Evaluation of the Penetration and Efficacy of Topical Anti-Aging Compounds

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To My Family

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Abstract

With an ever-growing topical anti-wrinkle market the cosmetic industry has focused on developing novel formulations. Despite the availability of methodology, demonstrations of anti-wrinkle efficacy are few and far between. This has been a growing area for technological advancement but the justification for extrapolation between *in vitro* studies and the mechanisms *in vivo* is limited.

The aim of this research was to determine the *in vitro* penetration and then the *in vivo* efficacy of some common anti-wrinkle ingredients. Niacinamide, genistein and palmitoyl tripeptide-5 were investigated. The flux for niacinamide from propylene glycol across human skin *in vitro* was found to be 3.773 ± 0.5138 μg/cm^2/hr. Genistein was found to have an average flux of 0.03625 ± 0.00544 μg/cm^2/hr from propylene glycol across human skin *in vitro.* Using an assay for the tripeptide capable of quantifiably detecting 6.581 μg/ml, no detectable uptake or penetration into or through human skin *in vitro* was recorded.

Two *in vivo* split face clinical studies, involving 20 participants in each, were undertaken. The studies assessed whether a cream containing niacinamide and a cream containing palmitoyl tripeptide-5 reduced the appearance, size and number of mild to moderate facial wrinkles compared to either skin left untreated or skin treated with a control (the product excluding niacinamide or palmitoyl tripeptide-5). At the conclusion of the study, the product containing niacinamide showed a significant reduction in the size (28.99 ± 9.09 %) and number of facial wrinkles (8.50 ± 2.80 %) compared to the control product and a significant reduction in the size (34.55 ± 11.49 %) and visible appearance of facