospholipids and play a roll in the oxidative process accountable for skin aging (Nicolaÿ & Paillet, 2002:269). The LOOH is

highly hydrophilic which can explain its migration into the ECM (Nicolaÿ & Paillet, 2002:270). Proteins such as elastin or collagen located in the ECM then undergo irreversible oxidative cross-linking or extensive degradation (Nicolaÿ & Paillet, 2002:270). This leads to the structural changes and loss of elasticity in aged skin. Enzymes can also be attacked by LOOH; rendering them without activity (Nicolaÿ & Paillet, 2002:270).

2.2.1.3 PROMOTION OF MATRIX METALLOPROTEINASE (MMP) EXPRESSION

According to Thibodeau (2002:170) MMPs are enzymes found in the skin and are accountable for the breakdown of macromolecules that form the skin ECM responsible for the three-dimensional integrity of the skin (Thibodeau, 2002:170). Both intrinsic as well as extrinsic aging can be characterised by elevated levels of MMPs.

During intrinsic skin aging MMP-1 (collagenase-1), MMP-2 (gelatinase-A) and MMP-3 (stromelysin-1) are up-regulated (Hornebeck, 2003:569). Fibroblast senescence, which will be discussed in Section 2.2.2.4, decreases the expression of the tissue inhibitor of metalloproteinase-1 (TIMP-1), both *ex vivo* and *in vivo* (Hornebeck, 2003:569). This inhibitor helps to counteract the degradative effects of the MMPs (Jenkins, 2002:806).

UV irradiation activates cytokine receptors and cell surface growth factors which results in the signal transduction through a protein kinase cascade. The transcription factor AP-1 is stimulated, which in turn regulates several MMP family members and Type I pro-collagen (Rittié & Fisher, 2002:705). TIMP-1 was also found to be induced by UV irradiation (Jenkins, 2002:806). Regardless of this, UV exposure is still accountable for the destruction of both the collagen and the elastic fibre network within the dermal tissue by encouraging a degradative environment (Jenkins, 2002:806).

Fisher *et al.* (1997:1423) proposed a model depicting the pathophysiology of dermal damage caused by UV irradiation leading to skin wrinkling. This model, however, does not account for the alterations in skin surface texture and skin pigmentation seen in photoaged skin (Fisher *et al.*, 1997:1423). This model can be seen in **Figure 2.5**. Exposure of the skin to levels of UV light (that cause no detectable sunburn) will induce the expression of MMPs in keratinocytes (KC) in the outer layers of the skin, as well as fibroblasts (FB) in connective tissue. The MMPs are responsible for the degradation of collagen in the ECM of the dermis. The destruction caused by the MMP is partially inhibited by the simultaneous induction of TIMP-1. Synthesis and repair follows the breakdown of collagen. This process is imperfect and leaves subtle, clinically undetectable deficits in the organisation and/or composition of the ECM (Fisher *et al.*, 1997:1426).

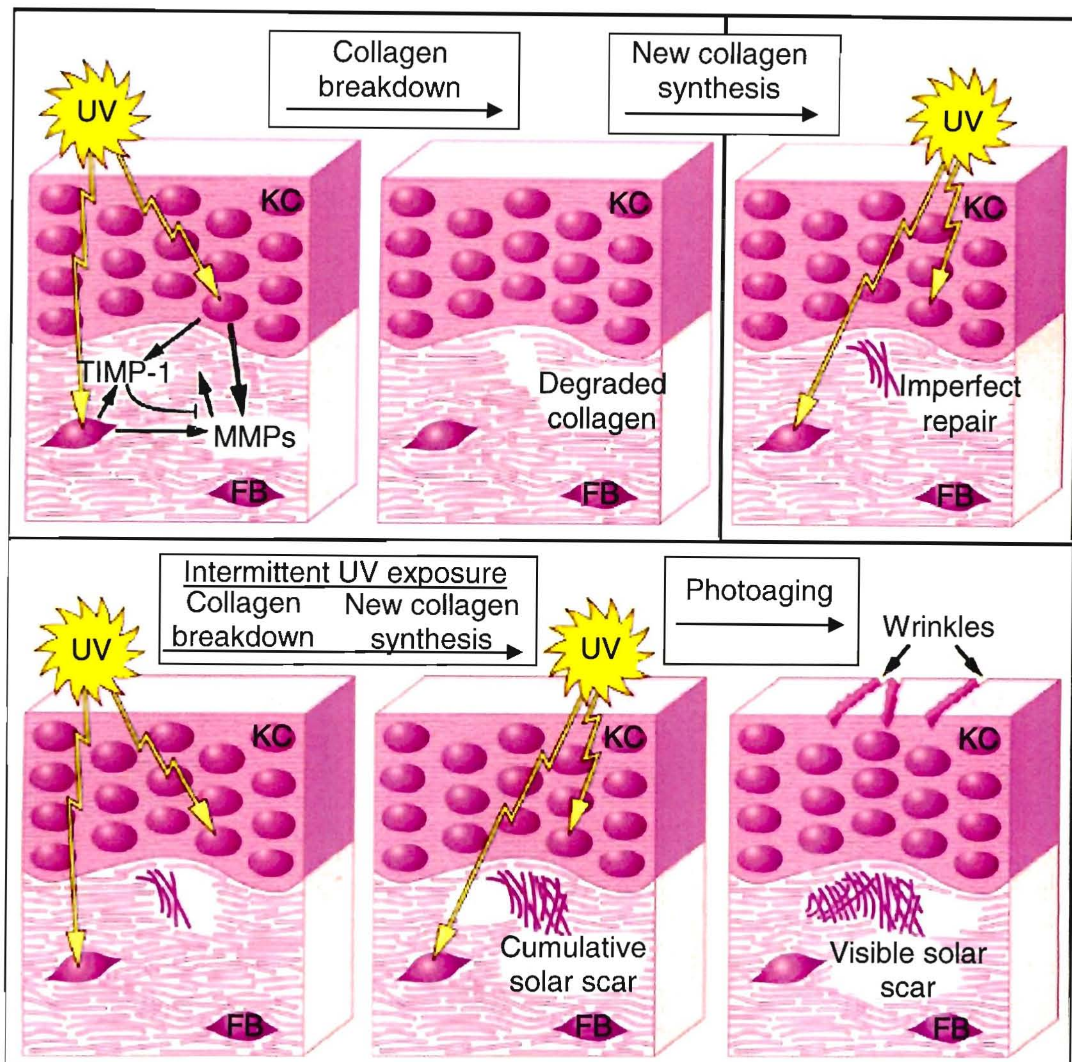

Figure 2.5: The hypothetical model of the pathophysiology of dermal damage and photoaging induced by UV irradiation. The blue background depict the processes observed by Fisher *et al.* (1997:1426) whereas the yellow backgrounds represent hypothetical processes that were consistent with their results.

As was seen in **Figure 2.3** both intrinsic and extrinsic aging stimulates AP-1 which up-regulates several MMPs (Rittié & Fisher, 2002:713):

- MMP-1 (collagenase1 or interstitial collagenase) responsible for initialising the degradation of Types I and III fibrillar collagens.
- MMP-9 (gelatinase B) which further degrades the collagen fragments formed by collagenases.
- MMP-3 (stromelysin 1) degrades Type IV collagen of the basement membrane and also activates proMMP-1.

Matrix degradation following UV irradiation is not only mediated by the activation of the transcription factor AP-1, but also by the inhibition of TGF-β. TGF-β inhibits the expression of certain enzymes responsible for collagen breakdown which includes MMP-1 and MMP-3 (Rittié & Fisher, 2002:714). **Figure 2.6** illustrates the keratinocyte producing the collagenases MMP-2 and MMP-9 (upper left corner) (Thibodeau, 2002:175). Both of these collagenases are located in the basal layer of the epidermis as well as in the upper layers of the dermis (papillary dermis) (Thibodeau, 2002:175). The lower left corner shows a fibroblast, responsible for the production of the chief types of skin collagen, particularly collagen Type I (Thibodeau, 2002:176). Dilation of the micro-capillaries is brought about by the slackening of the matrix due to disturbances in the integrity of the ECM (Thibodeau, 2002:176). This figure also depicts the different effects that intrinsic and extrinsic aging has on MMP.

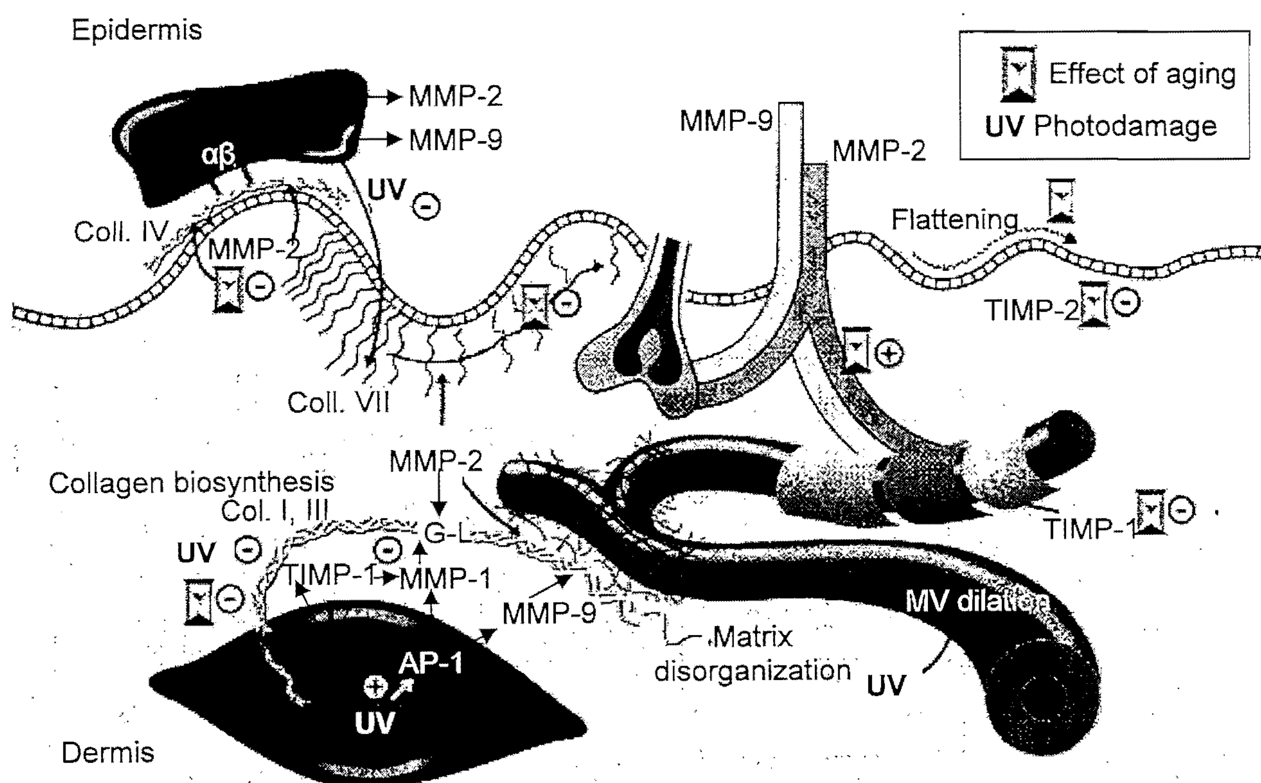

Figure 2.6: Action of MMP-2 on molecular and skin mechanisms at the dermal-epidermal junction (Thibodeau, 2002:175).

2.2.2 INTRINSIC AGING

Intrinsic aging is similar to that seen in most internal organs (Jenkins, 2002:801) and consists of genetic components that give rise to severe wrinkles and causes the skin to hang (Billek, 2002:115). The causes and mechanisms for intrinsic aging are far less well understood than for photoaging (Oender *et al.*, 2008:563).

2.2.2.1 MORPHOLOGIC AND HISTOLOGICAL CHANGES

Intrinsic aging (sun-protected, naturally aged skin) is responsible for certain structural and/or functional changes (Robert, 2007:115-119; Billek, 2002:111; Varani *et al.*, 2000:485) and can be characterised by the formation of rhagades and poor texture (Draelos, 2007:2). Aging skin has an irregular surface due to the appearance of clumped elastin, growth of benign neoplasms (adenexal tumours, seborrhoeic keratoses, etc.) and an increase in sebaceous gland size (Draelos, 2007:2). Collagenous fibres arrange into bigger bundles, intermolecular bonds increase and the skin's water-binding capacity decreases (loss of hydration) (Robert, 2007:117) to make the dermis stiff so the skin ultimately loses flexibility (Billek, 2002:112). In young dermis the elastic fibres run primarily vertical and are replaced by horizontally-running fibres during the aging process which leads to the loss of elasticity (Robert, 2007:117). There is also an increase in the production of fibronectin and protease activity (Robert, 2007:117). Histological features associated with the aging of human skin are listed in **Table 2.1** and includes epidermal, dermal and appendageal changes (Gilchrest, 1984:17).

Table 2.1: Histological features of aging human skin as described by Gilchrest (1984:19).

Epidermis	Dermis	Appendages
Flattened dermo-epidermal junction	Atrophy (loss of dermal volume)	Depigmented hair
Variable thickness	Fewer fibroblasts	Loss of hair
Occasional nuclear atypia	Fewer blood vessels	Abnormal nail plates
Fewer melanocytes	Fewer mast cells	Conversion of terminal to vellus hair
Variable cell size and shape	Shortened capillary loops	Fewer glands
Fewer Langerhans cells	Abnormal nerve endings	

2.2.2.2 PHYSIOLOGICAL CHANGES

Functions of the skin that decline with age include cell replacement, barrier function, chemical clearance, vitamin D production, thermoregulation, sebum production, vascular responsiveness, and sweat production (Gilchrest, 1984:25). According to Rhein *et al.* (2000:114) there is also a disturbance in the production and differentiation of keratinocytes due to aging. This causes an imbalance in the cycle of cell loss and replacement in the stratum corneum (SC) (Rhein *et al.*, 2000:114). The number of blood vessels in addition to the amount of nerve endings decreases with age; this can lessen the perception of pain and noxious stimuli (Rhein *et al.*, 2000:115). Rhein *et al.* (2000:118) states that the immune system changes with age. The thymus undergoes atrophy during the aging process and thus the number of T-cells available to protect the body is decreased (DiSalvo, 2000:231). Fewer immune cells are present in the skin, while

those remaining are less effective due to a lack of growth factors. This will leave the fungi, viruses and bacteria free to infect and kill the healthy cells (DiSalvo, 2000:234).

2.2.2.3 DERMATOLOGICAL DISORDERS ASSOCIATED WITH AGING

According to Gilchrest (1984:37) no skin disease exclusively occurs in the elderly, although some disorders may prevail more commonly in this age group. These conditions may also evolve differently in older patients. Makrantonaki & Zouboulis (2008:e158) summarised the age-associated diseases as given in **Table 2.2**.

Table 2.2: Age-associated skin diseases (Makrantonaki & Zouboulis, 2008:e158).

Common skin lesions (e.g. dry skin, senile purpura, freckling, telangiectasia, gruttate hypomelanosis, lentigines, stellate pseudoscars, solar comedones, colloid milia, lichen sclerosus et atrophicus)
Benign tumours (e.g. seborrhoeic keratoses, cherry angiomas)
Premalignant tumours (e.g. actinic keratosis, Bowen's disease)
Malignant tumours (e.g. basal cell carcinoma, squamous cell carcinoma, cutaneous lymphomas, angiosarcoma, malignant melanoma, Merkel cell carcinoma, Kaposi sarcoma, cutaneous metastases and sebaceous tumours)
Bullous dermatoses (e.g. bullous pemphigoid)
Pruritus
Infectious diseases (e.g. dermatophytosis, cellulites, zoster)
Lichen simplex chronicus
Vulvodynia, glossodynia, atrophic balanitis
Pressure ulcers, lower extremity ulcers

2.2.2.4 CELLULAR SENESCENCE

Cellular senescence is thought to be a tumour-suppressive mechanism and is an underlying cause of aging (Dimri *et al.*, 1995:9363). Normal somatic cells enter a state of changed function and irreversibly arrested growth after a set number of divisions (Dimri *et al.*, 1995:9363). A modification in telomere structure is due to the progressive telomere shortening with each cell division (Sharpless & DePinho, 2004:162; Hayflick, 1998:640). The lifespan of primary human cells are limited to a finite number (50 – 70 for human fibroblast) of cell divisions during telomere shortening (Song *et al.*, 2009:75). Telomeres consists of a tandemly repeated DNA sequence 5'-(TTAGGG)-3' and specific binding proteins situated at the distal ends of eukaryotic chromosomes and are crucial in order to protect the chromosome ends from ligation and degradation (Sugimoto *et al.*, 2006:43-44).

The addition of telomeric repeats to telomeres is catalysed by telomerase, a ribonucleoprotein enzyme (RNA-dependent DNA polymerase). Telomeres shorten with each cell division due to

the lack of telomerase activity in somatic cells (Sugimoto *et al.*, 2006:44). Normal somatic cells irreversibly stop proliferating and acquire altered functions along with a characteristic morphology when the telomeres reach a critically short length (Sugimoto *et al.*, 2006:44). Cells enter proliferative senescence when the telomeres become 'critically' short and they function as a biologic clock which informs cells whether they are young or old (Kosmadaki & Gilchrest, 2004:156). Dimri *et al.* (1995:9363) states that senescent cells accumulate *in vivo*, where their altered phenotype contributes to age-related pathology.

Sugimoto and co-workers (2006:45) found that the epidermis contains shorter telomeres than the dermis. During aging the telomere length in the epidermis and dermis were found to be reduced by an average telomere shortening rate of 9 and 11 bp/yr, respectively (Sugimoto *et al.,* 2006:45). Telomere length between sun-protected and sun-exposed sites did not differ significantly, and they were unable to show evidence that telomere shortening is associated with photoaged skin (Sugimoto *et al.*, 2006:43).

2.2.2.5 CROSS-LINKING OF THE EXTRACELLULAR MATRIX (ECM)

Progressive loss of skin tissue is a major characteristic of aging skin and analysis showed a loss of approximately 7 % per decade, with considerable individual variations (Ravelojaona *et al.*, 2008:369). Age-associated changes affect the composition, modification and turnover of the skin ECM components (Szauter *et al.*, 2005:449). The cell proliferation, differentiation, migration and apoptosis in the dermis and epidermis (keratinocytes) depend on the integrity of the ECM. It also serves as a reservoir for cytokines, hormones and growth factors and is known to mediate interactions of cells with these factors. The balance of these factors may be altered with the slightest change in structural integrity, composition and cross-linking state of the matrix (Szauter *et al.*, 2005:449).

With age, the ECM is known to become progressively more cross-linked of which some of the cross-links in the matrix fibres (fibrillar collagens) are chemical and others undergo translational modifications catalysed by enzymes, which include lysyl oxidase (LOX) (Szauter *et al.*, 2005:449). LOX is a copper- and lysyl-tyrosyl-containing amine oxidase which catalyses the cross-linkages in collagen fibres and elastin in the ECM (Szauter *et al.*, 2005:449). Five LOX genes have been discovered (Noblesse *et al.*, 2004:621). Fibroblasts in the dermis that produce different types of collagens and elastin are known to express LOX (Szauter *et al.*, 2005:449).

2.2.3 EXTRINSIC AGING

The term dermatoheliosis (sun-induced aging/photoaging) is used to describe the range of clinical and histological findings which characterise chronically sun-exposed skin in middle-aged and elderly adults (Gilchrest, 1984:97).

2.2.3.1 CHARACTERISTIC CHANGES

Photoaged skin is different form sun-protected, naturally aged skin and can be characterised by irregular pigmentation (hyper- and hypo-pigmentation), wrinkles and deep lines with a coarse, rough surface (Harnish *et al.,* 2002:145, Jenkins, 2002:804; Fisher *et al.*, 1997:1419) and a thickened appearance (actinic keratosis) due to distorted keratinocytes and corneocytes (Billek, 2002:115; Harnish *et al.*, 2002:145; Varani *et al.*, 2000:480). This type of aging leads to total disorganisation of the dermal matrix, but also involves changes in cellular biosynthetic activity (Jenkins, 2002:801). UV irradiation is also responsible for the acute and chronic changes in the DNA, lipid and protein building blocks (Billek, 2002:114).

During the process of photoaging there are two compartments affected, the epidermis and the dermis (Vioux-Chagnoleau *et al.*, 2006:S2; Billek, 2002:114). The epidermis is directly targeted by UV radiation, while the dermis can undergo solar elastosis and disorganisation of the dermal ECM (Vioux-Chagnoleau *et al.*, 2006:S2). UVA is more effective than UVB to generate ROS species (Pinnell & Madey, 1998:468).

UVA is present to a greater extent (more than 30 times) in sunlight than UVB. Epidermal damage are provoked by the UVB as it is almost entirely absorbed in the top 0.1 mm of the skin, whilst UVA penetrates through the skin into the deeper layers, also known as the dermis (Vioux-Chagnoleau *et al.*, 2006:57; Pinnell & Madey, 1998:468). The UVB exposure releases soluble epidermal factors, through an indirect mechanism, thereby inducing MMP-1. UVA exposure directly induces MMP-1 production in the dermal fibroblasts (Vioux-Chagnoleau *et al.*, 2006:56). Clinical features of actinically damaged skin are listed in **Table 2.3**.

Table 2.3: Features of actinically damaged skin (Gilchrest, 1984:98).

Clinical abnormalities	Histological abnormality	Presumed pathophysiology
Dryness (roughness)	Minimal SC irregularity	Altered keratinocyte maturation
Actinic keratoses	Nuclear atypia; loss of orderly, progressive keratinocyte maturation; irregular epidermal hyper- and/or hypoplasia; occasional dermal inflammation	Premalignant disorder
Irregular pigmentation		
Freckling	Reduced number of hypertrophied strongly dopa-positive melanocytes	Reactive hyperplasia and later loss of functional melanocytes
Lentigenes	Elongation of epidermal rete ridges; increase in number and melanization of melanocytes	
Guttate hypomelanosis	Absence of melanocytes	
Dermis		
Wrinkling		
Fine surface lines Deep furrows	None detected	Alterations in dermal matrix and fibrous proteins
Stellate pseudo-scars	Absence of epidermal pigmentation, altered dermal collagen	Loss of functional melanocytes. Reactive collagen deposition by fibroblasts
Elastosis (fine nodularity and/or coarseness)	Nodular aggregations of fibrous to amorphous material in the papillary dermis	Overproduction of abnormal elastin fibres
Inelasticity	Elastotic dermis	Altered elastin fibres
Telaniectasia	Ectatic vessels often with atrophic walls	Loss of connective tissue support
Venous lakes	Ectatic vessels often with atrophic walls	Loss of connective tissue support
Purpura (easy bruising)	Extravasated erythrocytes	Loss of connective tissue support for dermal vessel walls
Appendages		
Comedones	Ectatic superficial portion of the pilosebaceous follible	Loss of connective tissue support
Sebaceous hyperplasia	Concentric hyperplasia of sebaceous glands	Increased mitotic and functional responsiveness of glandular tissue

2.3 TREATMENT WITH COSMECEUTICAL ACTIVES: CALENDULA OIL AND L-CARNITINE-L-TARTRATE

This section describes the cosmeceutical actives studied. As was explained in the introductory chapter, the term cosmeceuticals can be used to describe a cosmetic product which exerts a pharmaceutical therapeutic advantage, but not necessarily a biological therapeutic advantage (Choi & Berson, 2006:163). The natural actives, calendula oil and L-carnitine-L-tartrate were employed due to their anti-aging effects on the human skin.

2.3.1 CALENDULA OIL

Figure 2.7: Calendula officinalis (MDidea, 2005:1, 3, 4, 7).

Calendula, also known as *Calendula officinalis* or Marigold (**Figure 2.7**), is generally considered as an herb of ancient medicinal use (Ćetković *et al.*, 2004:643). The botanical name comes from the Latin word *kalendulae*, which means the first day of the month in the Roman calendar. This name was assigned to these flowers because they tend to bloom at the beginning of almost every month (Centerchem, 2006:1). This plant is characterised by its yellow or golden-orange flowers and it grows throughout Europe and North America as a wild or a common garden plant (Ćetković *et al.*, 2004:644). The dried flower heads or the dried ligulate flowers (ray florets) are the plant parts used for cosmetic and pharmaceutical purposes (Hamburger *et al.*, 2003:329).

2.3.1.1 COMPOSITION

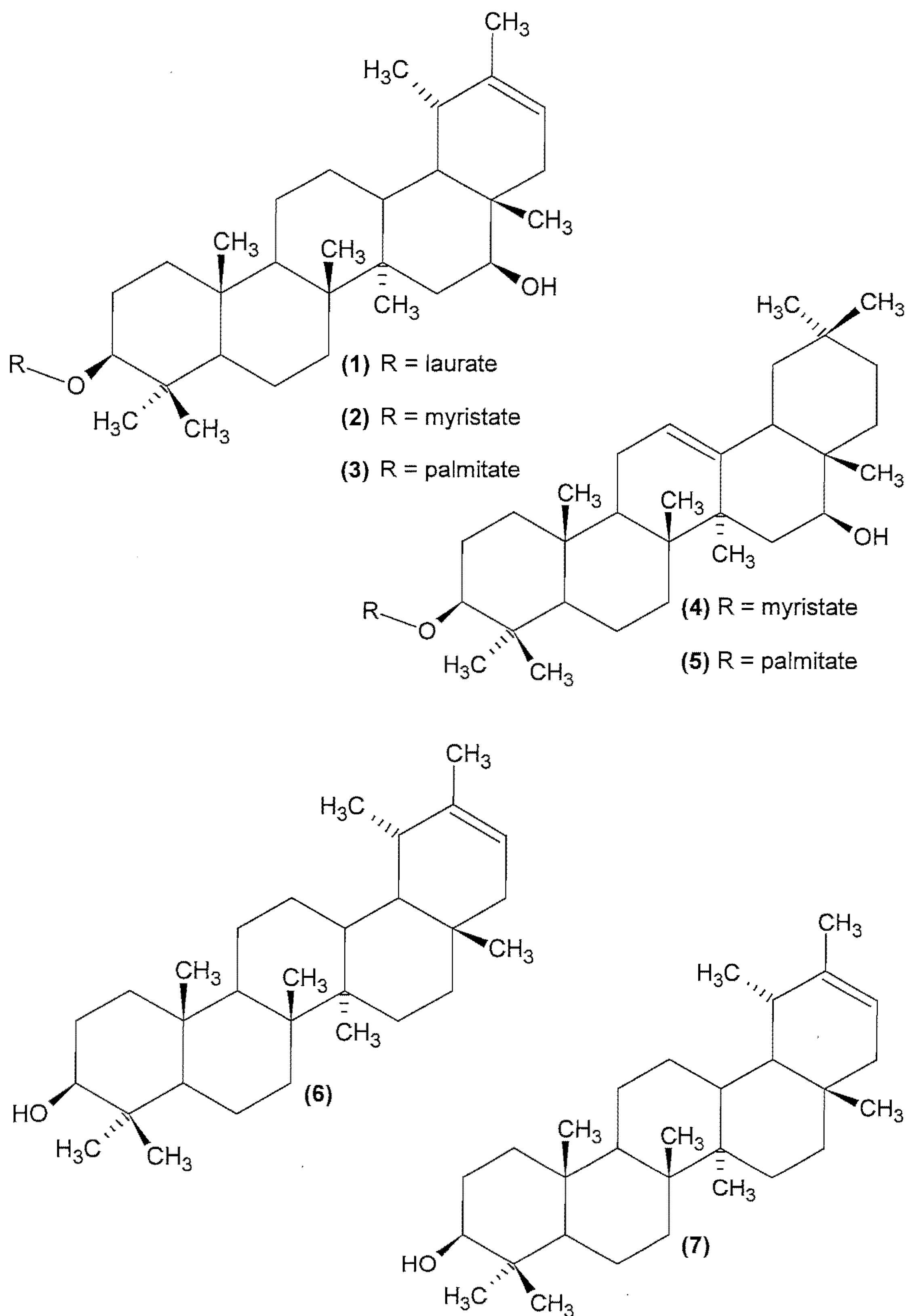

Figure 2.8: Structures of triterpenoids (Hamburger *et al.*, 2003:333).

Numerous investigations have proved *via* modern analytical methods that *Calendula officinalis* contain diverse classes of compounds. According to Fïume (2001:13) extracts contain the following ingredients: flavonoids, sugars, mucilages, carotenoids, sterols, phenolic acids, sterins, resins, quinones, polyprenylquinones, saponins, vitamins and essential oils. Lutein is an important carotenoid that can be found in calendula and possesses antioxidant activity (MDidea, 2005:6).

Hamburger *et al.* (2003:328) developed a method for the purification of the major anti-inflammatory triterpenoid esters that can be found in the flower heads; namely faradiol 3-O-laurate, palmitate and myristate (**Figure 2.8**).

Bakó *et al.* (2002:241) investigated with high pressure liquid chromatography (HPLC) analysis the carotenoid composition of parts of the fresh flowers, petals, leaves, pollens and stems of *C. officinalis* L. after extraction and saponification (Bakó *et al.,* 2002:247). They identified the carotenoids based on their ultra-violet/visible (UV-VIS) spectra, chromatographic behaviour, specific chemical tests and co-chromatography with authentic samples (Bakó *et al.*, 2002:248).

In **Figure 2.9** the structures of the different carotenoids identified are as follow: antheraxanthin: R = e, Q = c; auroxanthin: R = Q = f; α-carotene: R = a, Q = b; ß-carotene: R = Q = a; α-cryptoxanthin: R = c, Q = b; ß-cryptoxanthin: R = c, Q = a; flavoxanthin/chrysanthemaxanthin: R = f, Q = d; lutein: R = c, Q = d; lutein 5,6-epoxide: R = e, Q = d, luteoxanthin: R = e, Q = f; lycopene: R = Q = h; mutatoxanthin: R = f, Q = c; neoxanthin: R = g, Q = e; neochrome: R = g, Q = f; violaxanthin: R = Q = e; zeaxanthin: R = Q = c (Bakó *et al.,* 2002:242).

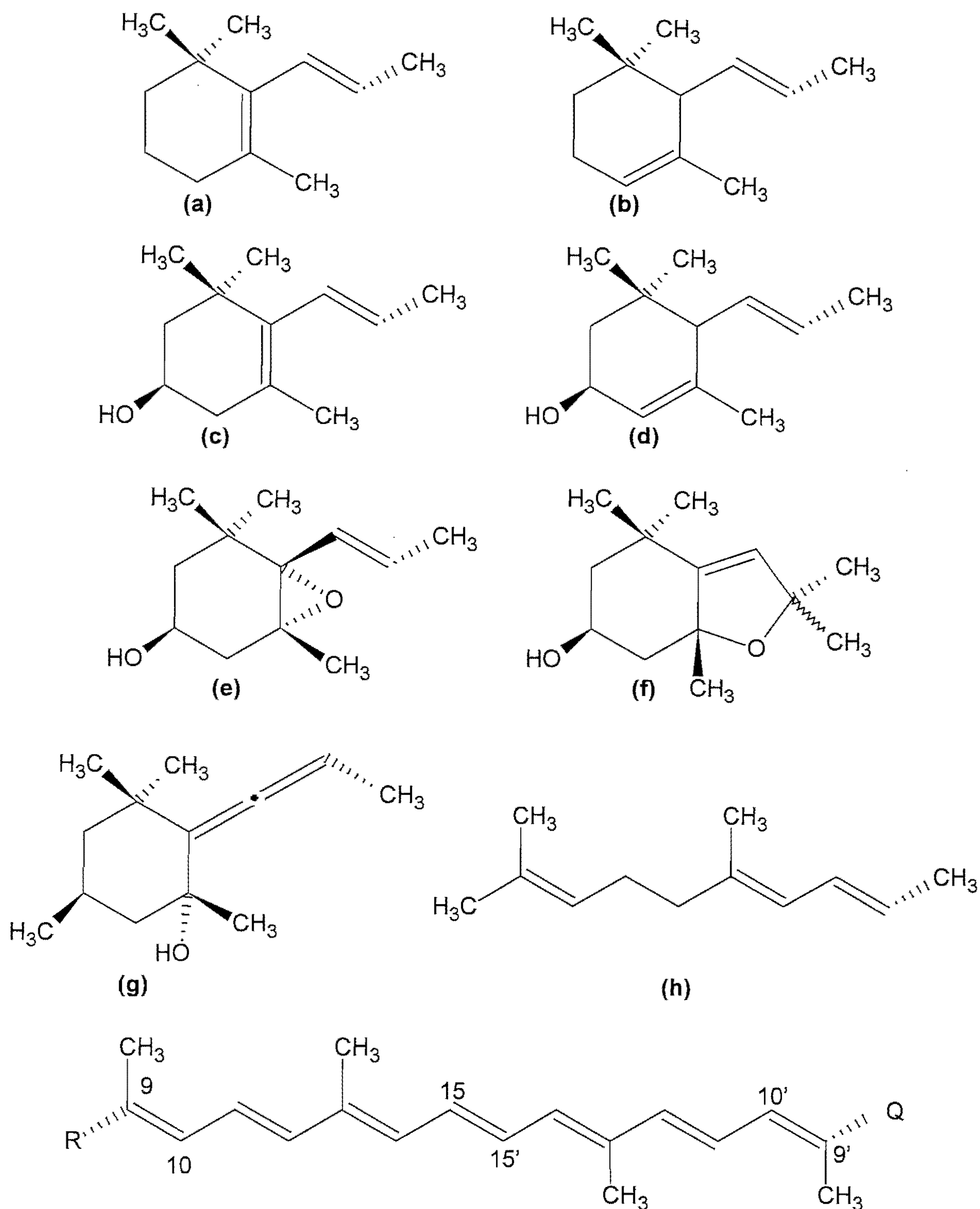

Figure 2.9: Structures of carotenoids as found in the steams, leaves, petals and pollens of *calendula officinalis* L. (Bakó *et al.*, 2002:242)

2.3.1.2 USE OF CALENDULA OIL

As was discussed in Section 2.2.1.2 oxidative stressors create inflammatory molecules that can lead to the formation of free radical species (Choi and Berson, 2006:163). Free radicals play a role in inflammation, photodamage and carcinogenesis (Choi & Berson, 2006:164). In

correlation with the concentration of the total flavonoids and phenolic compounds, calendula extracts scavenges hydroxyl, peroxyl and diphenylpicrylhydrazyl (DPPH) radicals in a concentration dependent manner (Ćetković *et al.*, 2004:648). Antioxidants play an important role in the protection of the skin from exogenous stressors (UV light, pollution, cigarette smoke) and endogenous stress (the by-products from cellular energy) (Choi & Berson, 2006:164; MDidea, 20056) thereby preventing or ameliorating premature aging and cancer (Fuchs *et al.*, 1989:769).

The triterpenoids found in the CO_2 extract of calendula flowers are the most important anti-inflammatory entities (Della Loggia *et al.*, 1994:516). The most active compound present is the free faradiol which equals indomethacin in activity (Della Loggia *et al.*, 1994:519). The free monools, Ψ-taraxasterol, taraxasterol, lupeol, and β-amyrin seem to be less active than the free diol (Della Loggia *et al.*, 1994:519). Inflammation caused by ROS can thus be relieved by the topical application of calendula. Other examples include the flavonoids, a sub-group of polyphenols which are plant-derived antioxidants that also show anti-inflammatory activity (Choi & Berson; 2006:165). Calendula shows numerous uses which include wound healing and it can be utilised as an antiseptic and antifungal. It is responsible for increasing the blood flow to the affected area. Acne, cold sores and diaper rash can also be treated with calendula (MDidea, 2005:3). According to the Medical Centre of the University of Maryland (2007:3), the topical effective concentration for a calendula ointment is between 2 – 5 %. **Table 2.4** summarises the different cosmetic applications together with the responsible entity.

Table 2.4: The cosmetic applications of calendula (Centerchem, 2006:7).

Action	Active	Cosmetic application
Immunomuodulatory	Polysaccharides	Anti-aging
Moisturising	Saponins Polysaccharides (mucilages)	Moisturising Soothing
Anti-inflammatory	Triterpenes	Anti-irritant
Re-epithelising and wound healing	Flavonoids Polysaccharides Triterpenes Carotenes	Anti-aging

2.3.2 L-CARNITINE L-TARTRATE

L-carnitine L-tartrate (**Figure 2.10**) is the more stable salt form of L-carnitine. Carnitine (3-hydroxy-4-*N*-trimethylammoniobutanoate) has been studied at length since its discovery 100 years ago (Steiber *et al.*, 2004:456). L-carnitine exists as an internal quaternary salt at

neutral pH (Held, 2004:41) and its status in humans vary by gender, body composition and overall diet (Steiber *et al.*, 2004:457). Food sources of methionine, lysine and carnitine account for approximately 75 % of the total body carnitine (Steiber *et al.*, 2004:457).

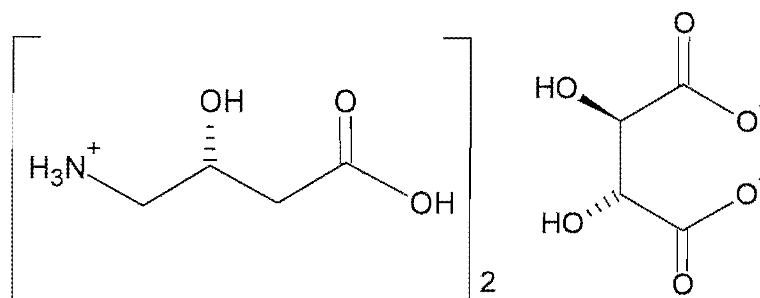

Figure 2.10: L-carnitine L-tartrate (Shenyang KoncepNutra Co. Ltd., 2005:1).

The total body carnitine pool is exceptionally dynamic with the carnitine moving between the gastrointestinal tract after absorption, the liver after biosynthesis, the kidney for elimination and tissues that entail carnitine for function, such as skeletal muscle and the heart (Brass, 2002:591). As metabolic shifts take place, the carnitine pool within a tissue is redistributed between carnitine and acylcarnitines (Brass, 2002:591).

2.3.2.1 BIOSYNTHESIS

The synthesis of carnitine is a multi-step process and takes place in the mitochondria of the heart, kidney, liver, muscle and brain (Steiber *et al.*, 2004:461). It is synthesised from the essential amino acids lysine and methionine in the rat, which involves the methylation of a lysine-derived carbon chain by methionine (Cox & Hoppel, 1973a:1081; Cox & Hoppel, 1973b:1083). The first sequence of reactions is as follow: lysine → protein-bound lysine → protein-bound 6-trimethyl-lysine → free 6-trimethyl-lysine (Cox & Hoppel, 1973b:1089). The initial step in the biosynthesis from trimethyl-lysine involves the addition of a hydroxyl group to the third carbon of trimethyl-lysine by the enzyme hydroxylase (trimethyllysine dioxygenase) (Steiber *et al.*, 2004:461; Sachan & Hoppel, 1980:529). 3-Hydroxy-N^6-trimethyllysine is the product of this reaction and it seems to be the only step in which the enzyme is located in the mitochondria (Steiber *et al.*, 2004:461; Sachan & Hoppel, 1980:533). Cofactors required during this step include O_2, $FeSO_4$, ascorbate and α-osoglutarate (Sachan & Hoppel, 1980:533).

The enzyme 3-hydroxy-N^6-trimethyllysine aldolase converts 3-hydroxy-N^6-trimethyllysine to 4-*N*-trimethylammoniobutanal and glycine (Steiber *et al.*, 2004:461; Hoppel *et al.*, 1980:517). Pyridoxal 5'-phosphate functions as a cofactor in this reaction, and this makes pyridoxine a requisite for the aldolase activity. 4-*N*-trimethylammoniobutanol dehydrogenase uses niacin in the form of NAD as a hydrogen acceptor in order to catalyse the formation of

4-*N*-trimethylammoniobutanoate (butyrobetaine) from 4-*N*-trimethylammoniobutanal (Steiber *et al.*, 2004:461).

4-*N*-trimethylammoniobutanoate is taken up predominantly by the kidney and the liver through a transport mechanism after it enters circulation (Steiber *et al.*, 2004:461). The activity of butyrobetaine dioxygenase is 3 – 16 fold higher in the kidney than in the liver, with the activity in the brain being 50 % that of the liver (Steiber *et al.*, 2004:461). In the cytosol of the liver and kidney, L-carnitine is formed by the hydroxylation of 4-*N*-trimethylammoniobutanoate on the third carbon; this reaction requires vitamin C, molecular oxygen and Fe^{2+} for activity (Steiber *et al.*, 2004:461). L-carnitine is transported through the circulation and is taken up by other tissues *via* an active sodium-dependant transporter (Steiber *et al.*, 2004:461).

2.3.2.2 FUNCTION

The uptake of L-carnitine in human fibroblasts is maintained by a plasmalemmal carnitine transporter which is dependant on the sodium gradient (Koeck & Kremser, 2003:149; Tein *et al.*, 1996:147). The transporter is essential for the efficient importation of carnitine into the cell in order to fulfil its vital intracellular functions (Tein *et al.*, 1996:154). Lamhonwah (2005:1967) states that there appears to be a family of carnitine transporters with different affinities for carnitine. In the mitochondria carnitine serves as an essential cofactor for long-chain fatty acid oxidation (Lamhonwah *et al.*, 2005:1969; Tein *et al.*, 1996:145). According to Steiber *et al.* (2004:462) fatty acids represent a foremost energy source for most tissues, and therefore requires the availability of carnitine for normal function (Brass, 2002:591). These fatty acids undergo β-oxidation (chain shortening) (Steiber *et al.*, 2004:462) in the mitochondrial matrix, and their stored energy is released and conserved as adenosine triphosphate (ATP) (Steiber *et al.*, 2004:462).

Carnitine transfers long-chain fatty acids as acylcarnitine esters across the inner mitochondrial membrane in order for intra-mitochondrial β-oxidation to take place (Lamhonwah *et al.*, 2005:1969). It plays a role in the upholding of the homeostasis in the acyl-CoA pools of the cell and is therefore responsible for supplying the cells with a vital source of free coenzyme A (CoA). It will keep the acyl-CoA/CoA pool constant even under conditions of very high acyl-CoA turnover (Lamhonwah, 2005:1966).

The mechanism by which carnitine works have been described by several authors. Long-chain fatty acids are activated on the outer membrane by the long-chain acyl-CoA syntetase (LCAS) and are impermeable through the mitochondrial inner membrane and thus cannot reach the site of β-oxidation and is transported into the mitochondrial matrix by a carnitine-dependent transporter (Steiber *et al.*, 2004:462; Brass, 2002:590). The transport system is composed of three proteins, each with different sub-mitochondrial localisation: acylcarnitine carnitine

translocase (CACT), carnitine palmitoyltransferase I (CPT-I) and carnitine palmitoyltransferase II (CPT-II) (Steiber *et al.*, 2004:462).

LCAS is present in the mitochondrial outer membrane and catalyses the first step which is the formation of acyl-CoAs (Steiber *et al.*, 2004:462). The next step has been suggested as the movement of activated fatty acids across the mitochondrial outer membrane by the voltage-dependent anion channel or porin (VDAC) (Steiber *et al.*, 2004:462). CPT-I (localised in the mitochondrial outer membrane) catalyses the conversion of the activated fatty acids to the respective acylcarnitine form, e.g. palmitoylcarnitine, with release of free CoA (Steiber *et al.*, 2004:462; Lamhonwah *et al.*, 2005:1969). Long-chain carnitine esters are the reaction products and are translocated into the mitochondrial matrix in an exchange reaction catalysed by CACT, an integral inner membrane protein (Steiber *et al.*, 2004:462).

In the matrix, CPT-II (situated on the inner side of the inner mitochondrial membrane) converts the acylcarnitine esters (e.g. palmitoylcarnitine) back to their respective CoA esters (e.g. palmitoyl-CoA) in the presence of free CoA (Lamhonwah *et al.*, 2005:1969; Steiber *et al.*, 2004:462). This involves the reversible transfer of a carboxylic acid moiety (acyl group) from a coenzyme A thioester (acyl-CoA) to form the analogous carnitine ester (acylcarnitine) (Brass, 2002:590).

Furthermore it was determined that topically applied carnitine shows promising effects. The positive outcome that carnitine has in burn injuries and ischemia was investigated by Arslan *et al.* (2003:221) by making use of the distally-burned dorsal skin flap model/experiment on rats. In a burn injury tissue has difficulty in surviving and this tissue also has an increased energy flow requirement (Arslan *et al.*, 2003:221). It was determined that carnitine increases the vascularity, as well as the cellularity (including mixed inflammatory cells and fibroblast proliferation) in burnt tissues (Arslan *et al.*, 2003:224). When compared to the untreated study group, necrosis was found to be more limited in the group treated with carnitine (Arslan *et al.*, 2003:225).

L-carnitine (ß-hydroxy acid) shows outstanding skin exfoliating properties at a lower pH and can therefore be held responsible for the increase in the epidermal turnover rate of the skin (Held, 2004:41). The epidermal turnover rate can be seen as the time needed by a new generation of cells to migrate from the basal to the upper horny layer in order to replace the outermost cells. When this rate is disturbed, such as during the aging process, dead cells tend to gather in the outer layers, decreasing the turnover rate and increasing the renewal time. The increase of the skin turnover rate together with the removal of the dead skin cells; will result in younger, healthier-looking skin (Held, 2004:41). Due to the hygroscopic nature of L-carnitine, even small

amounts of L-carnitine will leave the skin smooth, soft and moisturised (Held, 2004:40). L-carnitine was found to be most effective in a formulation containing 2 % (Held, 2004:40).

Carnitine can prove valuable in improvement of aging skin owing to its promising effects of fibroblast proliferation and skin moisturisation.

2.4 TRANSDERMAL PENETRATION

2.4.1 INTRODUCTION

The skin accounts for more than 10 % of the body mass and is the body's largest organ (Roberts & Walters, 1998:1; Black, 1993:145). It functions as a protective barrier between the internal organs and the harsh environment (Rittié & Fisher; 2002:706) in order to protect the sensitive protoplasmic jelly of the body's interior, averting the intrusion of chemicals, microbes and numerous forms of radiation, while keeping body fluids and tissues from spilling out (Zatz, 1993:12). Additionally the skin is essential for the thermal regulation of body temperature and has become modified to being able to withstand aridness (Rhein *et al.*, 2000:88). According to Lund (1994:231) the skin is more resistant to penetration by drugs than the other mucosal surfaces utilised as absorption sites yet it represents a suitable target for drug delivery (Thomas & Finnin, 2004:697).

2.4.2 STRUCTURES OF SKIN

Skin consists of three regions, namely the epidermis, dermis and the subcutaneous tissue (Rittié & Fisher; 2002:706).

2.4.2.1 EPIDERMIS

The outer layer of the skin, known as the SC or the horny layer (pH 5) (Rieger, 2002:121), forms the major resistance to most, though not all, substances (Rhein *et al.*, 2000:93; Zatz, 1993:12) and serves as a barrier against the outlet of water (Hadgraft, 2004:291). The SC is approximately 10 – 20 µm thick (Thomas & Finnin, 2004:699; Roberts & Walters, 1998:4) and is composed of 15 – 25 layers of flattened, stacked, hexagonal and cornified cells. The SC has a turnover rate of once every 2 – 3 weeks (Roberts & Walters, 1998:5).

The epidermis is a cell-rich superficial layer and is composed mainly of keratinocytes, melanocytes and Langerhans cells. The keratinocytes are the most abundant cells in the skin, while the melanocytes are responsible for pigment production (Rittié & Fisher; 2002:706). The Langerhans cells guard the skin from invaders that broke through the physical barrier and makes out a sheer 2 – 4 % of the cell population. Keratinocytes of the spiny and granular layers of the epidermis are responsible for sending chemical signals *via* growth factors (cytokines) to

the Langerhans cells and for making the cells rigid and strong (DiSalvo, 2000:226; Rhein *et al.*, 2000:88).

According to Kuwazuru *et al.* (2008:517) the epidermis can be divided into four layers where every layer represents a progressive stage in the life cycle of a keratinocyte and includes the (1) SC (horny layer); (2) granular layer; (3) prickle layer and (4) basal layer. During the upward movement of the newly formed keratinocyte it will flatten, lose its nucleus and die. Subsequently it will produce keratin which fills the SC (Rhein *et al.*, 2000:91) which means that the SC can be deemed as 'dead' on morphological grounds (Black, 1993:145).

The epidermis and the dermis are separated by a basement membrane and consist mainly of ECM proteins, produced by resident fibroblasts (Rittié & Fisher; 2002:706). The dermo-epidermal junction is very complex and supports the epidermis, while acting as a sift to prevent the movement of inflammatory cells and materials. The epidermis has downward finger-like extensions which allows the skin to be moveable and flexible (Rhein *et al.*, 2000:98).

2.4.2.2 DERMIS

The dermis, also known as corium, is approximately 2 mm thick with a pH of near 7 (Hadgraft & Finnin, 2006:362; Barry, 2002:502; Rieger, 2002:121). The dermis can be divided into three layers: papillary, sub-papillary and reticular layers (Kuwazuru *et al.*, 2008:517). The dermis provides immune, nutritive and several other support systems for the epidermis through the blood vessels, lymphatics and nerve endings contained within (Roberts & Walters, 1998:5; Ghosh & Pfister, 1997:3). It also plays a role in the regulation of pain, temperature and pressure (Roberts & Walters, 1998:5). The dermal vasculature is the most extensive of any organ system in the body and thus helps to regulate the core body temperature through vasodilatation and vasoconstriction (Gilchrest, 1984:3).

It is composed of collagenous fibres (70 %) (provides support and cushioning) and elastic connective tissue (responsible for the skin's elasticity) in a semi-gel matrix of mucopolysaccharides (Barry, 2002:502; Roberts & Walters, 1998:5). The main cell types present in the dermis is the melanocytes, fibroblasts and mast cells. The melanocytes are implicated in the production of melanin (pigment), the fibroblasts produce the connective tissue components of vitronectin, fibronectin, laminin and collagen, while the mast cells are involved in the inflammatory and immune responses (Roberts & Walters, 1998:5).

2.4.2.3 SUBCUTANEOUS FAT

Roberts & Walters (1998:6) states that the subcutaneous tissue (hypodermis) is the deepest layer of the skin and consists of mainly adipocytes (Gilchrest, 1984:3) and functions not only as

a shock absorber and heat insulator, but also as energy storage (Barry, 2002:502). It consists of a network of fat cells attached to the dermis by interconnecting collagen fibres (Roberts & Walters, 1998:6). It is noticeably diminished with age in most body sites (Gilchrest, 1984:3).

2.4.2.4 SKIN APPENDAGES

Skin appendages include the hair follicles with their associated sebaceous glands, eccrine sweat glands, apocrine sweat glands and the nails (Roberts & Walters, 1998:6). Hair follicles extend through the epidermis into the dermis with the base being well vascularised. Attached to the side of the follicles are sebaceous glands which secrete a lipid mixture known as sebum into the area between the sheath and the hair (Ho, 2004:50). Sweat glands have their origin in the dermis as a coiled and vascularised tube and extend to the skin surface where it excretes sweat to provide thermal regulation (Ho, 2004:50). Skin appendages may act as shunts to enhance penetration of compounds (Barry, 2002:507).

2.4.2.5 NATURAL ANTIOXIDANTS

The skin possesses natural antioxidants to protect itself from damage caused by UV induced ROS. These antioxidants are depleted after exposure to UV irradiation (Podda *et al.,* 1997:55). The lipophilic antioxidants include alpha-tocopherol, ubiquinol 9 and ubiquinone 9. The water soluble antioxidants consist of non-enzymic glutathione and ascorbic acid as well as enzymic antioxidants such as superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase (Fuchs *et al.*, 1989:769). Shindo *et al.* (1994:122) found that the concentration of antioxidants in the human skin was higher in the epidermis than in the dermis with the epidermis acting as the preliminary barrier to assault by oxidants (Shindo *et al.*, 1994:122).

2.4.2.6 DRUG TRANSPORT THROUGH THE SKIN

The goal of topical drug delivery is to reduce the flux of the drug through the skin while extending its retention in the skin (Hsieh, 1994:11). The compounds must still penetrate across the SC (Hsieh, 1994:12) through passive diffusion which occurs very slowly for most drugs. The tissue consists of closely-packed cells, containing both aqueous and lipid regions (Roberts & Walters, 1998:13; Lund, 1994:231). Water soluble drugs will pass through due to hydrated protein particles within the cell wall, whereas lipid-soluble drugs will permeate through the lipid regions of the cell membranes (Lund, 1994:231). Sweat glands and hair follicles perforate through this barrier of the skin (Pugh *et al.*, 1998:246).

The scheme behind the development of permeation enhancement technologies for the treatment of dermal conditions, such as skin aging, should be to use the SC and/or the epidermis as a drug reservoir for the slow release of drugs to the targeted pathological site(s)

(Hsieh, 1994:13). As stated by Ghosh & Pfister (1997:7) as little as 1 % and no more than 15 % of the topical drug applied is bioavailable from topical formulations, with drug levels in the local tissue being higher than systemic administration. Unfortunately systemic uptake of topically applied drugs is unavoidable.

2.4.3 PENETRATION PATHWAYS ACROSS THE STRATUM CORNEUM

Absorption of a permeant can be seen as a purely physical and passive process as the SC is dead and no active transport of permeants have been identified (Pugh *et al.*, 1998:246). Penetration of permeants can thus follow three possible routes (**Figure 2.11**): intercellular diffusion across the lipid intercellular matrix; transcellular diffusion which commends through the corneocytes and finally *via* the hair follicles and sweat pores (Pugh *et al.*, 1998:246).

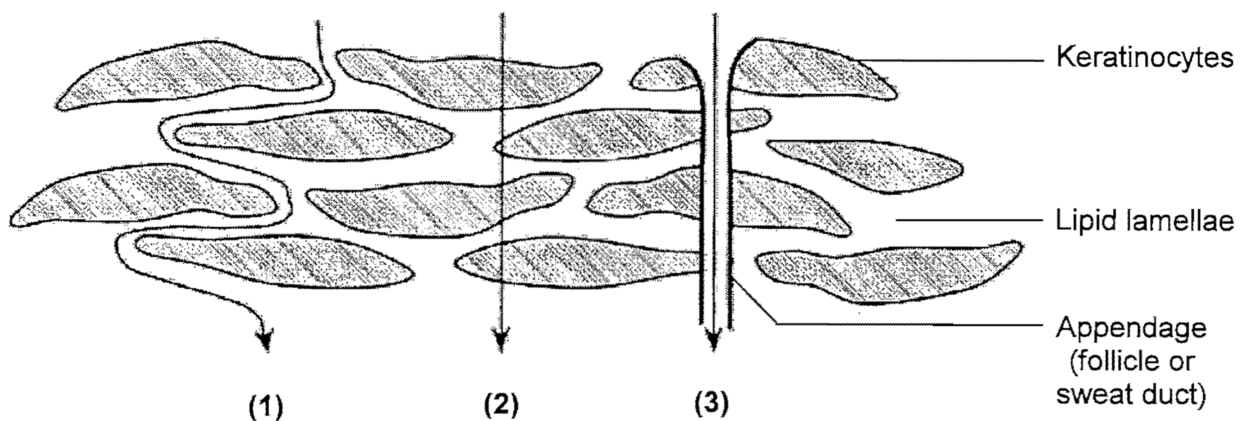

Figure 2.11: Skin permeation routes: **(1)** intercellular diffusion through lipid lamellae; **(2)** transcellular diffusion through the keratinocytes and lipid lamellae; and **(3)** diffusion through appendages such as the hair follicles and sweat glands (Ho, 2004:50).

2.4.3.1 INTERCELLULAR SPACES

Also known as the paracellular route, it is the predominating route of transport through the SC (Boddé *et al.,* 1989:13) where drug molecules are transported between or around the corneal cells (Hsieh, 1994:5). The diffusional path length is estimated to be 500 µm in length, which makes it longer than the thickness of the SC which is approximately 20 µm thick (Hadgraft, 2004:292).

2.4.3.2 TRANSCELLULAR PATHWAY

The transcellular pathway is through the tightly packed keratin-filled corneocytes (Guy & Hadgraft, 1988:756). Dayan (2007:34) states that this route is hydrophilic and it is made up of aqueous regions, surrounded by polar lipids to create the walls of micro-channels. Penetration

by the transepidermal route is reasonably rapid, albeit slower than intestinal track absorption. It is often accompanied by some extent of pilosebaceous penetration (Idson, 1975:907).

2.4.3.3 HAIR FOLLICLES AND SWEAT PORES

Several scientists believe that this route does not contribute to the permeability of a penetrant as the area of pores is very small (sebaceous glands represent not more than 0.1 % of the total skin surface), and the access of penetrant would be against the outward flow of secretes (Dayan, 2007:32; Pugh *et al.*, 1998:246), whereas others say that the appendages bypass the low diffusivity of the SC, thereby acting as diffusional shunts (Dayan, 2007:32). Every hair follicle has one or more connecting sebaceous glands. Their secretes are emptied into the follicular canal near the skin surface. These duct systems and canals are lined with stratified squamous epithelium which is promptly penetrated by certain compounds (Idson, 1975:907). Medicaments can pass into the areas below the membrane barrier through the microscopic spaces between the follicular wall and the hair shaft (Idson, 1975:907). This diffusion pathway is especially applicable to hydrophilic and high molecular weight penetrants (Ghosh & Pfister, 1997:6).

2.4.4 PHYSIOLOGICAL FACTORS INFLUENCING TRANSDERMAL DRUG DELIVERY

The biological variability inherent to skin complicates the quantitative prediction of the rate and extent of percutaneous penetration and absorption of topically-applied drugs (Riviere, 1993:113) and is described in this section.

2.4.4.1 HYDRATION

A major factor affecting the rate and extent of percutaneous absorption is the hydration state of the skin (Behl *et al.*, 1993:238; Riviere, 1993:117) with the SC being the layer primarily affected (Behl *et al.*, 1993:238). When water enters the skin, it becomes soft, swells and forms wrinkles – all these events will render the skin more permeable to several solutes (Behl *et al.*, 1993:238; Roberts & Walker, 1993:25). A proposed mechanism is where water acts as a plasticizer in its bound state in the SC (Roberts & Walker, 1993:25) and thus the SC becomes less compact in addition to the size of the pores increasing (Lund, 1994:231). Hydration of the SC causes water to gather near the outer surface of the protein filaments (Idson, 1975:908). This allows polar molecules to pass through the immobilised water, whereas the non-polar molecules dissolve and diffuse through the non-aqueous lipid matrix between the protein filaments (Idson, 1975:908).

2.4.4.2 SKIN AGE

Skin aging alters the ability of the human body to absorb topically applied drugs by diffusion through the skin and the underlying blood vessel, and this implies that aged skin presents as a less effective barrier (Ademola & Maibach, 1997:203). Infant skin is highly permeable because of the barrier function being incompletely formed (Ademola & Maibach, 1997:203). Children have a greater surface area per unit body weight, making them more susceptible to the toxic effects of drugs and chemicals (Barry, 2002:510).

2.4.4.3 GENDER AND RACE

Gender has not been found to be a critical factor influencing permeation of drugs (Behl *et al.*, 1993:247). The thickness of the SC differs between the Caucasian and Negroid races to such an extent that the thicker SC in the Negroid may lead to a slightly decreased percutaneous absorption rate for certain compounds (Jackson, 1993:180).

2.4.4.4 DAMAGE AND DISEASE OF THE SKIN

Damage and injury to the SC leads to enhanced permeability (Ademola & Maibach, 1997:204; Idson, 1975:909). Jackson (1993:178) states that diseased skin is damaged skin. Diseased skin includes transient infections or chronic conditions such as acne or psoriases. Any cut, scratch, crack or split due to excessive dryness will leave a person exposed to the harsh environment (Jackson, 1993:179). Skin may be breached by an epidermal break, which penetrates into the dermis, subcutaneous layer or even the muscle tissue (Jackson, 1993:178). Diffusive water loss is increased when the SC is damaged (Idson, 1975:909). The rate of topical absorption could also be influenced by certain systemic disease states (Riviere, 1993:116).

2.4.4.5 TEMPERATURE

According to Barry (2002:511) the penetration rate of a compound through the SC can change 10 - fold for a large temperature variation, as the diffusion coefficient decreases as the temperature falls. The temperature of the SC is between 30 – 37 °C. Transient temperature changes have insignificant effects on the transport properties of the skin, whereas temperatures reaching above 65 °C for extended periods (> 1 min) will give rise to structural alterations (Ademola & Maibach, 1997:205). Fluctuations in temperature and penetration rates can be prevented by wearing adequate clothing on most of the body (Barry, 2002:511).

2.4.4.6 ANATOMICAL SITE

According to Lund (1994:231) the area of the body selected also plays a physiological role due to the variation of the thickness of the SC. The SC is thinnest in the post-auricular and facial regions, while thickest in the plantar and palmer regions (Lund, 1994:231). Other properties of the skin that changes with the region on the human body, includes sebum production, and the concentration of hair follicles and glands (Amsden & Goosen, 1995:1975).

2.4.4.7 SKIN METABOLISM

Reduced enzymatic degradation of the drug is one of the advantages of transdermal drug delivery. Nonetheless, the skin has a capacity to metabolise a large number of compounds and is capable to undertake most of the biotransformations occurring in the liver, such as conjugation, reduction, oxidation and hydroxylation (Amsden & Goosen, 1995:1975). Metabolism occurs mainly in the viable epidermis and metabolites may be more or less toxic than the parent drug, or either inactive or active (Amsden & Goosen, 1995:1975). Hydrophilic drugs were found to penetrate the skin more when the metabolic rate was higher (Bando *et al.*, 1997:759).

2.4.5 PHYSICOCHEMICAL PROPERTIES

The skin forms a lipid layer along which individual molecules must migrate by means of diffusion (Cevc, 2004:681). The chemical-physical characteristics of the permeant plays a role in the possible routes of permeation of a drug through the skin (Amsden & Goosen, 1995:1978; Guy & Hadgraft, 1988:753) which will be described in this section.

2.4.5.1 DRUG SOLUBILTY AND MELTING POINT

Drug lipophilicity is an important factor to consider during skin permeation studies (Behl *et al.*, 1993:240). According to Gettings *et al.* (1998:462) a permeant should be reasonably soluble in both hydrophobic and hydrophilic media to ensure optimum percutaneous absorption as the skin can be regarded as a bilaminate membrane which is made up of adjoining lipoidal (SC) and aqueous (viable tissue) layers (Guy & Hadgraft, 1988:753). The potential of a compound to form a reservoir in the SC is dependable on its solubility constraint (Goosen *et al.*, 1998:208). According to Naik *et al.* (2000:319) the ideal aqueous solubility for a drug to ideally permeate the skin is more than 1 mg/ml. The aqueous solubility of calendula oil is unknown and it was found to be insoluble in water, ethanol and methanol due to its high lipophilicity. Calendula oil is not suspected to permeate the skin. The solubility of L-carnitine L-tartrate was determined to be 16.63 mg/ml in PBS (pH 7.41) at a temperature of 32 °C, which makes it ideal to penetrate the skin. Several authors (Hadgraft & Finnin, 2006:365; Cleary, 1993:28) state that the lower the

melting point the greater ability the drug has to permeate the skin. A melting point below 200 °C is necessary for ideal permeation (Naik *et al.*, 2000:319).

2.4.5.2 DRUG CONCENTRATION

As stated by Idson (1975:912) the permeation rate of a drug through a membrane can be examined by relating to Fick's general law of diffusion. This law states that the driving force that causes substances to move from a high to a low concentration is proportional to the concentration gradient. The amount of the drug absorbed percutaneously increases in accordance to an increase in the concentration of the drug in the vehicle during a given period of time (Lund, 1994:232).

2.4.5.3 MOLECULAR SIZE AND SHAPE

It is believed that the higher the molecular weight of the molecule, the slower the drug permeation through the skin. This slower diffusion of larger molecules is due to their larger molecular volume, though this factor is not as important as the lipophilicity of the drug (Behl *et al.*, 1993:240). According to Cevc (2004:681) molecules larger than a few hundred Dalton (> 500 Dalton) (Naik *et al.*, 2000:319) cannot diffuse through the skin, while small compounds will diffuse relatively quickly and thus permeate the skin the best (Hadgraft & Finnin, 2006:365).

2.4.5.4 PARTITION COEFFICIENT

The partition coefficient is a physical property that measures the ratio of the solubility of the drug in the oil (usually octanol) and in the aqueous phase (Shargel & Yu, 1993:285), and is also known as the octanol-water partition coefficient (log P) (Thomas & Finnin, 2004:699). It is one of the most essential factors to influence the tissue distribution of a drug (Shargel & Yu, 1993:285). Roberts & Walters (1998:13) state that a compound will permeate the skin comparatively fast when that compound has a log P of between 1 and 3, which indicates an ability to dissolve in both water and oil. Compounds with a low log P value show low permeability as they have minute partitioning into the skin lipids, whereas compounds with a high log P also have low permeability due to their lack of ability to partition out of the SC (Thomas & Finnin, 2004:699). The octanol-PBS partition coefficient (Log D) value for calendula oil was undetermined; whereas the octanol-PBS partition coefficient value for L-carnitine L-tartrate was determined to be -1.35. This value does not fall in the ideal range; permeation may not be optimal.

2.4.5.5 DIFFUSION COEFFICIENT (D)

The diffusion coefficient can be defined as the transport of matter due to the movement of a substance within a substrate (Rieger, 1993:38) and it measures the penetration rate of a

molecule under specified conditions (Barry, 2002:512). This parameter is included in Fick's law and is assumed to create a linear concentration gradient of the permeant within the SC (Rieger, 1993:38). The diffusion coefficient of a drug in the skin or a topical vehicle at a constant temperature depends on the properties of the diffusion medium and the drug, as well as the interaction between them (Barry, 2002:512).

2.4.5.6 STATE OF IONIZATION

Ionized drugs show a higher aqueous solubility than the unionized species, therefore the maximum flux through the skin may take place at a pH where ionization is high (Hadgraft & Valenta, 2000:243). Charged compounds should encounter high resistance due to the non-polar nature of the SC (Zatz, 1993:28). It is stated that the permeability coefficient of the unionized form exceeds that of the charged species. This can be attributed to the unionized compound having a greater solubility in the horny layer as well as being poorly soluble in the aqueous donor solvent (Zatz, 1993:29). The pH of the skin may also influence permeation as a pH of 3 provides the skin with a weak positive charge, thus favouring the passage of anions. Above pH 3 the skin is negatively charged, favouring the passage of cations (Amsden & Goosen, 1995:1985).

2.4.6 SKIN'S MATHEMATICS

According to Barry (2002:506) the key hypothesis underlying the mathematical theory for isotropic materials (which have identical structural and diffusional properties in all directions) is that the rate of transfer of diffusing material per unit area of a section is proportional to the concentration gradient measured normal to the section. Fick's laws describe, in part, the process of diffusion and permeation (Rieger, 1993:37) and are relevant whenever the rate of diffusion is controlled by the chemical or physical nature of the skin (Rieger, 1993:38).

2.4.6.1 FICK'S FIRST LAW

Hsieh (1994:11) states that the percutaneous absorption of the majority of drugs is a passive diffusion process and can be described by Fick's first law of diffusion. This is the most basic diffusion equation and describes the steady state flux (J) per unit area (Hadgraft, 2004:292).

$$J - KD(c_{app} - c_{rec})/h \qquad \textbf{(Equation 2.1)}$$

where K is the partition of the permeant between the applied formulation and the skin, D is the diffusion coefficient in the intercellular channels and h the diffusional path length. The applied concentration of the permeant in the vehicle is shown as c_{app}, while the concentration of the permeant in the receptor phase is depicted as c_{rec} (Hadgraft, 2004:292).

This equation can be simplified to **Equation 2.2**, because $c_{rec} << c_{app}$ (Hadgraft, 2004:292).

$J - k_p c_{app}$ **(Equation 2.2)**

where k_p (= KD/*h*) is the permeability coefficient. A compound's flux will be to a maximum when c_{app} is equal to the solubility. These equations show the importance of the physicochemical properties: solubility, diffusion coefficient and partition coefficient (Hadgraft, 2004:292).

2.4.6.2 FICK'S SECOND LAW

In real systems a molecule that enters the SC can move in three directions. The diffusion coefficients in these three directions are assumed to be equal in simplified solutions of Fick's second law. This results in Fick's second law being commonly presented as (Rieger, 1993:42):

$$\frac{dC}{dt} = D\left[\frac{d^2C}{dx^2} + \frac{d^2C}{dy^2} + \frac{d^2C}{dz^2}\right]$$ **(Equation 2.3)**

This law shows that the rate of change of the concentration of a diffusing species in a given volume element of the barrier membrane is proportional to the rate of change in concentration gradient at that point (Rieger, 1993:42). The steady state (zero-order) flux situation described, applies to a unidirectional flow where the concentration in the donor compartment remains constant while the receptor compartment is retained at zero concentration (sink conditions) (Rieger, 1993:42).

2.4.7 PENETRATION ENHANCERS

In this section the major ways of overcoming the impermeable human skin barrier are described. Most compounds are not permitted to permeate through the skin and this factor makes it necessary to enhance the permeation characteristics of the drugs of choice (Behl *et al.*, 1993:248) by making use of permeation enhancers (Büyüktimkin *et al.*, 1997:357).

The properties of an ideal enhancer include the following: (1) it should be tasteless, odourless and colourless; (2) it should be specific in its action; (3) it should be pharmacologically inert; (4) it should be chemically and physically stable; (5) it should be non-allergenic, non-irritant and non-toxic and (6) its action should be reversible, and rapid for a predictable duration of time (Hadgraft *et al.*, 1993:175).

A brief overview of the best known enhancement techniques will be discussed as a complete discussion is beyond of the scope of this dissertation. Chemical or physical approaches can also be used (Behl *et al.*, 1993:248).

2.4.7.1 CHEMICAL

Table 2.5: Chemical penetration enhancers

Penetration Enhancer	Description	Mechanism
Water	Water is the most natural penetration enhancer (Robert & Walker, 1993:1). One of the most eminent factors in determining the rate of penetration absorption of a given compound is the hydration state of the SC (Büyüktimkin *et al.*, 1997:372). The percutaneous flux of a diversity of drugs is enhanced with increased hydration of the SC (Büyüktimkin *et al.*, 1997:372; Zhang *et al.*, 2006:67).	Water opens up the compact structure of the horny layer (Barry, 2006:8). Regardless of the variation in the humidity of the environment, the skin maintains consistent water content of approximately 5 – 15 %. Therefore normal skin forms a partially hydrated tissue, with the SC minimising the transepidermal water loss (TEWL) as well as preventing external ingress (Zhang *et al.*, 2006:67). The balance between water retention and the water loss factors of the tissue, determines the normal physiological water content of the SC (Zhang *et al.*, 2006:68).
Fatty acids	Fatty acids are composed of an aliphatic hydrocarbon chain and a terminal carboxyl group (Babu *et al.*, 2006:144). Fatty acids are used mainly for the penetration enhancement of lipophilic drugs (Babu *et al.*, 2006:138). Fatty acids include saturated fatty acids with a linear hydrocarbon chain (valeric, palmitic, stearic, etc.), saturated fatty acids with a branched hydrocarbon chain (isostearic, neodecanoic, etc.) and unsaturated fatty acids (oleic acid, α-linolenic acid, erucic acid, etc.) (Babu *et al.*, 2006:139).	Fatty acids disrupt the lipid organisation in the SC (Barry, 2001:106), and it is believed to increase the partitioning and diffusivity of drugs across the SC (Babu *et al.*, 2006:138), thereby increasing its permeability (Barry, 2001:106). It was concluded that unsaturated fatty acids showed better permeation enhancement than their corresponding saturated fatty acids (Kanikkannan, 2006:29). A parabolic relationship has been observed between the carbon chain length and skin permeation enhancement (Kanikkannan, 2006:29).

Chemical penetration enhancement involves the enhancement of delivery through the lipid matrix between the corneocytes (Chan, 2005:19). Chemical permeation enhancers consist of two categories: those that alter the partitioning into the SC and those that impact diffusion across the SC. The former class of permeation enhancers works by altering the solubility properties of the skin, thus increasing the solubility of the drug within the SC. The latter class disrupts the ordered nature of the skin lipids, increasing the fluidity and consequently assists permeation of the drug (Thomas & Finnin, 2004:700; Behl *et al.*, 1993:250). This group includes solvents such as alcohols, water, pyrolidones, 1-dodecylazacycloheptan-2-one (Azone) and dimethyl sulfoxide (DMSO). Urea, fatty acids such as oleic acid, sugar esters and surfactants are also classified under this group (Amsden & Goosen, 1995:1981; Møllgaard,

1993:229). The enhancers that may have played a role during this study are discussed in **Table 2.5**, and include fatty acids, which make out a vital part of the Pheroid™ composition as discussed in Section 2.5.

2.4.7.2 PHYSICAL

Physical means of enhancing were not used during this study, although the physical enhancing of drug molecules through the skin comprises microneedles, thermal poration, electroporation, sonophoresis and iontophoresis (Chan, 2005:18).

2.4.7.3 DELIVERY SYSTEMS

One or more features of the following dosage forms (**Table 2.6**) have been incorporated in the design of Pheroid™ (Grobler *et al.*, 2008:287). Pheroid™ will be discussed in detail in Section 2.5.

Table 2.6: Delivery systems incorporated in the design of Pheroid™

Penetration enhancer	Description	Pheroid™
Liposomes	Liposomes are microscopic vesicles and consist of aqueous compartments surrounded by membrane-like lipid layers. Amphiphilic phospholipids with a hydrophilic head and lipophilic tail make out the lipid layers (Mezei, 1994:175). Advantages include their acting as a depot, releasing the entrapped compound slowly and gradually (Mezei, 1994:178). Liposomes can also accommodate both water- and oil-soluble compounds (Mezei, 1994:177). Liposomes work by interacting with the skin (Mezei, 1994:194) in order to enhance the penetration of the drug into the skin (Mezei, 1994:195).	Pheroid™ contains a lipid bilayer, but does not contain phospholipids or cholesterol. In contrast to liposomes, Pheroid™ are formed by a self-assembly process, which is similar to that of low-energy emulsions and micro-emulsions, no lyophilisation or hydration of the lipid components is needed (Grobler *et al.*, 2008:288).

Emulsions and micro-emulsions or nano-emulsions	Emulsions include two types: water-in-oil (W/O) and oil-in-water (O/W), with the last named the predominant type. The oil and water are dispersed through each other and is held together by applying physical energy and chemical emulsifiers with hydrophilic and lipophilic ends (Jackson, 1993:182). Nano-emulsions has a very small droplet size which has some benefits, this includes the system remaining dispersed with no separation, creaming or sedimentation (Tadros *et al.*, 2004:303), and due to the large surface area it allows rapid penetration of active compounds (Tadros *et al.*, 2004:304).	According to Grobler *et al.* (2008:288) Pheroid™ as in emulsions, are distributed within a dispersion medium, but it exists not only of liquid phases, but additionally a dispersed gas phase, associated with the fatty acid dispersed phase.
Polymeric microspheres	Polymeric microspheres are carrier systems in which the drug can be encapsulated by the polymer coat (reservoir devices) or entrapped within the polymer network (matrix devices). The drug can be dispersed or dissolved within the device (Grobler *et al.*, 2008:288). The polymeric microspheres have a diameter in the order of sub-micron to several microns, with the dispersion medium being water in general (Kawaguchi, 2000:1172). It is small in size and volume with a large specific surface area, ability to diffuse and forms a stable dispersion (Kawaguchi, 2000:1173).	Pheroid™ show reservoir characteristics, such as can be seen in the case of polymeric microspheres, due to the specific ratio of pegylated to ethylated fatty acids used (Grobler *et al.*, 2008:289).
Macromolecular microspheres	It can be produced from natural and synthetic polymers. Numerous hydrophilic and lipophilic drugs can be entrapped or incorporated in biodegradable microspheres (Zeng *et al.*, 1995:154). These microspheres are bioadhesive and can be tailored to adhere to any mucosal tissue, and can be used for controlled release, as well as targeted delivery (Vasir *et al.*, 2003:29).	The natural depots formulated in the Pheroid™, are similar to the structure of macromolecular microspheres (Grobler *et al.*, 2008:289).

2.5 PHEROID^{TM} TECHNOLOGY IN AID OF OPTIMAL DELIVERY OF CALENDULA OIL AND L-CARNITINE L-TARTRATE

2.5.1 INTRODUCTION

The patented Pheroid^{TM} delivery system is based on what was previously known as Emzaloid^{TM} technology (Grobler *et al.*, 2008:284). It is comprised mainly of plant and essential fatty acids (Grobler, 2004:4) and is able to enhance the absorption of various categories of drugs (Grobler, 2004:3).

2.5.2 CHARACTERISTICS OF THE PHEROID^{TM} DELIVERY SYSTEM

2.5.2.1 STRUCTURAL CHARACTERISTICS

The Pheroid^{TM} delivery system is a colloidal system that contains unique and stable lipid-based sub-micron- and micron-sized structures, known as Pheroid^{TM}, which are homogeneously distributed in a dispersion medium (Grobler *et al.*, 2008:285). Pheroid^{TM} can be formulated into a wide variety of structures (Grobler *et al.*, 2008:285), and the basic Pheroid^{TM} has a vesicular structure which ranges from 200 – 440 nm in size (Grobler *et al.*, 2008:283). Pheroid^{TM} consists mainly of ethylated and pegylated polyunsaturated fatty acids, which include omega-3 and omega-6 fatty acids, but excluding arachidonic acid (Grobler *et al.*, 2008:285). These fatty acids are compatible with the fatty acids found in humans since they are oriented in the *cis*-formation (Grobler *et al.*, 2008:285) and an affinity exists between the Pheroid^{TM} and the cell membrane, thereby enhancing penetration and delivery (Grobler, 2004:6). Linoleic (LA) and linolenic acid are inherent components of Pheroid^{TM} and are primary constituents in all formulations (Grobler *et al.*, 2008:305). These inherent essential fatty acids were chosen in an effort to develop a skin friendly carrier, as they are essential in the formation of the skin barrier and are obtained from nutritional sources (Grobler *et al.*, 2008:305).

Nitrous oxide (N_2O) forms the dispersed gas phase to the respective oil and water phases, and contributes to the following: (1) miscibility of the fatty acids in the dispersal medium; (2) self-assembly process of the Pheroid^{TM} and the stability of the formed Pheroid^{TM} (Grobler *et al.*, 2008:289). Tocopherol or tocopherol-based molecules (vitamin E or vitamin E derivatives) make out the base of all topical formulations based on Pheroid^{TM} technology. These molecules function as anti-oxidants and emulsion stabilizers (Grobler *et al.*, 2008:293).

The type and diameter of the Pheroid^{TM} can be determined by taking into account the following parameters: (1) rate of delivery; (2) administration route; (3) required capacity dependable on the amount and size of the active compound to be entrapped (Grobler *et al.*, 2008:285).

The structural and functional characteristics of Pheroid™ can be manipulated by:

- Addition of sunscreen formulations.
- Addition of charge-inducing agents, or cryo-protectants.
- Addition of non-fatty acids or phospholipids such as cholesterol.
- Changing the hydration medium (ionic strength, pH) and the fatty acid composition or concentrations.
- Changing the character and concentration of the active compound.
- Changing the method of preparation (Grobler *et al.*, 2008:292).

2.5.2.2 FUNCTIONAL CHARACTERISTICS

The efficacy of the delivery of an active compound to skin includes various processes and depends on the target of delivery (Grobler *et al.*, 2008:293).

2.5.2.2.1 PLIABILITY

Extremely elastic and fluid structures are formed due to the use of a gas and the pliable pegylated tails added to the fatty acids (Grobler *et al.*, 2008:294). The interior volume is stably maintained (Grobler, 2004:7) and Pheroid™ are not shattered under moderate pressure or extravascation (Grobler *et al.*, 2008:294). Pheroid™ vesicles are ideal for quick release, due to the fluidity of the membranes, while maintaining the possibility of sustained release from pro-Pheroid™ depots (Grobler, 2004:7).

2.5.2.2.2 ENTRAPMENT EFFICIENCY (EE)

An efficiency of more than 90 % has been the objective for all products in development. The entrapment efficiency of Pheroid™-based products is commonly determined by CLSM and visualised through fluorescence labelling (Grobler *et al.*, 2008:294). The size, charge and solubility of the active compound determine the number of molecules entrapped within one Pheroid™ (Grobler *et al.*, 2008:294). Pheroid™ is polyphilic and thus drugs which is lipophilic, hydrophilic or even insoluble in nature can be entrapped (Grobler, 2004:7).

2.5.2.2.3 PENETRATION EFFICIENCY

It has continually been confirmed that Pheroid™ rapidly traverses the SC with its entrapped compound (Grobler *et al.*, 2008:297) thereby enhancing the percentage of active compound delivered to the skin (Grobler *et al.*, 2008:296). Efficiency can be determined by comparing the Pheroid™ to an existing commercial product (Grobler *et al.*, 2008:296).

2.5.2.2.4 CELLULAR UPTAKE OF PHEROID™ AND ENTRAPPED COMPOUNDS

The fluidity of the Pheroid™ membrane is thought to be responsible for efficient dermal and transdermal delivery, as was described in Section 2.5.2.2.1. The formulation of the Pheroid™ as well as the mechanism of uptake may influence the uptake of the Pheroid™. Factors that influence the permeation of the Pheroid™ formulation include:

- Size and morphology of the Pheroid™.
- Molecular geometry, concentration and ration of the various fatty acids.
- Hydration medium.
- pH of the preparation.
- Character and concentration of the active compound.
- State of the Pheroid™.
- Presence of charge-changing molecules or molecules that influence the electrostatic milieu (Grobler *et al.*, 2008:299).

There exists an affinity between the Pheroid™ and the cell membrane due to the fact that Pheroid™ consists of fatty acids, which results in enhanced penetration and delivery (Grobler, 2004:6).

2.5.2.2.5 DISTRIBUTION, TARGETING AND METABOLISM

It reduces the volume of distribution, consequently increasing the concentration of the active compound at the target site (Grobler, 2004:7). By using different combinations of fatty acids, the Pheroid™ can to some extent be targeted at sub-cellular level (Grobler, 2004:6). Due to the smaller volume of distribution, an enhanced but narrow therapeutic index can be achieved, whilst there is a decrease in aspecific toxicity (Grobler, 2004:7). Pheroid™ can be metabolised in the mitochondria or the peroxisomes of the cell, depending on their composition. This will result in the release of the active compound (Grobler *et al.*, 2008:300). Pheroid™ are known to protect drugs from metabolism (Grobler, 2004:7).

2.5.3 THERAPEUTIC ENHANCEMENT OF CALENDULA OIL

The goal of this study was to determine whether calendula oil will diffuse through the skin or whether it will be contained within the skin layers and what the possible beneficial effect there would be when it is entrapped in the Pheroid™ delivery system. Earlier studies conducted showed that entrapment in the Pheroid™ enhanced the effect of essential oils on the skin (Grobler *et al.*, 2008:300). In order to examine the enhancement of 0.1 % (v/v) *Calendula officinalis* (marigold) oil added to the Pheroid™ a proof of concept study was performed (Grobler

et al., 2008:300). It was determined by surface evaluation of the skin (Visioscan VC 98), as well as by measuring the hydration state of the skin with a Corneometer CM 825 on a healthy volunteer with pronounced skin sensitivity as well as a dry skin (Grobler *et al.*, 2008:300).

The hydration state of the skin, scaliness and surface wrinkles on the forehead and cheek of the volunteer were the parameters ascertained. The Pheroid™ itself showed a considerable hydrating effect and a definitive decrease in wrinkles was observed on the forehead, whilst the cheek showed no change in wrinkles (Grobler *et al.*, 2008:301). It was concluded in terms of the parameters investigated that the Pheroid™-entrapped essential oil showed a rapid response and therapeutic value (Grobler *et al.*, 2008:301).

2.6 SUMMARY

Aging of the human skin is a very complex process and anti-aging treatment and/or prevention has become a very vital part of the cosmeceutical market. Aging can be divided into two components: intrinsic and extrinsic or photoaging aging. Intrinsic aging is determined by a person's genetics; whereas extrinsic aging is due to environmental factors, such as UV exposure. The process of intrinsic aging is far less well understood than extrinsic aging. These two components overlap during a person's lifetime and there are readily distinct differences between them on the macromolecular level, although recently it has been found that they share important molecular features, which include pro-collagen breakdown, generation of ROS and the promotion of MMP enzymes.

Two cosmeceutical actives, calendula oil and L-carnitine L-tartrate, were chosen due to their beneficial effects on aging skin. Calendula oil functions as an anti-oxidant and is responsible for scavenging radicals associated with skin aging. It also shows anti-inflammatory, re-epithelising and moisturising effects, which can be advantageous as an anti-aging regime. Calendula is insoluble in methanol, ethanol and water due to its high lipophilicity. It is speculated that this natural oil will not permeate the skin, but it is hoped that it would possibly concentrate in the epidermis and dermis, which are the target site for anti-aging treatment.

Carnitine increases fibroblast proliferation and shows excellent exfoliating properties to give the skin a youthful appearance. L-carnitine L-tartrate shows excellent solubility in PBS at a pH of 7.41 (16.63 mg/ml), which makes it favourable for drug bioavailability. Unfortunately the Log D (-1.35) value indicates that the compound has a high affinity for the water phase, which will cause minute partitioning into the skin lipids.

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PHEROID™ TECHNOLOGY FOR THE TOPICAL APPLICATION OF SELECTED COSMECEUTICAL ACTIVES

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Abstract

There are two types of skin aging (intrinsic and extrinsic) which share important molecular features, while having distinct differences on the macromolecular level. Both lead to increased production of reactive oxygen species (ROS), enhanced matrix metalloproteinase expression (MMP) and decreased procollagen synthesis. The cosmeceutical actives, calendula oil and L-carnitine L-tartrate was employed in this study due to their anti-aging effects. The Pheroid™ delivery system was tested against phosphate buffered solution (PBS), in order to determine its enhancement efficacy and the topical delivery of the cosmeceutical actives to the target sites, i.e. epidermis and dermis. Entrapment of L-carnitine L-tartrate within the Pheroid™ delivery system was confirmed with confocal laser scanning microscopy (CLSM); while entrapment could not be established for calendula oil. Calendula oil samples were methylated with boron trifluoride (BF_3) - methanol (MeOH) reagent and qualitatively analysed by gas chromatography mass spectrometry (GC/MS). It is inconclusive whether Pheroid™ enhanced its delivery. L-carnitine L-tartrate was analysed by liquid chromatography mass spectrometry (LC/MS) and it was found that Pheroid™ enhanced the permeation of this active, although the PBS showed a higher concentration of the active in the target sites. The presence of carnitine in human skin was also investigated.

Keywords: Calendula oil, L-carnitine L-tartrate, Pheroid™, Skin aging, Topical delivery

1 Introduction

Aging is extraordinarily complex and is inevitable for every living organism. This phenomenon is an irreversible process which begins and accelerates at maturity, and it results in an increasing number of deviations from the ideal state and a decreasing rate of return to the ideal state (Gilchrest, 1984). Interest on the impact of aging on the function and appearance of the skin is growing due to an increasing life expectancy in developed countries (Rittié and Fisher, 2002). According to Jenkins (2002) cutaneous aging is a very complex biological phenomenon which consists of two components: intrinsic and extrinsic aging. Intrinsic aging is largely determined by a person's genetics, while extrinsic or photoaging is caused by environmental exposure, predominantly UV light.

According to Billek (2002) both intrinsic and extrinsic aging overlap during a person's lifetime and both are responsible for the dysfunction of the skin's natural repair and self-protection. Jenkins (2002) concluded that there are readily distinct differences between intrinsically and photoaged skin on the macromolecular level, although recent evidence indicates that they share important molecular features (Rittié and Fisher, 2002). This includes decreased procollagen synthesis, connective tissue damage and changed signal transduction pathways that promote matrix-metalloproteinase (MMP) expression to give rise to elevated levels of these enzymes (Varani et al., 2000; Rittié and Fisher, 2002;). Both types of aging also cause increased production of reactive oxygen species (ROS) which leads to the impaired function of the skin (Rittié and Fisher, 2002).

During photoaging UVB is almost completely absorbed in the top 0.1 mm of the skin and it thus induces epidermal damage, while UVA penetrates through the skin into the deeper layers (dermis) (Pinnell and Madey, 1998; Vioux-Chagnoleau et al., 2006). The composition, modification and turnover of the skin's extracellular matrix (ECM) components are affected by the age-associated alterations (Szauter et al., 2005). The integrity of the ECM determines the cell proliferation, differentiation, migration and apoptosis in the dermis and epidermis (keratinocytes); these age-related matrix changes are likely determinants of alterations in dermal functions (Norsgaard, 1996, Szauter et al., 2005; Berge et al., 2008). The target site for

anti-aging treatment can thus be considered as the epidermis and dermis layers of the skin (topical delivery).

Two cosmeceutical actives, calendula oil (marigold) and L-carnitine L-tartrate, were employed during this study due to their anti-aging effects. The term "cosmeceutical" is attributed to Dr. Albert Kligman and can be regarded as a hybrid category of products lying on the spectrum between drugs and cosmetics (Choi and Berson, 2006). Calendula extracts contain the following ingredients: sugars, carotenoids, saponins, sterols, phenolic acids, flavonoids, sterins, resins, quinones, mucilages, polyprenylquinones, vitamins and essential oils (Fïume, 2001). According to Ćetković et al. (2004) marigold extracts scavenges hydroxyl, peroxyl and DPPH (diphenylpicrylhydrazyl) radicals in a concentration dependent manner which correlates with the concentration of the total phenolic compounds and flavonoids (Ćetković et al., 2004). This anti-oxidant activity can prove valuable during anti-aging treatment of the skin.

L-carnitine L-tartrate was employed in this study as it is the more stable salt form of L-carnitine. Carnitine serves as an essential cofactor for long-chain fatty acid oxidation in the mitochondria (Tein et al., 1996; Lamhonwah et al., 2005). It is responsible for providing the cells with a critical source of free coenzyme A (CoA) by playing a role in the maintenance of the homeostasis in the acyl-CoA pools of the cell (Lamhonwah and Tein, 2005).

Additionally carnitine shows promising effects on the skin when applied topically. Arslan et al. (2003) used the distally-burned dorsal skin flap model/experiment on rats to demonstrate the positive effect that carnitine has in ischemia and burn injuries. During this study they saw that carnitine increases the cellularity (including mixed inflammatory cells and fibroblast proliferation) (Arslan et al., 2003) as well as the vascularity in the burnt tissues (Arslan et al., 2003). They also found that the necrosis was more limited than in the study group which were untreated with carnitine (Arslan et al., 2003). At a lower pH, L-carnitine (ß-hydroxyacid) shows excellent skin exfoliating properties. These properties are responsible for the acceleration in epidermal turnover rate in the skin (Held, 2004). The removal of the dead skin cells; together with the increasing rate of skin turnover; will result in younger, healthier-looking skin (Held, 2004). Even small amounts of L-carnitine will leave the skin soft, smooth and moisturised because of its

hygroscopic nature (Held, 2004). The promising effects of fibroblast proliferation and carnitine's ability to moisturise the skin can prove valuable in improvement of aging skin.

The patented Pheroid™ delivery system, formerly known as Emzaloid™, was employed as a carrier system for the selected cosmeceutical actives in order to determine whether it will enhance their penetration to the target site. Pheroid™ is a colloidal system that contains remarkable and stable lipid-based submicron- and micron-sized structures, called Pheroid™, which are equivalently distributed in a dispersion medium (Grobler et al., 2008). It contains the essential fatty acids linoleic and linolenic acid, as well as oleic acid emulsified in water saturated with nitrous oxide (Saunders et al., 1999). These fatty acids are oriented in the *cis*-formation, which makes it compatible with the fatty acids found in man, making Pheroid™ a skin friendly carrier system (Grobler et al., 2008).

Previous studies were performed wherein it was found that the effect of essential oils on the skin is enhanced by entrapment in Pheroid™ (Grobler et al., 2008). A proof of concept study was performed in order to investigate the enhancement of 0.1 % (v/v) *Calendula officinalis* (marigold) oil added to the Pheroid™ (Grobler et al., 2008). Parameters determined during this study were the hydration state of the skin, scaliness and surface wrinkles on the cheek and forehead of the volunteer. They determined that the Pheroid™ itself showed a significant hydrating effect. There was a definitive decrease in wrinkles observed on the forehead, whereas the cheek showed no change in wrinkles (Grobler et al., 2008). It was concluded that the Pheroid™-entrapped essential oil showed a fast response and therapeutic efficacy in terms of the parameters investigated (Grobler et al., 2008).

2 Materials and methods

2.1 Materials

The cosmeceutical actives calendula oil and L-carnitine L-tartrate were obtained from Sharon Bolel Chemical Marketing and Brunel Manufacturing, respectively. Potassium dihydrogen phosphate (KH_2PO_4) and sodium hydroxide (NaOH) used for the preparation of phosphate buffered solution (PBS) were supplied by Merck Laboratory Supplies (Midrand, South Africa). Reagents used during the gas chromatography mass spectrometry (GC/MS) method include: boron trifluoride-methanol complex (BF_3, 1.3 M in methanol), dried methanol (MeOH), sodium sulphate anhydrous (Na_2SO_4) and isooctane obtained from Merck Laboratory Supplies (Midrand, South Africa), while the sodium chloride (NaCl) were from Labchem (Edenvale, South Africa) and the nitrogen gas (N_2) and helium gas (He) were obtained from Afrox (Potchefstroom). Toluene and chloroform was obtained from VWR International (England). Formic acid (CH_2O_2) supplied by Saarchem (E. Merck, Johannesburg, South Africa) and acetonitrile (CH_3CN) from Merck Laboratory Supplies (Midrand, South Africa) were used in the mobile phase of the liquid chromatography mass spectrometry (LC/MS) analysis. Water used during this study was purified by a Milli-Q Academic purification system (Millipore, Milford, USA). Pheroid™ was prepared by the Unit of Drug Research and Development, North West University.

2.2 Methods

2.2.1 Skin preparation

Excised human skin from Caucasian female patients who underwent cosmetic abdominoplastic surgery was used (Leveque et al., 2004). Ethical approval for the procurement and exploitation of the skin was provided by the Research Ethics Committee of the North-West University under reference number 04D08. In order to ensure anonymity, the identities of the patients were masked and informed consent was obtained beforehand. The skin was frozen at -20 °C within 24 h after removal. Full thickness membranes were utilised during this study and it contained all the skin layers (stratum corneum, nucleate epidermis and dermis) (Williams, 2003). A scalpel was used to separate the skin from the adipose and connective tissue. Care was taken to ensure that the skin was not damaged or ruptured in any way as this could lead to erroneous results. The skin was subsequently punched into circles with a diameter of approximately ±

15 mm. The skin circles were then placed in a bath filled with high pressure liquid chromatography (HPLC) water, and positioned onto Whatman® filter paper with the stratum corneum side facing upwards and was consequently left to air dry. The skin circles were then placed in aluminium foil sheets and kept frozen at -20 °C until used. The frozen skin circles were thawed at room temperature prior to the diffusion study and were examined for any defects before it was mounted onto the diffusion apparatus.

2.2.2 Entrapment of calendula oil and L-carnitine L-tartrate in Pheroid™

Entrapment of L-carnitine L-tartrate in Pheroid™ was viewed with the aid of confocal laser scanning microscopy (CLSM). Entrapment of calendula oil could not be established as it was not capable to differentiate between the Pheroid™ and the calendula oil. The phospholipid components of the Pheroid™ were stained with phenoxazine dye nile red (Haugland, 2005). The sample was then placed on a glass slide and covered with a glass cover-slip and sealed with an adhesive to prevent the Pheroid™ from drying out. The CLSM used to capture the images was a Nikon PCM 2000 confocal laser scanning microscope, with a DXM 1200 digital camera for real-time imaging and ApoPlanar oil immersion objective with numerical aperture (NA) of 1.4. The microscope was equipped with a red helium/neon laser (excitation wavelength of 505 nm and emission wavelength of 515 nm) and green argon laser (excitation wavelength of 488 nm and emission wavelength of 515 nm).

2.2.3 Preparation of donor solutions

The donor solutions applied included calendula oil and L-carnitine L-tartrate. Calendula oil was either applied as is or encapsulated 50:50 in Pheroid™. The L-carnitine L-tartrate was dissolved in PBS buffer (pH 7.4) and in Pheroid™ both at a concentration of 2 % as was previously tested by Lonza, where it showed significant hydrating effects on the skin (Held, 2004). All the solutions were stirred in a water bath at 32 °C for 24 h in order for solvent saturation to take place. Pheroid™ encapsulation commenced as was previously described. PBS buffer (pH 7.4) was prepared according to the BP standards.

2.2.4 Preparation of reagents for fatty acid esterification

Alcoholic NaOH was prepared by dissolving 2.0 g NaOH in dry MeOH and diluting it further to a volume of 100 ml to give a 0.5 M solution. A saturated solution of NaCl was prepared by

dissolving 36 g NaCl in 70 ml of HPLC grade water, heating it to approximately 60 °C and letting it cool to about room temperature. It was then made up to a volume of 100 ml with HPLC grade water. Reagents were prepared fresh every day.

2.2.5 Franz cell diffusion method

Vertical Franz diffusion cells with a receptor capacity of approximately 2 ml and a diffusion area of 1.075 cm^2 were used for the *in vitro* permeation studies. These diffusion cells consist of a donor (top) and a receptor (bottom) compartment where the drug solution and the receptor fluid (PBS, pH 7.4) were placed, respectively. A small magnetic stirring bar was placed in each receptor compartment in order to maintain stirring throughout the experiment. The full-thickness skin was thawed and mounted between the receptor and donor compartments with the stratum corneum (SC) facing upwards. Dow Corning high vacuum grease was applied to each Franz Cell in order to prevent any leakage. A horseshoe clamp was then used to clamp the donor and receptor compartments together. The donor compartments were filled with 1 ml of the donor solution and subsequently covered with Parafilm® to prevent evaporation for the duration of the experiment. The receptor compartments were filled with PBS (pH 7.4) with care to prevent air bubbles under the skin. In order to control temperature the receptor compartments were placed directly in a 37 °C water bath in order to attain a skin temperature of 32 °C (Cleary, 1993). Diffusion studies performed with calendula oil were left for 8 h to allow permeation to take place after which the receptor compartments were withdrawn completely and tape stripping was performed as described later on. Samples obtained from the receptor compartment were extracted by the single-phase chloroform:MeOH:water extraction system as described later on and all samples obtained (lipid extracted and tape stripping) were treated with anhydrous Na_2SO_4 to ensure no water was present. Samples were then methylated by BF_3 in MeOH and analysed on the GC/MS. For the L-carnitine L-tartrate dissolved in PBS (pH 7.4) diffusion study the entire content of the receptor phases were withdrawn and replaced with fresh PBS (pH 7.4) at a temperature of 37 °C after 2, 4, 6, 8 and 10 h. The receptor compartment during the L-carnitine L-tartrate encapsulated in Pheroid™ diffusion study was withdrawn and replaced at 20 min, 40 min, 80 min, 2, 4, 6 and 8 h. It was suspected that the Pheroid™ delivery system would promote the permeation of the L-carnitine L-tartrate, and thus the withdrawal times were

started and ended earlier. This also ensured that the preferred amount of at least 5 data points would be achieved. Tape stripping was performed as described and all samples directly underwent LC/MS analysis. A control diffusion study with PBS (pH 7.4) and Pheroid™ containing no active (L-carnitine L-tartrate) in the donor phase was performed wherein the receptor compartments were only withdrawn after a period of 8 h. The control study consisted of 15 Franz cells with 8 cells containing PBS (pH 7.4) and 7 cells containing Pheroid™ vesicles in the donor compartments. This control diffusion study was performed when it was discovered that carnitine may possibly be found in the skin naturally. The amount of carnitine found in the skin was determined with LC/MS analysis; whereafter a correction factor was determined.

2.2.6 Tape stripping method

Tape stripping is a technique used to remove the outermost layer of the skin, the stratum corneum as well as the epidermis, and it has been found very useful in dermatopharmaceutical research (Purdon et al., 2006). This technique removes the corneocyte aggregates of the stratum corneum in a stepwise approach (Purdon et al., 2006). The tape stripping method was performed as previously described by Pellet et al. (1997). After completion of the diffusion study and the removal of the donor and receptor phases the diffusion cells were carefully taken apart. The piece of skin was pinned on a piece of Parafilm®, stapled to a solid surface. The exposed diffusional area (≈1.075 cm^2) is clearly marked by the indentation from the diffusion cells (≈5 mm diameter). The skin was then dabbed dry with tissue, and pieces of tape (3M Scotch® Magic™ Tape) were then cut to cover the diffusional area, making sure it did not overlap the areas outside of the diffusion cell imprints. The first tape strip was discarded, as it is regarded as part of the cleaning procedure and may be contaminated with the drug formulation. The following 15 tape strips (referred to as the epidermis) were placed in a vial. All the tape strips were placed in an appropriate amount of PBS (pH 7.4) or toluene for L-carnitine L-tartrate and calendula oil, respectively. These were kept overnight at 4 °C. An indication of the complete removal of the stratum corneum is when the viable epidermal layer glistens. The excess skin was cut away from the flange imprints of the diffusion cells, and the remaining skin (dermis) was cut into pieces to enlarge surface area. It was then placed in the appropriate amounts of PBS (pH 7.4) or toluene for L-carnitine L-tartrate and calendula oil, respectively and were kept

overnight at 4 °C. Samples were filtered, prepared where necessary and assayed by GC/MS (calendula oil) or LC/MS (L-carnitine L-tartrate).

2.2.7 Lipid extraction

All the receptor compartment samples of the calendula oil diffusion studies underwent lipid extraction. A method for lipid extraction was developed by the Biochemistry Department of the North-West University in Potchefstroom. This procedure was based on the single-phase chloroform:MeOH:water extraction system of Bligh and Dryer (1959) as modified by White et al. (1979). The water phase in this case was the samples obtained from the receptor compartments. Each sample underwent the following procedure: 2.5 ml chloroform, 5 ml MeOH, 1 ml HPLC grade water and 1 ml sample were added together, vortexed for 30 seconds and left to stand for 2 h to equilibrate. Chloroform (2.5 ml) and HPLC grade water (2.5 ml) was then added, vortexed and left to stand for 30 min in order for the phases to separate. Pasteur pipettes were used to transfer the bottom phase into a tube. The bottom phase then underwent methylation by BF_3 and MeOH and was analysed by GC/MS.

2.2.8 Methylation with boron trifluoride (BF_3) in methanol (MeOH)

In order to determine the complete fatty acid composition of a lipid (calendula oil) with GC/MS analysis, the fatty acid components must first be converted to the simplest convenient volatile derivative, in this case methyl esters (Christie, 1993). BF_3 is a Lewis acid and serves as a powerful acidic catalyst for the esterification of fatty acids when it is in the form of its coordination complex with MeOH (Christie, 1993). The initial step in the mechanism of action (Figure 1) involves the protonation of the acid to give an oxonium ion (1). This ion can undergo an exchange reaction with MeOH to give an intermediate (2) which in turn is able to lose a proton in order to become an ester (3) (Christie, 1993).

Figure 1: Acid-catalysed transesterification of lipids (Christie, 1993).

Methylation with BF_3 was performed according to Horwitz and Latimer (2006). Tape stripping samples as well as the lipid extracted samples were all vortexed with a small aliquot of anhydrous Na_2SO_4. This ensured that no water was present during the methylation reaction, as water is a stronger electron donor than the MeOH, and will not promote the formation of the

intermediate in the reaction; consequently esterification will not proceed fully (Christie, 1993). Table 1 illustrates the different sample amounts, as well as the different amounts of reagents used. These differences can be attributed to some sample volumes being too small as well as a lack of the necessary reagents.

Table 1: Amounts of samples and reagents used during methylation with BF_3 in MeOH.

An amount of methanolic NaOH solution was added to the sample volumes, vortexed and the air replaced with nitrogen. Nitrogen was used throughout, due to the air being responsible for the quick oxidation of the oil. Samples were then incubated on a heating block at 100 °C for 5 min and afterwards cooled to about room temperature. BF_3 reagent was added; it was blanketed with nitrogen and incubated further at 100 ± 2 °C for 30 ± 2 min. It was then allowed to cool to approximately 30 – 40 °C. Isooctane was added and it was blanketed with nitrogen and shaken vigorously for 30 seconds. Saturated NaCl solution was immediately added and it was blanketed again with nitrogen and agitated *via* vortex for roughly 45 seconds. Samples were then cooled to room temperature. After the isooctane layer has separated from the aqueous layer, it was removed to a clean tube, blanketed with nitrogen and capped. The aqueous phase was extracted once more with isooctane, vortexed for roughly 45 seconds and when the layers separated it was combined with the other extract. The methylated samples were then placed in vials and assayed by GC/MS.

2.2.9 Gas chromatography mass spectrometry (GC/MS) analysis of the calendula oil

A qualitative GC/MS method was utilised during this study. Methylated samples (calendula oil) were analysed by GC/MS using a Shimadzu QP2010 single quadrupole instrument with positive electron ionization. A 30 m length x 0.32 mm and 0.5 µm film thickness Zebron ZB-Wax column (Phenomenex, Torrance, CA) was used for all analyses. The marker compound used to determine the presence of calendula oil was linoleic acid. Table 2 represents the chromatographic conditions and the mass spectrometer settings.

Table 2: Chromatographic conditions and mass spectrometer settings.

2.2.10 Liquid chromatography mass spectrometry (LC/MS) analysis of L-carnitine L-tartrate

A LC/MS method was developed and validated for L-carnitine L-tartrate by using an Agilent 1100 series HPLC with binary gradient pump, autosampler and vacuum degasser coupled to an Applied Biosystems API 2000 triple quadrupole mass spectrometer and Analyst 1.4.2 data acquisition and analysis software. The column liquid chromatography included a Synergi Max-RP C12 column (150 x 2 mm, 4 µm particle size, Phenomenex, Torrance, CA). The mobile phase consisted of 10 volumes of CH_3CN containing 0.1 % CH_2O_2 and 90 volumes of distilled water containing 0.1 % CH_2O_2 with a flow rate of 250 µl/min isocratic. The total run time was 5 min. The peak eluted at approximately 1.12 min. Mass spectrometry was performed on a mass spectrometer with a turbo electro-spray ion source and was used in positive ionization mode. The mass spectrometer included the following source settings. The turbo ion-spray interface was maintained at 350 °C with the curtain gas at a rate of 10 ml/min. Ion-spray voltage was 5500 V; declustering potential was 26 V; entrance potential was 10 V and the focusing potential was 370 V. The multiple reaction monitoring (MRM) pair monitored was m/z 162.158/58.1 and 162.158/43.1 with a 150 millisecond dwell time.

2.2.11 Determination of aqueous solubility and log D values of L-carnitine L-tartrate

The aqueous solubility of L-carnitine L-tartrate was determined by preparing saturated solutions in PBS buffer with a pH of 7.4. The solutions were stirred with magnetic bars in a 32 °C water bath for 24 h. An excess amount of L-carnitine L-tartrate was inserted in order to keep the solution saturated at all times. The solutions were then filtered, diluted and analysed by LC/MS. This experiment was performed in triplicate.

The experimental *n*-octanol-PBS partition coefficient (log D) was performed as follows: pre-saturated *n*-octanol and PBS buffer (pH 7.4) were prepared by vigorously stirring equal amounts of each for 24 h after which the two layers were separated. L-carnitine L-tartrate (30 mg) was dissolved in 3 ml pre-saturated *n*-octanol (10 mg/ml) and was shaken for 10 min. Pre-saturated PBS (3 ml) was placed into the L-carnitine L-tartrate pre-saturated *n*-octanol and was agitated for 45 min and then centrifuged at 4000 rpm for 30 min. The aqueous phase was analysed as is, while the *n*-octanol phase was diluted with MeOH. The two phases were analysed

separately by LC/MS. The logarithmic ratio of the concentration in the *n*-octanol phase to the concentration in the PBS was used to calculate the log D.

2.2.12 Transdermal and statistical data analysis

The GC/MS method employed for the detection of the calendula oil was qualitative; therefore it was only observed whether or not the calendula oil was present in the samples. A response factor was determined with which estimated average values could be determined.

In the case of L-carnitine L-tartrate the average cumulative amount per area was plotted against time. The average (mean) flux was obtained from the slope of the linear portion of the curve. The Pheroid^{TM} plot showed a biphasic character with a steady-state flux between 40 min and 2 h, and then again between 2 and 8 h. Statistical analysis of the data obtained from L-carnitine L-tartrate involved the examination of the mean and median flux values. The mean flux values were visualised by a box-plot. The bootstrap method (a computer simulation statistical technique) was used to get an estimate of the sampling distribution of the flux values and to get a 95 % confidence interval for the true flux value. The tape stripping data was analysed using a two-way ANOVA, the nonparametric Mann-Whitney U test and box-plots.

3 Results and discussion

3.1 Entrapment of L-carnitine L-tartrate in Pheroid™ vesicles

Figure 2 illustrates the CLSM micrographs of the vesicular structures of the Pheroid™ delivery system and the entrapment of the L-carnitine L-tartrate inside the lipid structures. The Pheroid™ was stained with nile red; whereas the L-carnitine L-tartrate was auto-fluorescing. Micrograph **(a)** is the placebo Pheroid™ vesicles containing no L-carnitine L-tartrate. Micrographs **(b)** to **(d)** portray the same image of the entrapped L-carnitine L-tartrate (2 %) within the lipid structure of the Pheroid™. Micrograph **(b)** is the actual image and illustrates the yellowish L-carnitine L-tartrate present within the red spherical Pheroid™. Micrograph **(c)** illustrates the L-carnitine L-tartrate as green 'spots'. Micrograph **(d)** represents the Pheroid™ with omission of the L-carnitine L-tartrate molecules. The entrapment of the L-carnitine L-tartrate within the Pheroid™ can be confirmed by micrographs **(b)** to **(d)**.

Figure 2: CLSM micrographs of L-carnitine L-tartrate and the Pheroid™ delivery system. Micrograph **(a)** is the control, **(b)** is the L-carnitine L-tartrate entrapped within the Pheroid™, **(c)** is the L-carnitine L-tartrate and **(d)** is the Pheroid™.

3.2 Aqueous solubility and log D values of L-carnitine L-tartrate

The aqueous solubility for a drug to ideally permeate the skin is more than 1 mg/ml (Naik et al., 2000), whereas a log P of between 1 and 3 indicates an ability to dissolve both in water and oil, ensuring that the compound would permeate the skin comparatively fast (Roberts & Walters, 1998). The solubility of L-carnitine L-tartrate was determined to be 16.63 mg/ml in PBS (pH 7.41) at a temperature of 32 °C. The log D for L-carnitine L-tartrate was determined to be -1.35. When looking at the solubility of L-carnitine L-tartrate it was expected that it will permeate through the skin, in contrast to the log D value which indicates that permeation may not be optimal.

3.3 Calendula oil

3.3.1 *In vitro* permeation study

As was anticipated, results indicated that no linoleic acid was found in any of the receptor compartments of the Franz cells (PBS and Pheroid™). The calendula oil is a highly lipophilic substance and it was found to be insoluble in ethanol as well as in MeOH. It was only soluble in other organic solvents such as toluene. The lipophilic nature of this oil can explain its restricted penetration into the receptor compartment. Lipophilic drugs tend to partition into the skin lipids, but show difficulty in crossing the aqueous headgroup regions of the skin lipids and also to partition out of the stratum corneum into the viable epidermis (Hadgraft & Finnin, 2006). The viable epidermis (200 µm thick) lies directly underneath the stratum corneum, and is metabolically active and exceedingly aqueous in nature (Hadgraft & Finnin, 2006), in contrast to the high lipophilicity of the calendula oil.

3.3.2 Tape stripping

The analytical method (GC/MS) for determining the presence of calendula oil (linoleic acid) in the tape stripping samples could only be performed on a qualitative basis. A response factor was determined with which estimated average values could be determined wherein 1 µg/ml is equal to a peak area of 81691.78. From this response factor it was determined that when the calendula oil was applied as is, the dermis contained more linoleic acid (3246.7 µg/5ml) than the epidermis (339.07 µg/5ml). Of the 11 Franz cells, 8 epidermis samples and 11 dermis samples showed the presence of linoleic acid. In the case of Pheroid™ it was found that the epidermis and dermis contained 592.95 µg/5ml and 3018.2 µg/2ml, respectively. Linoleic acid was detected in both the epidermis as well as the dermis in 11 out of 11 Franz cells used, of which one Franz cell was a control. In both the epidermis and dermis the peak area of the control Franz cells was smaller than the other peak areas where calendula oil was present. In regards to the response factor it was determined that the epidermis and dermis contained 196.01 µg/5ml and 1214.32 µg/2 ml in the case of the control cells. These results cannot effectively be compared to each other due to the different amount of sample used before methylation with BF_3 in MeOH, as well as the difference in the amount of toluene used to 'extract' the calendula oil from the epidermis and dermis. Another factor that influenced the results was the presence of

the marker, linoleic acid, in the structure of the Pheroid™ delivery system. It is thus inconclusive whether it was in fact the calendula oil or the Pheroid™ that was detected, and it is uncertain whether the Pheroid™ enhanced the oil's penetration.

3.4 L-carnitine L-tartrate

3.4.1 Control study

After preliminary studies with L-carnitine L-tartrate it was suspected that carnitine may reside in the skin naturally; therefore a control diffusion study without L-carnitine L-tartrate was performed. The skin samples employed were all from the same donor. All the receptor compartments were filled with PBS (pH 7.4). The average carnitine detected in the receptor compartments after a period of 8 h was found to be 0.343 $\mu g/cm^2$ and 0.441 $\mu g/cm^2$ for PBS and Pheroid™, respectively. After 8 h tape stripping was performed as described. The epidermis in both the PBS and Pheroid™ presented with an omissible small amount of carnitine which could not be quantified. The dermis contained 0.035 µg/ml and 0.086 µg/ml for the PBS and Pheroid™, respectively. These results were employed as a correction factor during diffusion studies and tape-stripping performed with L-carnitine L-tartrate.

The presence of carnitine in the skin can be hypothesised by looking at the layers of the epidermis. The epidermis consists of four layers namely the stratum corneum (horny layer), stratum granulosum (granular layer), stratum spinosum (spiny layer) and the stratum basale (basal layer) (Rhein et al., 2000). Each of these layers represents a progressive stage in the life cycle of a keratinocyte. As the newly formed keratinocyte moves upward it will flatten, lose its nucleus and die, after which it will produce keratin which fills the stratum corneum (Rhein et al., 2000); thus the stratum corneum can be termed 'dead' on morphological grounds (Black, 1993). According to Selby (1955) the basal cell cytoplasm contains mitochondria and therefore it can be hypothesised that carnitine (present in all living cells) may also be synthesised here as it is normally synthesised in the mitochondria of the heart, kidney, liver, muscle and brain (Steiber et al., 2004). The omissible amount of carnitine found in the epidermis can be due to the epidermis being in part alive and in part dead (stratum corneum). The dermis contains a quantifiable amount of carnitine due to the living cells present.

3.4.2 *In vitro* permeation study

The *in vitro* permeation of L-carnitine L-tartrate with the aid of the Pheroid™ delivery system was investigated and compared against L-carnitine L-tartrate dissolved in PBS buffer (pH 7.4). Figures 3 and 4 illustrate the average cumulative amount per area against time for PBS (pH 7.4) and Pheroid™, respectively.

Figure 3: Average cumulative amount of L-carnitine L-tartrate dissolved in PBS (pH 7.4) that penetrated the skin as a function of time illustrating the average flux.

Figure 4: Average cumulative amount of L-carnitine L-tartrate encapsulated in Pheroid™ that penetrated the skin as a function of time illustrating the average flux.

The average (mean) flux is represented by the slope of the linear portion of the curve. L-carnitine L-tartrate demonstrated a biphasic flux pattern when encapsulated in Pheroid™. The average flux obtained for L-carnitine L-tartrate in PBS and Pheroid™ for the time interval of 2 and 8 h were as follows: 0.018 $\mu g/cm^2.h$ and 0.0361 $\mu g/cm^2.h$, respectively. Between 40 min and 2 h Pheroid™ showed a flux of 0.009 $\mu g/cm^2.h$. When comparing the flux between 2 and 8 h it can be seen that the flux obtained when L-carnitine L-tartrate was encapsulated in Pheroid™ was twice as high as when it was dissolved in PBS (pH 7.4).

The flux values determined in the different Franz cells are influenced by skewed distributions around the central location; therefore the mean flux could give an erroneous estimation of the true flux value. Calculation of the median flux would give a better representation of the true flux value for L-carnitine L-tartrate dissolved in PBS (pH 7.4) or encapsulated in Pheroid™ as it is not affected by a distortion in the spread of the data (Gerber et al., 2008). Figure 5 depicts a box-plot which is a quick way of examining one or more sets of data graphically.

Figure 5: Box-plots depicting the flux of L-carnitine L-tartrate dissolved in PBS (pH 7.4) (left) and encapsulated in Pheroid™ (right).

The median flux is the 50th percentile or the centre of all the flux data and was found to be higher for Pheroid™ (0.0393 $\mu g/cm^2.h$) than for PBS (0.0142 $\mu g/cm^2.h$) between the time period

of 2 and 8 h. It can be seen from Figure 5 that the distribution for PBS is more symmetrical than for Pheroid™ which is distributed more to the left side. From the box-plot it is clear that Pheroid™ enhances the permeation of L-carnitine L-tartrate more than when it is dissolved in PBS.

An alternative approach to calculate the flux value would be to make use of a model for time versus concentration (Figure 6). Hence, a simple least squares straight line regression fit is performed with time as independent variable and concentration as dependent variable. The slope represents the flux value and was found higher for Pheroid™ (0.03612142 $\mu g/cm^2.h$) than for PBS (0.0180011 $\mu g/cm^2.h$). These flux profiles should be and are similar to the values found when the average cumulative amount per area was plotted against time.

Figure 6: Linear plot illustrating the average flux values for L-carnitine L-tartrate in both PBS and Pheroid™.

The data were further analysed using a statistical resampling method, known as bootstrapping (Efron and Tibshirani, 1993). This approach involves resampling points from one's own data, with replacement, so as to create a series of bootstrap samples of the same size as the original data (Felsenstein, 1985). The result is a large number of datasets that might have been observed and can be used to estimate the distribution of a statistic, like the flux value. Confidence limits exist of two numbers to form an interval between which the value of the parameter (flux when L-carnitine L-tartrate is dissolved in PBS or when encapsulated in Pheroid™) is likely to fall in. Using the bootstrap, the confidence limits on a statistic can be constructed by the percentile method. For example, a 95 % confidence interval involves taking the 2.5 % and 97.5 % percentile of the distribution of bootstrap estimates of the statistic (Felsenstein, 1985). Figure 7 contains the kernel density estimators (Wand and Jones, 1995) of the sampling distribution of flux from 5000 bootstrap samples for PBS and Pheroid™ with 90 % and 95 % bias corrected bootstrap percentile confidence limits for the true flux values.

Figure 7: Kernel density estimators of the flux sampling distribution for PBS and Pheroid™.

The plots in Figure 7 show that the confidence limits for PBS were much smaller than for Pheroid™. This means that estimated values with PBS can be performed easier than in the case of Pheroid™, as the data are more concentrated around its average. Less data points are thus needed for PBS than for Pheroid™.

3.4.3 Tape stripping

The concentration of L-carnitine L-tartrate found in the epidermis (0.270 μg/ml) and dermis (2.403 μg/ml) was found higher for PBS than for Pheroid™ (0.111 μg/ml and 1.641 μg/ml for the epidermis and dermis, respectively). A probable explanation could be that Pheroid™ enhanced the permeation of the L-carnitine L-tartrate through the skin into the receptor compartment (higher flux value), consequently leading to a lesser amount of L-carnitine L-tartrate delivered to the target site, i.e. the epidermis and dermis.

Figure 8 represents box-plots obtained from the tape stripping. As can be observed, the dermis showed higher concentration values (μg/ml) than the epidermis and several outliers were present. These results proved to be in accordance with what was expected as the smaller amount of carnitine found in the epidermis can be due to the epidermis being in part alive and in part dead (stratum corneum) with carnitine being present only in the mitochondria of living cells (Steiber et al., 2004; Black, 1993). The living cells present in the dermis are responsible for the higher amount of carnitine detected.

Figure 8: Box-plots obtained from tape stripping data.

A two-way ANOVA (analysis of variance) was employed to determine whether there is an interaction between the medium (PBS and Pheroid™) and the skin (epidermis and dermis). The p-value for medium/skin interaction was 0.442 which according to the null hypothesis means that there was no interaction between the skin and the medium (PBS and Pheroid™). This is also evident from the box-plot representation given in Figure 8. With no interaction present, the main factors, medium and skin, were tested (using the Mann-Whitney nonparametric test) for significance. It was found that only the skin factor (with levels: epidermis and dermis) is significant at a 5 % level, indicating a statistical significant difference between the data of the

dermis and the epidermis. It can thus be concluded that the PBS and Pheroid^{TM} did not influence the results.

4 Conclusion

Calendula oil (linoleic acid) was not detected in the receptor compartment, indicating that no calendula oil penetrated through the full-thickness skin in the case of both PBS and Pheroid^{TM}. Even though linoleic acid was observed in the dermis and epidermis of the PBS and Pheroid^{TM} studies, it is inconclusive whether Pheroid^{TM} enhanced the oil's penetration into the delivery target site. In the future the amount of sample used for methylation as well as the amount of toluene for extraction of the epidermis and dermis should be uniform, so that results can be compared. Methylation with BF_3 and MeOH proved to be a successful method to convert the fatty acids to their simplest convenient volatile derivatives, in this case methyl esters. Furthermore a quantitative GC/MS method should be developed and a different marker should be chosen to prevent the components of the Pheroid^{TM} from interfering with the results. Definite entrapment of the L-carnitine L-tartrate within the Pheroid^{TM} was illustrated by the confocal micrographs. The tape stripping was not influenced by the Pheroid^{TM} delivery system, but it showed significant differences between the various skin layers (epidermis or dermis). The amount of L-carnitine L- tartrate found in both the epidermis and dermis was found higher for PBS than for Pheroid^{TM}, indicating that PBS delivered L-carnitine L-tartrate to the target delivery site more than in the case of Pheroid^{TM}. When comparing the average (mean) and median flux value in Pheroid^{TM} with that in PBS it was observed that the Pheroid^{TM} proved to be advantageous by increasing flux. Future investigations may be done on the presence of carnitine in the human skin.

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FIGURE LEGENDS:

Figure 1: Acid-catalysed transesterification of lipids (Christie, 1993).

Figure 2: CLSM micrographs of L-carnitine L-tartrate and the Pheroid™ delivery system. Micrograph **(a)** is the control, **(b)** is the L-carnitine L-tartrate entrapped within the Pheroid™, **(c)** is the L-carnitine L-tartrate and **(d)** is the Pheroid™.

Figure 3: Average cumulative amount of L-carnitine L-tartrate dissolved in PBS (pH 7.4) that penetrated the skin as a function of time illustrating the average flux.

Figure 4: Average cumulative amount of L-carnitine L-tartrate encapsulated in Pheroid™ that penetrated the skin as a function of time illustrating the average flux.

Figure 5: Box-plots depicting the flux of L-carnitine L-tartrate dissolved in PBS (pH 7.4) (left) and encapsulated in Pheroid™ (right).

Figure 6: Linear plot illustrating the average flux values for L-carnitine L-tartrate in both PBS and Pheroid™.

Figure 7: Kernel density estimators of the flux sampling distribution for PBS and Pheroid™.

Figure 8: Box-plots obtained from tape stripping data.

TABLES

Table 1: Amounts of samples and reagents used during methylation with BF_3 in MeOH.

Sample	Amount of sample used	NaOH (ml)	BF_3 (ml)	NaCl (ml)	Isooctane (ml)
Calendula oil					
Lipid extracted (receptor compartment)	1 ml	1.5	2	2.5	2
Epidermis (5 ml)	260 µl	1.5	2	2.5	2
Dermis (5 ml)	260 µl	1.5	2	2.5	2
Calendula oil entrapped in Pheroid™					
Lipid extracted (receptor compartment)	1 ml	1.5	2	2.5	2
Epidermis (5 ml)	500 µl	3	4	5	2
Dermis (2 ml)	100 µl	3	4	5	2

Table 2: Chromatographic conditions and mass spectrometer settings.

Chromatographic conditions	
Carrier gas	Helium at constant velocity of 40 cm/minute
Flow rate	250 μl/min
Injection mode	1:10 split, 1.0 μl injected
Injection temp.	200 °C
Temp. program	Start at 80 °C, ramp at 4 °C/min to 245 °C, hold 20 min
Mass spectrometer settings	
Electron ionisation	Positive ion mode, full scan, 100 – 450 amu
Transfer line temp.	200 °C
Ion source temp.	250 °C
Detector potential	70 kV

FIGURES

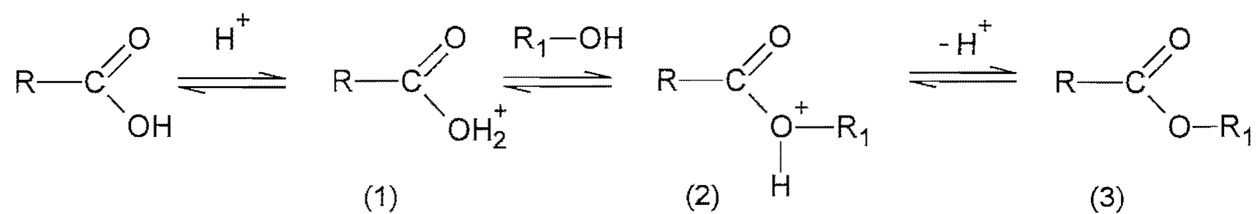

Figure 1: Acid-catalysed transesterification of lipids (Christie, 1993).

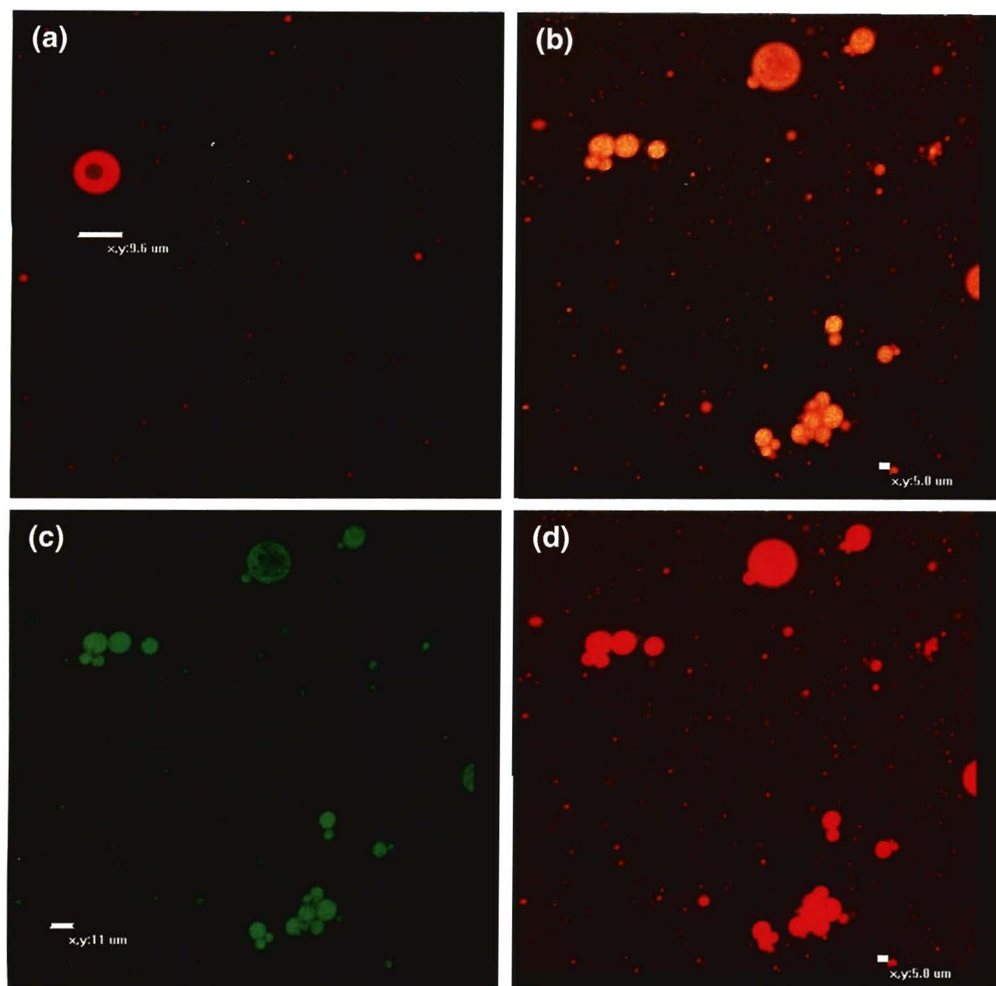

Figure 2: CLSM micrographs of L-carnitine L-tartrate and the Pheroid^{TM} delivery system. Micrograph **(a)** is the control, **(b)** is the L-carnitine L-tartrate entrapped within the Pheroid^{TM}, **(c)** is the L-carnitine L-tartrate and **(d)** is the Pheroid^{TM}.

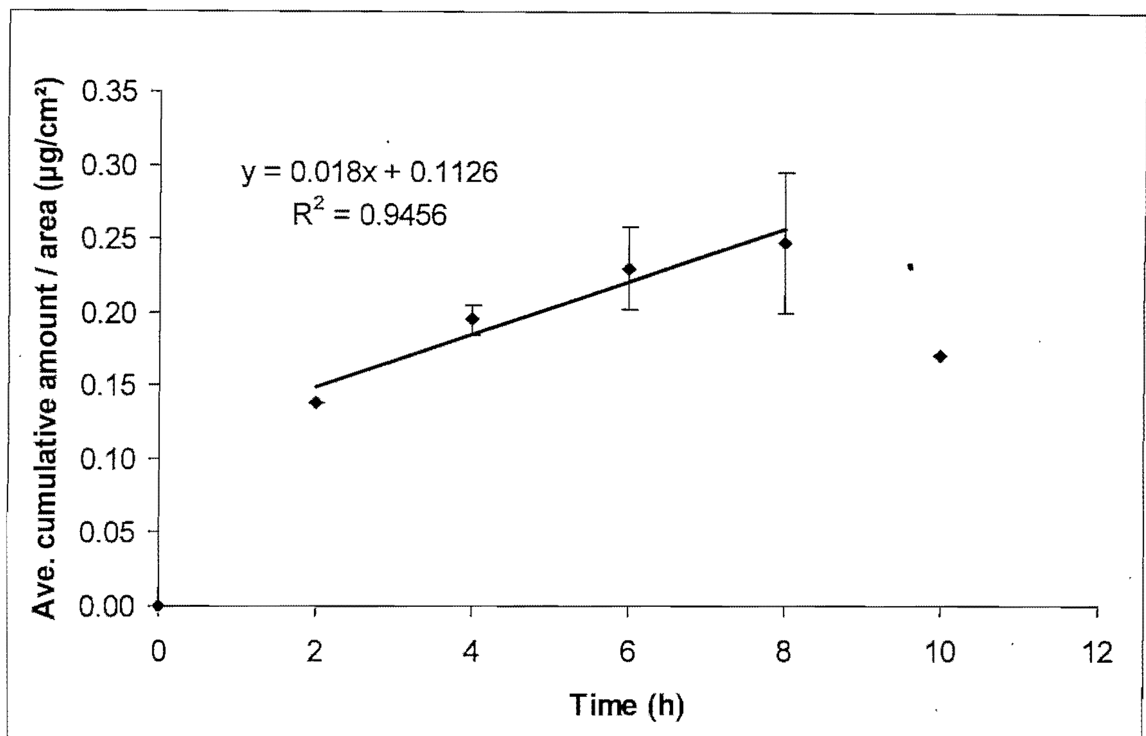

Figure 3: Average cumulative amount of L-carnitine L-tartrate dissolved in PBS (pH 7.4) that penetrated the skin as a function of time illustrating the average flux.

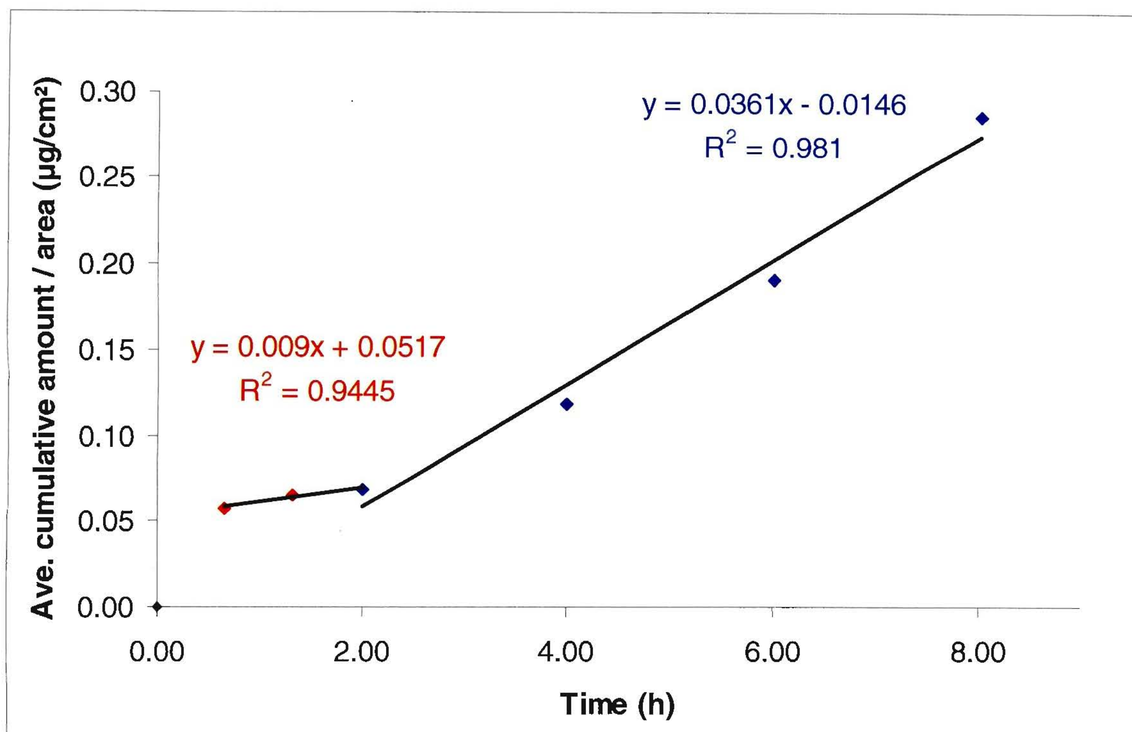

Figure 4: Average cumulative amount of L-carnitine L-tartrate encapsulated in Pheroid™ that penetrated the skin as a function of time illustrating the average flux.

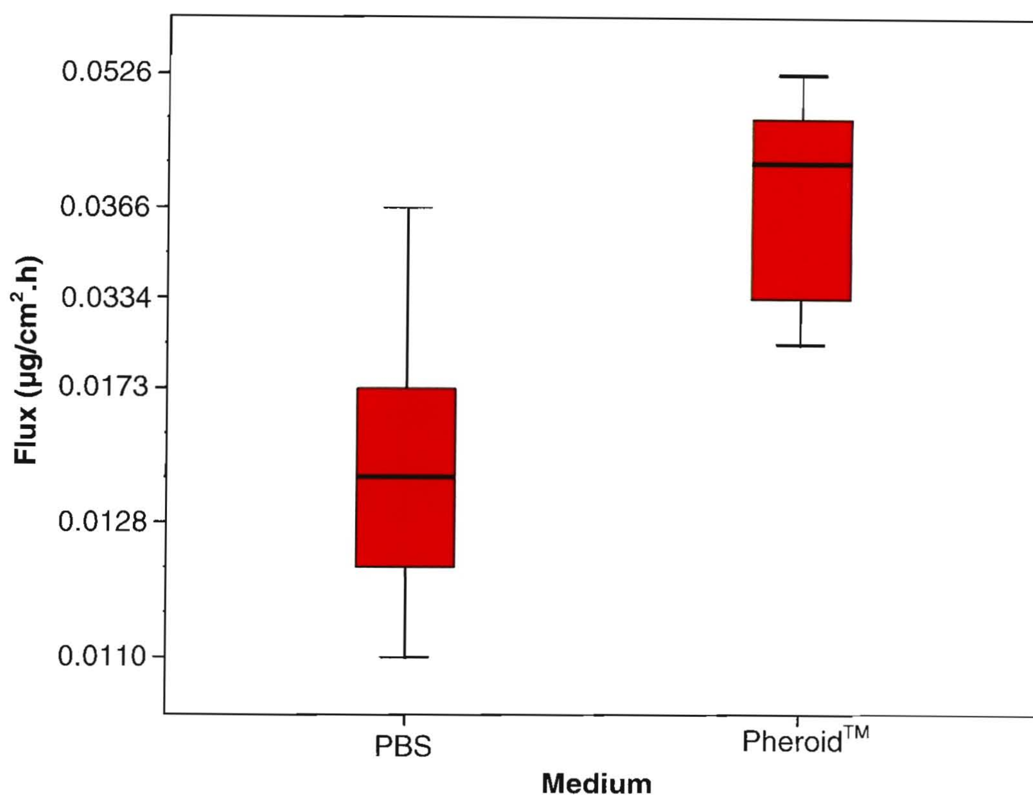

Figure 5: Box-plots depicting the flux of L-carnitine L-tartrate dissolved in PBS (pH 7.4) (left) and encapsulated in Pheroid^{TM} (right).

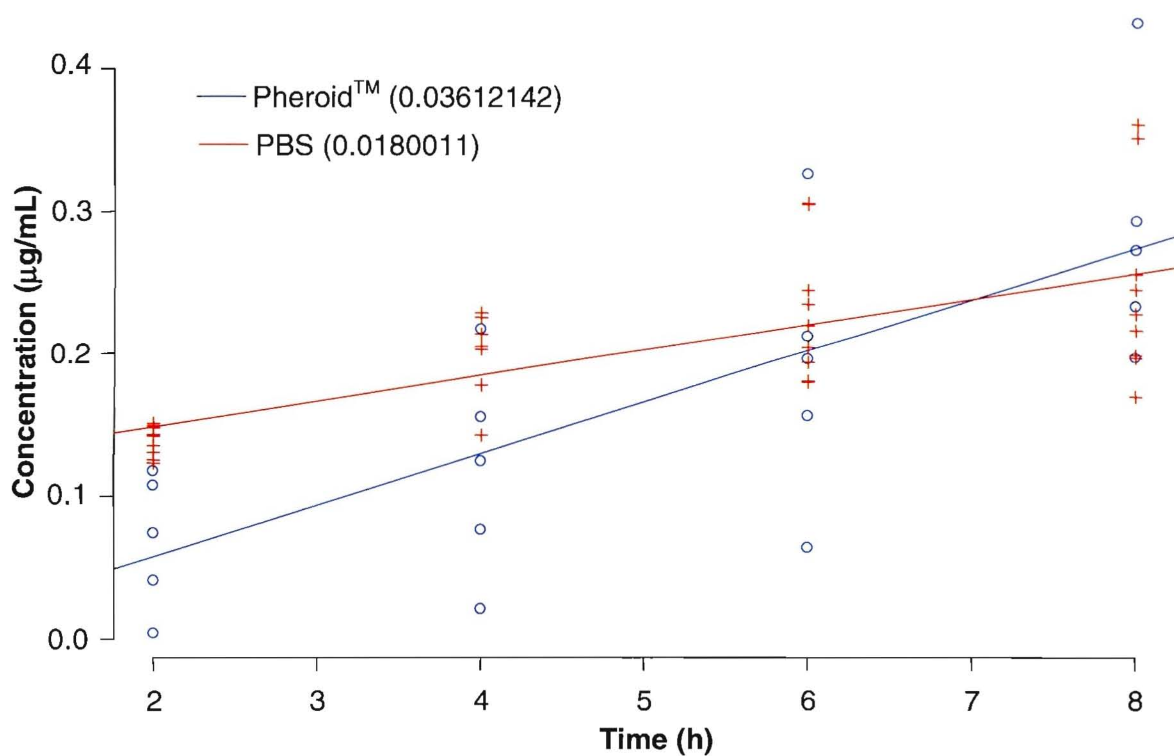

Figure 6: Linear plot illustrating the average flux values for L-carnitine L-tartrate in both PBS and Pheroid™.

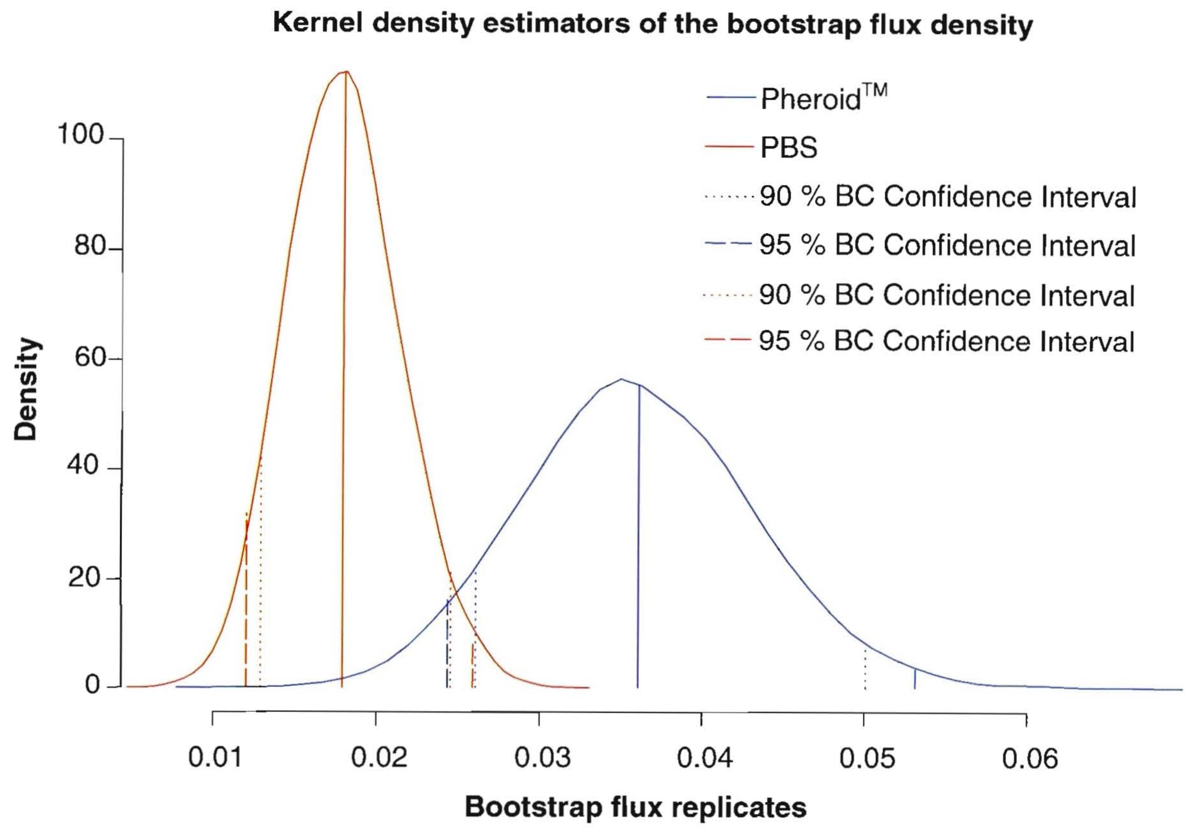

Figure 7: Kernel density estimators of the flux sampling distribution for PBS and Pheroid™.

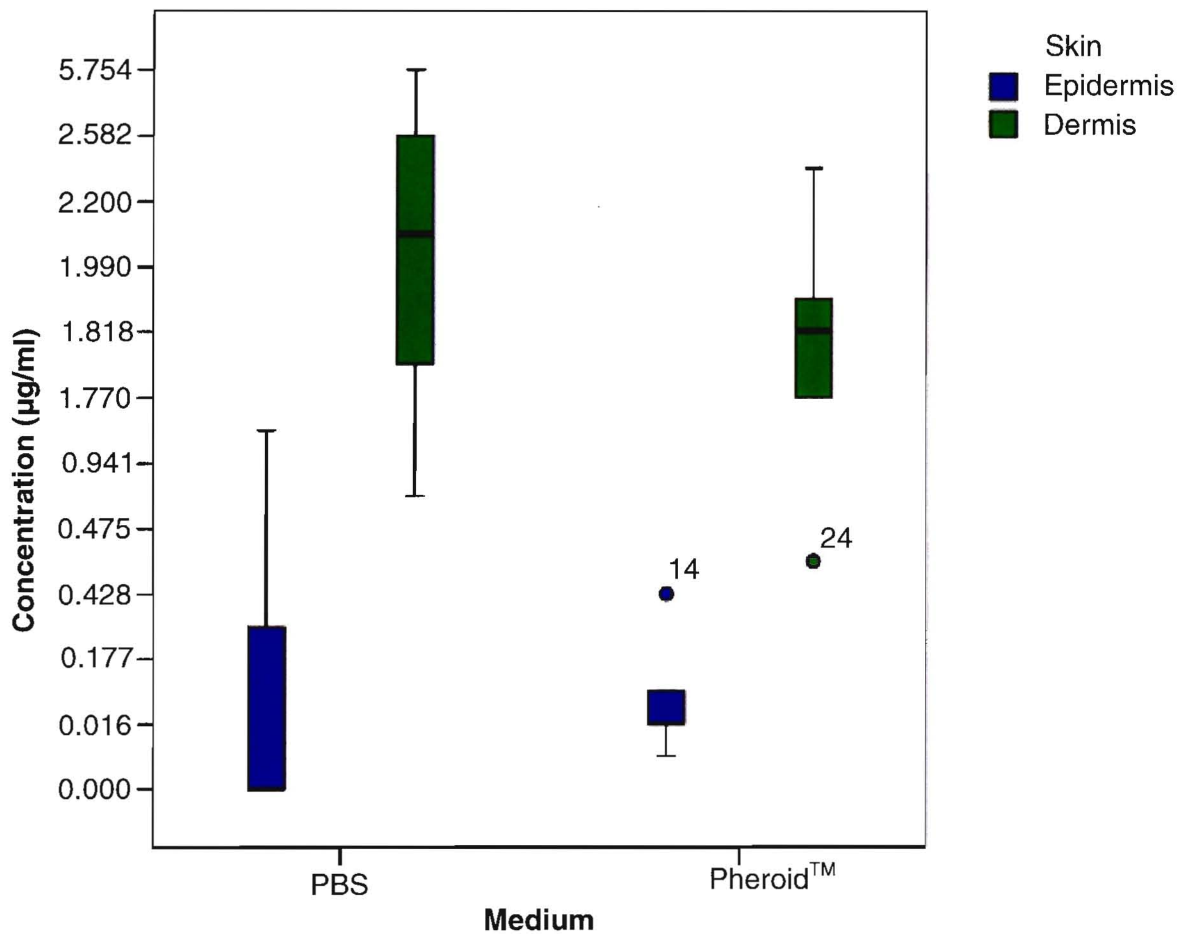

Figure 8: Box-plots obtained from tape stripping data.

CHAPTER 4: FINAL CONCLUSIONS AND FUTURE PROSPECTS

Aging is a foremost factor associated with many complex diseases which include cardiovascular diseases, diabetes, cancer and neurodegenerative disorders. After decades of research many biological processes associated with aging and hundreds of genes involved have been identified, but many fundamental questions are still unanswered and/or under debate (Xue *et al.*, 2007:1). Technological and medical development has lead to a considerable increase in general life expectancy with the number of people living to a relatively old age ever-increasing (Giacomoni, 2005:S45).

The aging of the skin has exceedingly direct effects on the daily life and psychological and social well-being of an individual (Giacomoni, 2005:S46). As the largest organ of the body, one of the skin's major functions is to provide humans with an almost completely impermeable barrier to the external environment (Black, 1993:145). Delaying of the skin aging process will consequently not only help us to keep a more youthful appearance but will most likely have advantageous effects for the whole organism (Giacomoni, 2005:S46).

The aging process of the skin can be divided into intrinsic and extrinsic (photo-) aging (Chung, 2001:1218). Intrinsic aging is due to the passage of time; whereas extrinsic aging can be attributed to repeated exposure to UV radiation. These two types can be seen as distinct entities rather than analogous skin aging processes (Rhie *et al.*, 2001:1212). Varani *et al.* (2000:480) found that photoaged skin and naturally aged, sun-protected skin share important molecular characteristics, which include reduced collagen production, elevated MMP levels and connective tissue damage. Both also lead to the production of ROS which ultimately leads to the impaired function of the skin (Rittié & Fisher, 2002:709). During the aging process the epidermal and dermal layers of the skin are affected, making it the target site for topical drug delivery (Vioux-Chagnoleau *et al.*, 2006:S2).

Cosmeceutical actives calendula oil and L-carnitine L-tartrate were investigated in this study due to their effectiveness to treat and/or prevent skin aging. This can be attributed to calendula oil's ideal properties to scavenge DPPH, peroxyl and hydroxyl radicals (Ćetković *et al.*, 2004:648) and L-carnitine L-tartrate's ability to promote the skin's turnover rate and to increase fibroblast proliferation (Held, 2004:41; Arslan *et al.*, 2003:224). The skin-friendly carrier system, Pheroid™, was tested in order to determine whether it will enhance the actives' uptake into the layers of the skin or through the skin.

Entrapment of calendula oil within the Pheroid™ could not be confirmed as it is undistinguishable from the components of the Pheroid™. Unfortunately the GC/MS method for determining the methyl ester forms of the fatty acids present in the calendula oil was only

qualitative, indicating only whether or not the calendula oil (with linoleic acid as the marker compound) was present. Methylation of the fatty acids with BF_3/MeOH proved to be successful and can prove valuable for future investigations. As was expected, calendula oil was not observed in the receptor compartment samples of the Franz cells due to its high lipophilicity. This indicates that no calendula oil diffused through the skin layers.

Linoleic acid was observed in the tape stripping samples (epidermis and dermis), but regrettably it is undetermined whether the Pheroid™ delivery system enhanced this essential oil's penetration, due to the fact that linoleic acid is one of the key components of the Pheroid™. This made it impossible to distinguish between the fatty acids of the calendula oil and the Pheroid™.

A further aim of this study was to determine the aqueous solubility and log D value of L-carnitine L-tartrate. The aqueous solubility was found to be 16.63 mg/ml in PBS (pH 7.4) at a temperature of 32 °C, indicating that it may pass through the skin, as the ideal solubility for a drug to permeate the skin is more than 1 mg/ml (Naik *et al.*, 2000:319). In contrast, the log D value fell outside of the ideal range (log P between 1 and 3) (Hadgraft, 2004:292) as it was determined to be -1.35.

Entrapment of L-carnitine L-tartrate inside the Pheroid™ vesicles was established visually by means of CLSM. A LC/MS method was developed and validated to accurately quantify the L-carnitine L-tartrate present in the receptor compartment (of the Franz cells) and in the tape stripping (epidermis and dermis) samples. Pheroid™ proved to be beneficial for increasing the average (mean) and median flux value (0.0361 μg/cm^2.h and 0.0393 μg/cm^2.h respectively) when compared to PBS (average of 0.0180 μg/cm^2.h, median of 0.0142 μg/cm^2.h). In contrast, the PBS delivered a higher degree of L-carnitine L-tartrate to the target site of delivery as it achieved a concentration of 0.270 μg/ml in the epidermis and 2.403 μg/ml in the dermis; whereas Pheroid™ showed a lower concentration of 0.111 μg/ml and 1.641 μg/ml in the epidermis and dermis respectively.

Future prospects for further investigation and aspects that need to be considered with the objective of possible formulation of a product include the following:

- A quantitative GC/MS method should be developed and validated in order to accurately determine the amount of calendula oil in the receptor phase and in the layers of the skin (epidermis and dermis).
- A different marker compound for calendula oil should be chosen in order to insure that the components of the Pheroid™ delivery system do not impede the results.
- Uniform sample and reagent amounts should be utilised for methylation with BF_3/MeOH.

- Equal amounts of the PBS (L-carnitine L-tartrate) and toluene (calendula oil) should be used wherein the epidermis and dermis samples are to be left overnight.
- The presence of carnitine in the human skin should be further investigated.
- *In vivo* testing should be performed in order to determine the possible beneficial and adverse reactions which may be associated with the use of the product.

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APPENDIX A: INTERNATIONAL JOURNAL OF PHARMACEUTICS GUIDE FOR AUTHORS

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The International Journal of Pharmaceutics publishes innovative papers, reviews, mini-reviews, rapid communications and notes dealing with physical, chemical, biological, microbiological and engineering studies related to the conception, design, production, characterisation and evaluation of drug delivery systems in vitro and in vivo. "Drug" is defined as any therapeutic or diagnostic entity, including oligonucleotides, gene constructs and radiopharmaceuticals.

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Note: In 2004, a new section was started on pharmaceutical nanotechnology. For more details, see Editorials in 279/1-2, 281/1, and 288/1.

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(e) Keywords

A maximum of 6 keywords or short phrases suitable for indexing should be supplied. If possible keywords should be selected from Index Medicus or Excerpta Medica Index. Authors may also wish to refer to the Subject Index published in International Journal of Pharmaceutics, for example, Vol. 287/1-2, pp. 205-219.

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The text should be divided into main sections, such as the following: 1. Introduction. 2. Materials and methods. 3. Results. 4. Discussion. Acknowledgements. References. Figure legends. Tables and Figures. These sections must be numbered consecutively as indicated. Subdivisions of a section should also be numbered within that section, for example, 2.1. Materials, 2.2. Relative humidity measurement, 2.3. Sample preparation, etc.

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Standard nomenclature should be used throughout; unfamiliar or new terms and arbitrary abbreviations should be defined when first used. Unnecessary or ambiguous abbreviations and symbols are to be avoided. Data should be expressed in SI units.

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Figure legends, tables and footnotes should be typed on separate sheets, lines double spaced. Footnotes, to be numbered consecutively in superscript throughout the text, should be used as little as possible.

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Crowe, J.H., Crowe, L.M., Carpenter, J.F., Aurell Wistrom, C., 1987. Stabilisation of dry phospholipid bilayers and proteins by sugars. Biochem. J., 242, 1-10.

Crowe, J.H., Crowe, L.M., Carpenter, J.F., Rudolph, A.S., Wistrom, C.A., Spargo, B.J., Anchordoguy, T.J., 1988. Interactions of sugars with membranes. Biochim. Biophys. Acta, 947, 367-384.

Crowe, L.M., Crowe, J.H., Womersley, C., Reid, D., Appel, L., Rudolph, A., 1986. Prevention of fusion and leakage in freeze-dried liposomes by carbohydrates. Biochim. Biophys. Acta, 861, 131-140.

Crowe, L.M., Mouradian, R., Crowe, J.H., Jackson, S.A., Womersley, C., 1984b. Effects of carbohydrates on membrane stability at low water activities. Biochim. Biophys. Acta, 769, 141-150.

Examples of presentation for various types of publication

Langerman, L., Chaimsky, G., Golomb, E., Tverskoy, M., Kook, A.I., Benita, S., 1990. A rabbit model for evaluation of spinal anesthesia: chronic cannulation of the subarachnoid space. Anesth. Analg., 71, 529-535.

Timsina, M.P., Martin, G.P., Marriott, C., Ganderton, D., Yianneskis, M., 1994. Drug delivery to the respiratory tract using dry powder inhalers. Int. J. Pharm., 101, 1-13.

Gibaldi, M. and Perrier, D., 1982. Pharmacokinetics, 2nd Ed., Dekker, New York.

Deppeler, H.P., 1981. Hydrochlorothiazide. In: Florey, K. (Ed.), Analytical Profiles of Drug Substances, Vol. 10, Academic Press, New York, pp. 405-441.

US Pharmacopeia XXII, 1990. US Pharmacopeial Convention, Rockville, MD, pp. 1434-1435.

Mueller, L.G., 1988. Novel anti-inflammatory esters, pharmaceutical compositions and methods for reducing inflammation. UK Patent GB 2 204 869 A, 23 Nov.

Du Plessis, J., 1992. Topical liposomal delivery of biologically active peptides. Ph.D Thesis, Potchefstroom University for CHE, South Africa.

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Appendix B: Validation of the LC/MS experimental method for L-carnitine L-tartrate

B.1 PURPOSE OF THE VALIDATION

The objective of the validation of the analytical procedure is to demonstrate that it is sensitive and reliable in the determination of the amount of L-carnitine L-tartrate that permeated the skin.

B.2 CHROMATOGRAPHIC CONDITIONS

Analytical instrument: The LC/MS analysis of L-carnitine L-tartrate was performed by using an Agilent 1100 series HPLC with a binary gradient pump, auto sampler and vacuum degasser coupled to an Applied Biosystems API 2000 triple quadruple mass spectrometer and Analyst 1.4.2 data acquisition and analysis software.

Column: A Synergi Max-RP C-12 column, 150 x 2 mm, 4 µm (Phenomenex, Torrance, CA) was used during the analysis.

Mobile phase: The mobile phase consisted of 10 volumes of acetonitrile containing 0.1 % formic acid and 90 volumes of distilled water containing 0.1 % formic acid.

Flow rate: 250 µl/min

Injection volume: 5 µl

Retention time: The analyte elutes at approximately 1.12 minutes

B.3 MASS SPECTROMETER SETTINGS

Turbo Spray electron ionisation in the positive ion mode was used with Multiple Reaction Monitoring (MRM) Q1/Q3 mass were 162.158/58.1 and 162.158/43.1 with a 150 millisecond dwell time.

Declustering potential: 26 V
Focusing potential: 370 V
Entrance potential: 10 V
Curtain gas: 10 ml/min
Collision gas: 5

Ion spray voltage: 5500 V
Temperature: 350 °C
Ion source gas 1: 20 ml/min
Ion source gas 2: 25 ml/min

Collision Energy: **162.158/58.1:** 59 V **162.158/43.1:** 39 V
Collision Exit Potential: **162.158/58.1:** 6 V **162.158/43.1:** 4 V

B.4 CALIBRATION CURVE

A calibration curve for L-carnitine L-tartrate was established by making use of the following standard solutions with the concentrations of: 0.1; 0.2; 1.0; 2.0 and 3.0 µg/ml.

B.5 VALIDATION PARAMETERS

B.5.1 LINEARITY

The linearity of an analytical method is its capacity (within a given range) to elicit test results which are directly proportional to the amount (concentration) of analyte in the sample. The linearity of L-carnitine L-tartrate was determined by performing linear regression analysis on the plot of the peak area ratios versus concentration (µg/ml) of the standards, prepared as described.

The regression value (r^2) obtained for both ion pairs (162.158/58.1 & 162.158/43.1) indicate a high degree of linearity and therefore demonstrates the good stability of the analysis system. **Figure B.1** and **Figure B.2** illustrate the linear regression curves obtained for the different ion pairs. They should not differ from each other more than 10 % for the method to be valid. All experimental data was quantified by making use of the 162.158/58.1 ion pair standard curves.

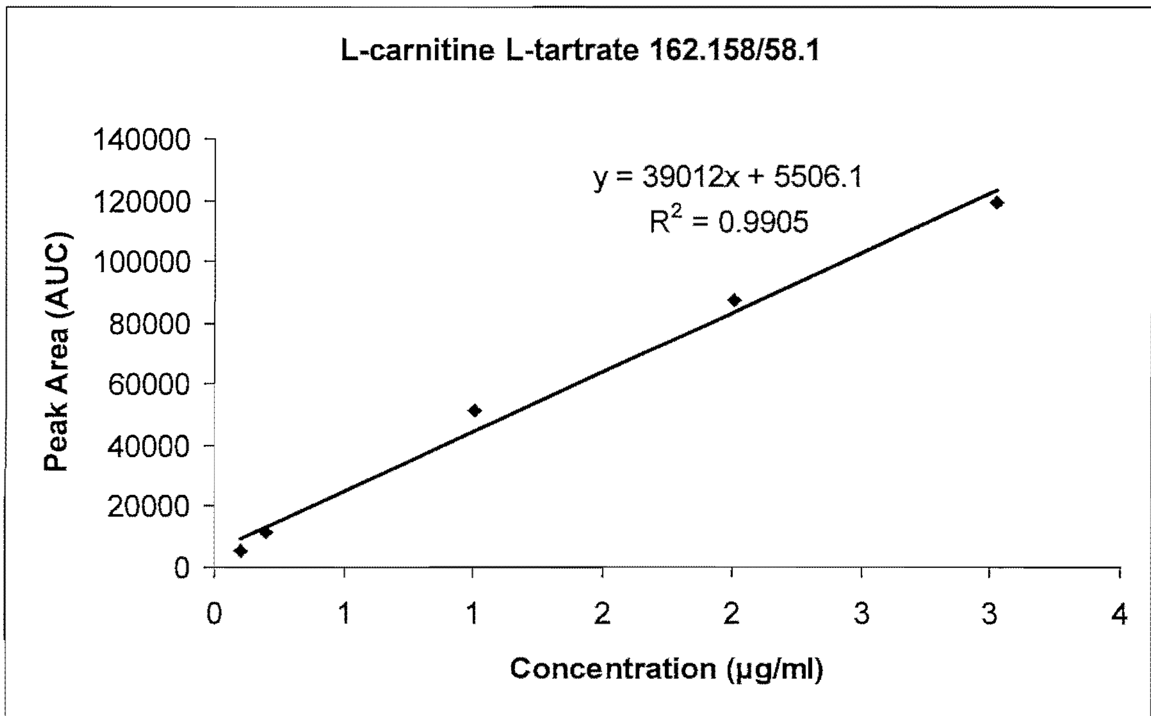

Figure B.1: Linear regression curve for L-carnitine L-tartrate standards (162.158/58.1 ion pair).

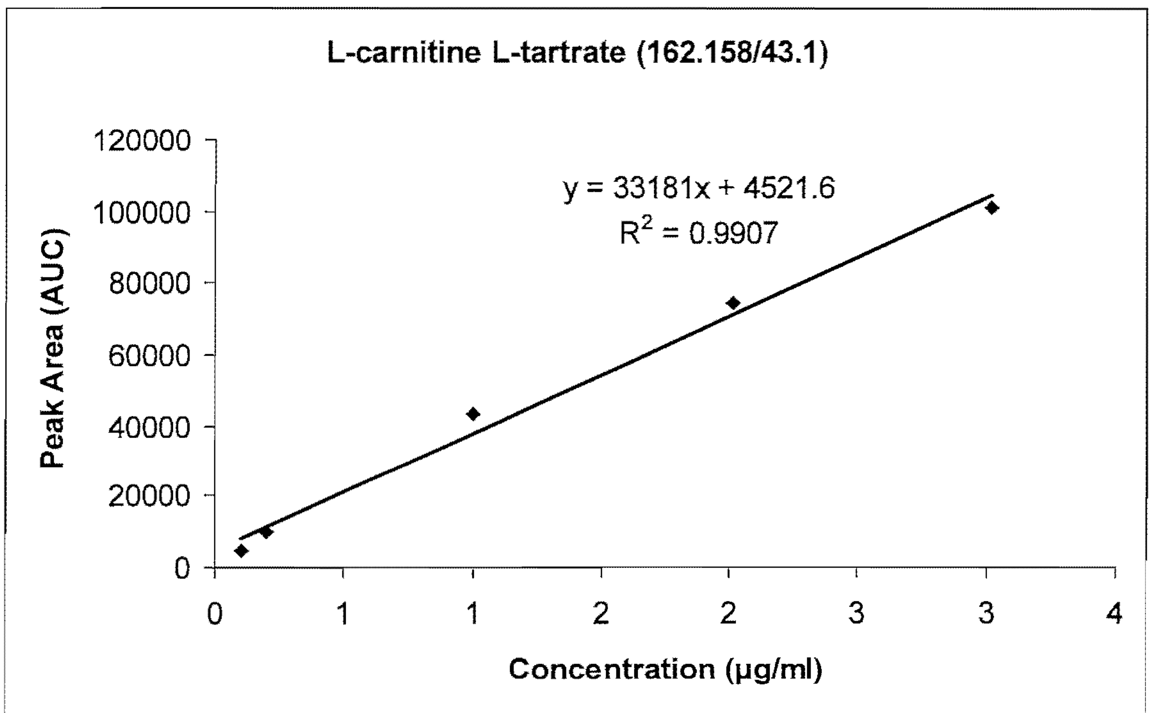

Figure B.2: Linear regression curve for L-carnitine L-tartrate standards (162.158/43.1 ion pair).

Table B.1 and **Table B.2** give the peak area ratio values of the L-carnitine L-tartrate standards for the 162.158/58.1 and 162.158/43.1 ion pairs, respectively.

Table B.1: Peak area ratio values of L-carnitine L-tartrate standards (162.158/58.1).

Standard (µg/ml)	Peak area ratio
0.10	5483.33
0.20	11600.00
1.01	51166.67
2.01	87533.33
3.02	119000.00
r^2	0.99
Intercept	5506.07
Slope	39012.43

Table B.2: Peak area ratio values of L-carnitine L-tartrate standards (162.158/43.1).

Standard (µg/ml)	Peak area ratio
0.10	4626.67
0.20	9706.67
1.01	43000.00
2.01	74633.33
3.02	100933.33
r^2	0.99
Intercept	4521.59
Slope	33180.61

B.5.2 ACCURACY AND PRECISION

The accuracy of an analytical procedure represents the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. Precision can be described as the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous substance under prescribed conditions. Precision was investigated by measuring the degree of intraday (repeatability) variation and interday (reproducibility) under normal operating conditions.

B.5.2.1 ACCURACY AND INTRADAY PRECISION

LC/MS analyses was performed on three different samples of known standards (1.0, 1.5, 2.0 µg/ml) of L-carnitine L-tartrate in triplicate during the same day (n = 3). Standard solutions

(1.0; 1.5 and 2.0 μg/ml) were prepared as described with and additional dilution of 3 ml of the mother solution to 100 ml with HPLC grade water to yield a concentration of 1.5 μg/ml.

Recovery proved to be between 96.24 % and 108.71 % for the 162.158/58.1 ion pair; and between 96.98 % and 115.23 % for the 162.158/43.1 ion pair. **Table B.3** and **Table B.4** give the accuracy of the different ion pairs. According to the USP standards accuracy of an analytical procedure should be established across its range.

Table B.3: Accuracy parameters of L-carnitine L-tartrate (162.158/58.1).

Conc.* Spike (μg/ml)	**AUC** 1**	**AUC** 2**	**Mean**	**Recovery (μg/ml)**	**%**
0.998	45300	47200	46250	1.04	104.65
0.998	44100	44100	44100	0.99	99.13
0.998	45600	44300	44950	1.01	101.31
1.497	62300	64600	63450	1.49	99.22
1.497	65100	62600	63850	1.50	99.90
1.497	63600	64100	63850	1.50	99.90
1.996	81700	79200	80450	1.92	96.24
1.996	81200	83700	82450	1.97	98.81
1.996	85900	84700	85300	2.05	102.47
				Mean	100.18
				SD***	2.40
				% RSD****	2.39

*Conc refers to concentration (μg/ml)

**AUC refers to the area under the curve

***SD refers to standard deviation

****%RSD refers to relative standard deviation

Table B.4: Accuracy parameters of L-carnitine L-tartrate (162.158/43.1).

Conc.* Spike (µg/ml)	AUC** 1	AUC** 2	Mean	Recovery (µg/ml)	%
0.998	39000	40300	39650	1.06	106.08
0.998	38100	38200	38150	1.01	101.55
0.998	40900	36900	38900	1.04	103.82
1.497	55800	55300	55550	1.54	102.73
1.497	54400	53000	53700	1.48	99.01
1.497	54700	54800	54750	1.51	101.12
1.996	70700	66800	68750	1.94	96.98
1.996	69700	71900	70800	2.00	100.08
1.996	72500	71500	72000	2.03	101.89
				Mean	101.47
				SD* **	2.17
				% RSD****	2.14

*Conc refers to concentration (µg/ml)

**AUC refers to the area under the curve

***SD refers to standard deviation

****%RSD refers to relative standard deviation

B.5.2.2 INTER-DAY PRECISION

LC/MS analysis was performed on 3 different samples of a known concentration of L-carnitine L-tartrate (1 µg/ml) on three different days (n = 3). The results can be seen in **Table B.5** and **Table B.6**. The recovery was found to be ranging between 94.52 % and 118.98 % for the 162.158/58.1 ion pair, and between 95.8 % and 121.72 % for the 162.158/43.1 ion pair.

Table B.5: Interday precision parameters of L-carnitine L-tartrate standards (162.158/58.1).

Concentration (µg/ml)	Day 1	Day 2	Day 3	Between days
	104.65	118.98	104.33	
	99.13	116.81	94.52	
	101.31	109.68	105.98	
Mean	101.70	115.16	101.61	106.15
SD*	1.97	3.97	5.06	6.37
% RSD**	1.93	3.45	4.98	6.00

*SD refers to standard deviation

**%RSD refers to relative standard deviation

Table B.6: Interday precision parameters of L-carnitine L-tartrate standards (162.158/43.1).

Concentration (µg/ml)	Day 1	Day 2	Day 3	Between days
	106.08	121.72	103.59	
	101.55	117.97	95.8	
	103.82	111.08	104.04	
Mean	103.82	116.92	101.14	107.29
SD*	1.60	4.41	3.78	6.90
% RSD**	1.54	3.77	3.74	6.43

*SD refers to standard deviation

**%RSD refers to relative standard deviation

B.5.3 SENSITIVITY

Through employing the limit of detection, as well as the lowest limit of quantification the sensitivity of the analytical method can be assessed. The % RSD for both measurements should be < 15 %.

B.5.3.1 LIMIT OF DETECTION (LOD)

LOD is defined as the lowest concentration of an analyte in a sample that can be detected, but not necessarily quantified as an exact value, under the stated experimental conditions. Based on a signal to noise ratio of 3:1, where the analyte peak is approximately three times the height of the baseline noise, the LOD for L-carnitine L-tartrate was determined to be 0.01 µg/ml.

B.5.3.2 LIMIT OF QUANTIFICATION (LOQ)

LOQ is defined as the lowest concentration of an analyte in a sample that can be quantitatively determined with acceptable precision and accuracy (% RSD < 15 %) under the stated experimental conditions. The LOQ of L-carnitine L-tartrate was determined to be 0.1 µg/ml.

B.5.4 SYSTEM REPEATABILITY

A concentration of 1 µg/ml was injected six consecutive times into the chromatograph, under the same conditions, in order to evaluate the repeatability of the peak area and retention time. The variations in response (% RSD) are given in **Table B.7** and **Table B.8** for the 162.158/58.1 and 162.158/43.1 ion pairs, respectively.

Table B.7: Variations in response (% RSD) concerning the peak area and retention time of the 162.158/58.1 ion pair.

Injection no	Peak area	Retention times (min)
1	45300	1.06
2	47200	1.24
3	44100	1.07
4	44100	1.24
5	45600	1.07
6	44300	1.05
Mean	45100	1.12
SD*	1106.04	0.08
% RSD**	2.45	7.48

*SD refers to standard deviation

**%RSD refers to relative standard deviation

Table B.8: Variations in response (% RSD) concerning the peak area and retention time of the 162.158/43.1 ion pair.

Injection no	Peak area	Retention times (minutes)
1	39000	1.05
2	40300	1.24
3	38100	1.06
4	38200	1.23
5	40900	1.07
6	36900	1.05
Mean	38900	1.12
SD*	1360.15	0.08
% RSD**	3.50	7.52

*SD refers to standard deviation

**%RSD refers to relative standard deviation

B.5.5 SELECTIVITY

Specificity is the ability to assess unequivocally the analyte in the presence of components that may interfere with analyte detection. The method is selective when no interfering peaks with the same retention time as the drug are detected. A blank injection of PBS was injected and illustrated no interference.

A standard curve (0.20; 0.50; 0.75; 1.00; 1.50 and 2.00 µg/ml) was injected after preparing it by making use of PBS (pH 7.4) buffer instead of HPLC water. It was determined that the PBS (pH 7.4) buffer had no effect on the linearity of the analytical method. **Figure B.3** and

Figure B.4 illustrate the linear regression curves obtained for the different ion pairs. The PBS (pH 7.4) buffer showed no significant difference from the linear regression as previously described.

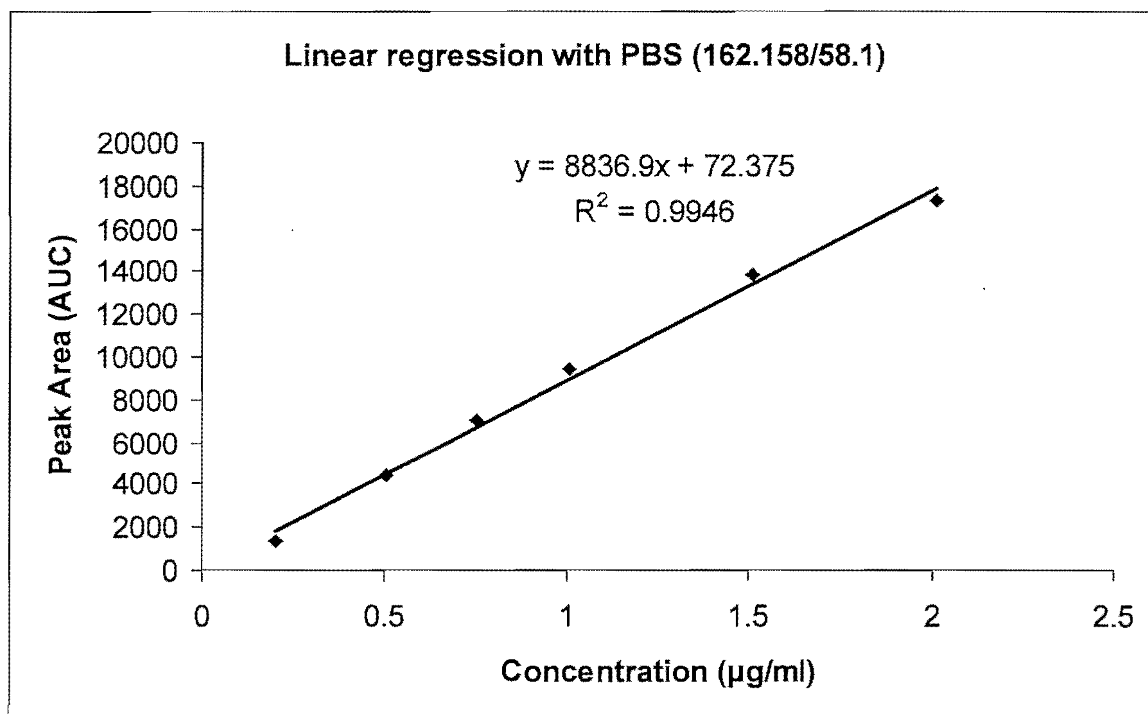

Figure B.3: Linear regression curve for L-carnitine L-tartrate standards in PBS buffer (pH 7.4) of the 162.158/58.1 ion pair.

Figure B.5 and **Figure B.6** depict LC/MS chromatograms which illustrates that PBS shows no interference. **Figure B.5** illustrates the retention time of the L-carnitine L-tartrate and the peaks obtained when L-carnitine L-tartrate was dissolved in PBS (pH 7.4) buffer where only the L-carnitine L-tartrate can be seen. **Figure B.6** illustrates the chromatogram when a blank injection with PBS (pH 7.4) is made, there are no interfering peaks.

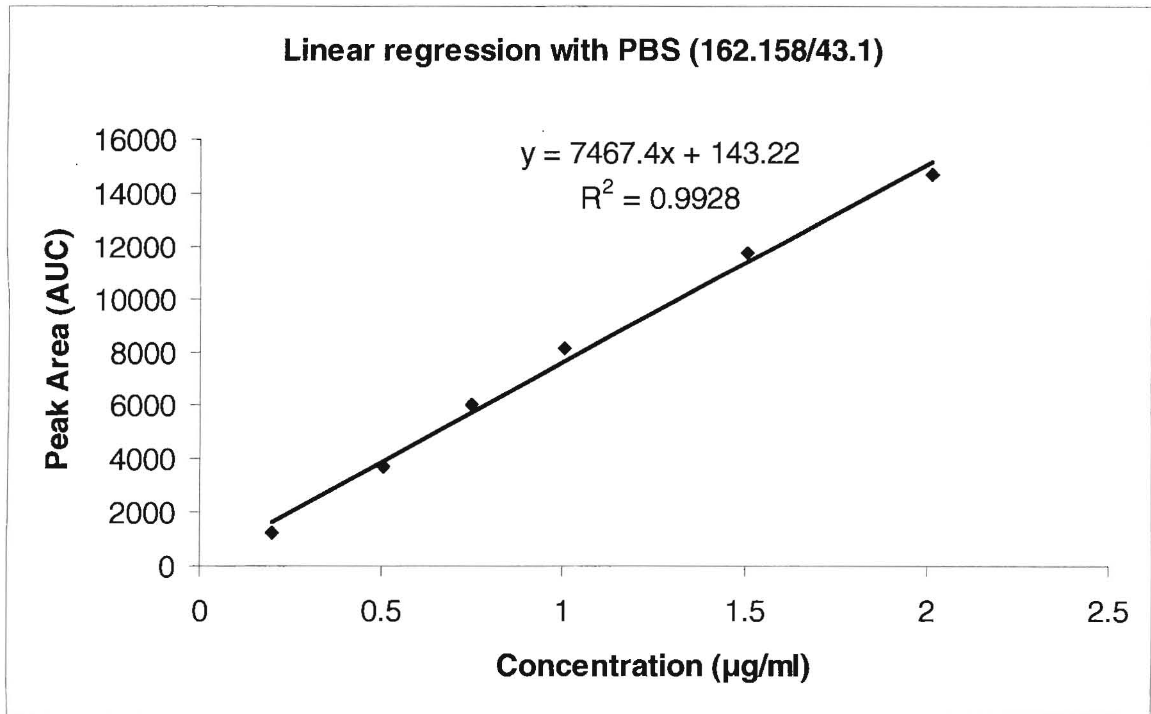

Figure B.4: Linear regression curve for L-carnitine L-tartrate standards in PBS buffer (pH7.4) of the 162.158/43.1 ion pair.

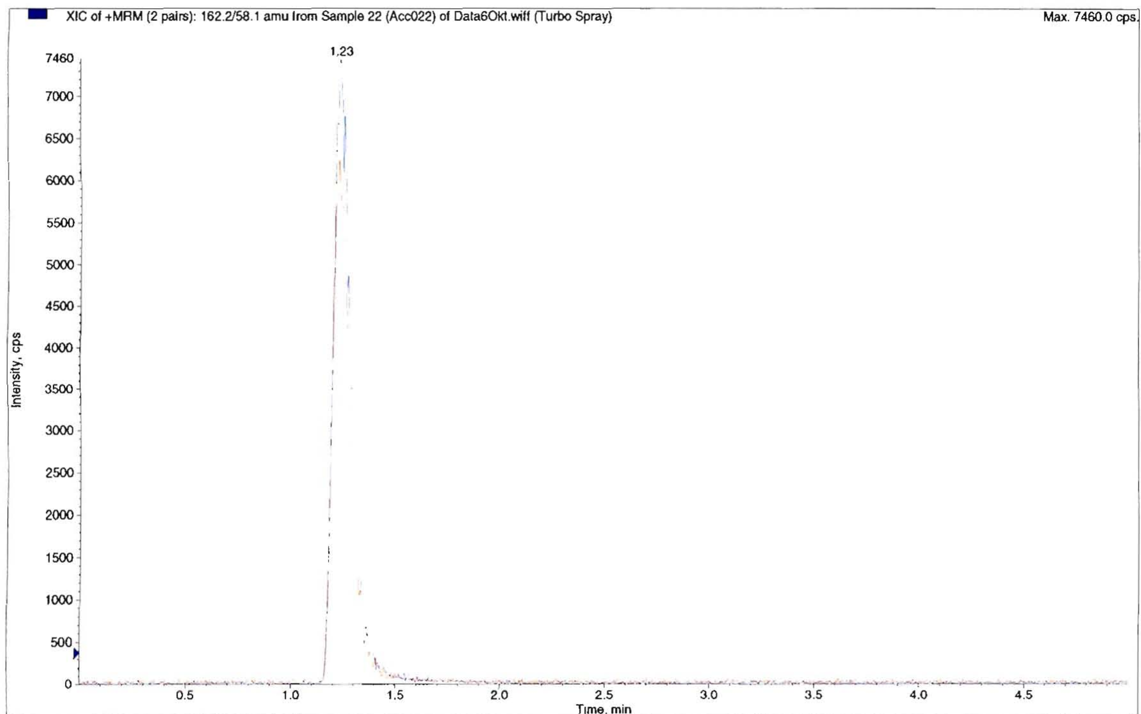

Figure B.5: LC/MS chromatogram illustrating the retention time of L-carnitine L-tartrate (1 µg/ml) when dissolved in HPLC water.

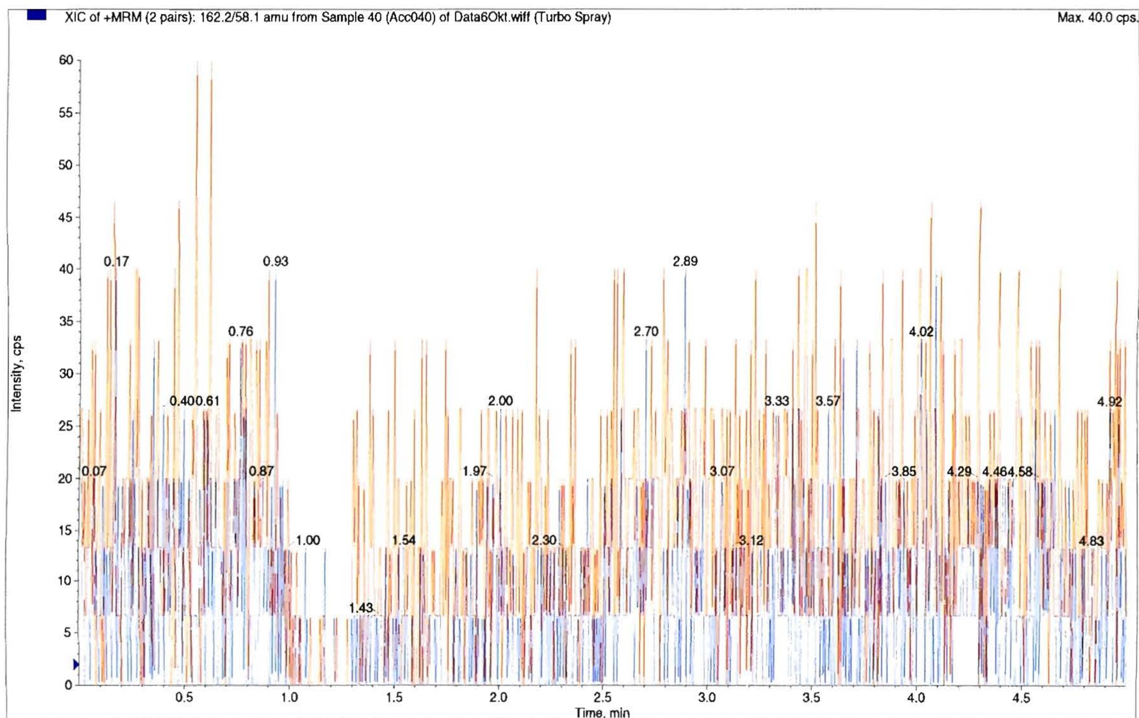

Figure B.6: LC/MS chromatogram illustrating the peak obtained with a blank injection of PBS (pH 7.4).

B.6 CONCLUSION

The LC/MS method developed has been found to be reliable and sensitive enough for the determination of the concentration of L-carnitine L-tartrate.

APPENDIX C: DIFFUSION STUDY DATA

C.1 L-CARNITINE L-TARTRATE

C.1.1 PERMEATION PROFILES

In order to determine whether Pheroid™ enhanced the flux of L-carnitine L-tartrate it was compared against when the cosmeceutical active was dissolved in PBS (pH 7.4). **Figure C.1** and **Figure C.2** illustrate the cumulative amount per area plotted against time for each Franz cell during the PBS and Pheroid™ diffusion studies, respectively.

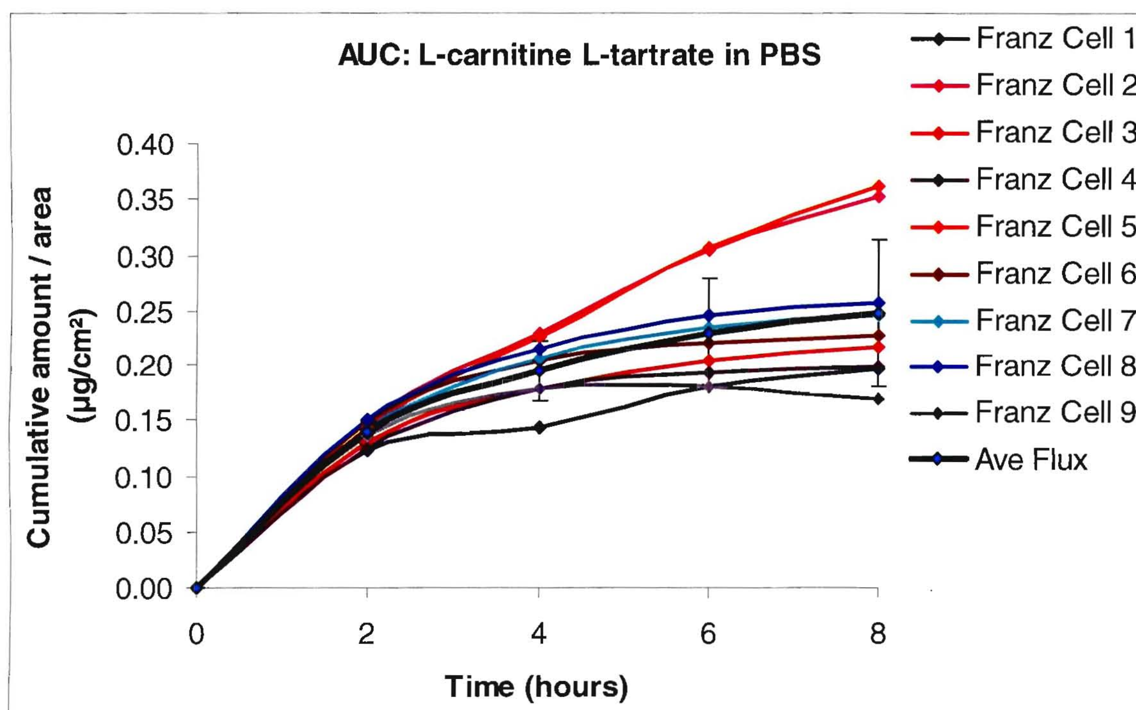

Figure C.1: Cumulative amount per area plotted against time for each Franz cell when L-carnitine L-tartrate was dissolved in PBS (pH 7.4) buffer.

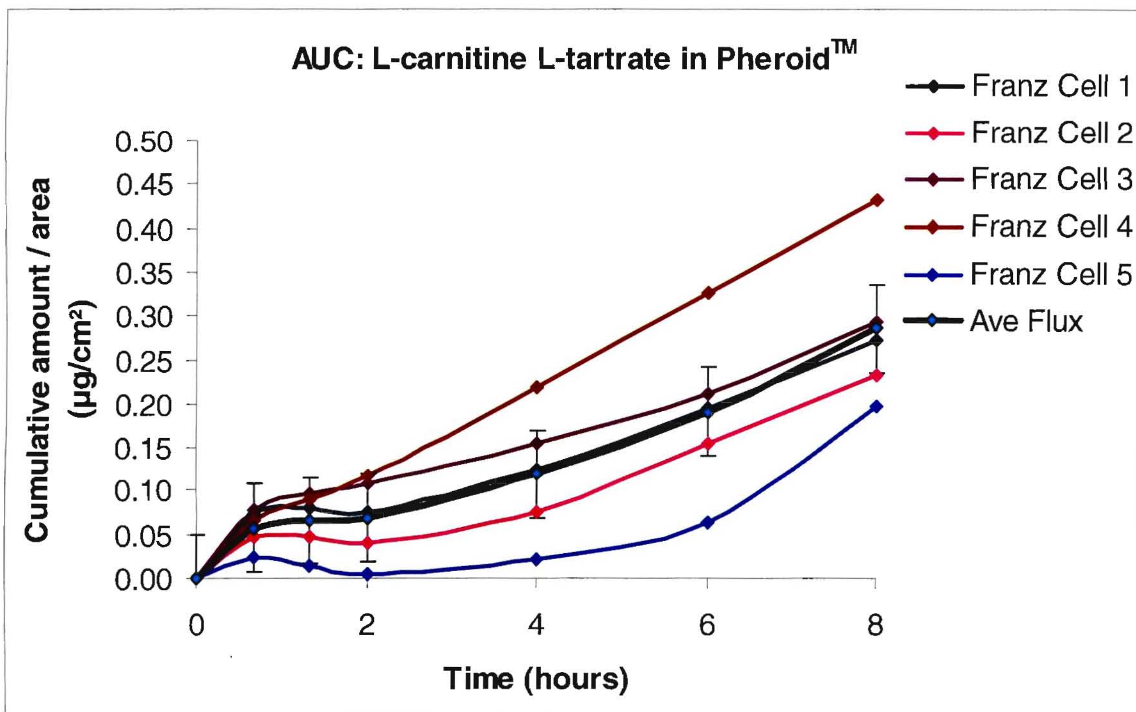

Figure C.2: Cumulative amount per area plotted against time for each Franz cell when L-carnitine L-tartrate was encapsulated in Pheroid™.

C.1.2 CONTROL STUDY DATA

During Franz cell diffusion studies with L-carnitine L-tartrate it was discovered that the skin may contain natural carnitine resources. **Table C.1** gives the correction factors of the control diffusion study performed. This correction factor was incorporated during the data processing for determination of the flux values and the tape stripping values (epidermis and dermis).

Table C.1: Correction factors as determined by the control diffusion study due to the carnitine found in the skin.

	PBS (pH 7.4)	Pheroid™
Carn*-Skin Corr** (µg/cm²)	0.343	0.441
Carn-skin Corr/h (µg/cm²)	0.069	0.074
Tape-Strip (µg/ml)	0.006	0.001
Tape-Strip (dermis) (µg/ml)	0.035	0.086

*Carn refers to L-carnitine L-tartrate

**Corr refers to the correction factor determined

APPENDIX D: PHOTOS OF APPARATUS USED DURING DIFFUSION STUDIES AND SAMPLE ANALYSIS

Photo D.1: Vertical Franz diffusion cell with donor and receptor compartments

Photo D.2: Horseshoe clamps

Photo D.3: Grant water bath

Photo D.4: Assembled Franz diffusion cells

Photo D.5: Dow Corning vacuum grease

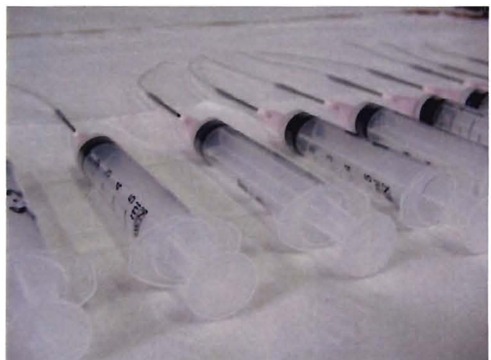

Photo D.6: Syringes used to withdraw samples from receptor compartments

Photo D.7: Variomag® magnetic stirring plate

Photo D.8: HPLC vials wherein samples were placed for analyses

Photo D.9: Milli-Q water purifying system

Photo D.10: Synergi C12 column, 150 x 2 mm, 4 µm (Phenomenex, Torrance, CA) for LC/MS

Photo D.11: Agilent 1100 series HPLC coupled to an Applied Biosystems API 2000 triple quadrupole mass spectrometer

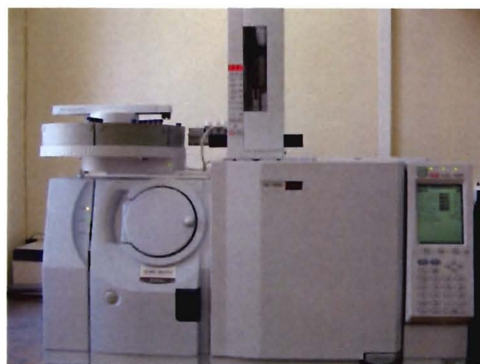

Photo D.12: Shimadzu QP2010 single quadrupole GC/MS with electron ionisation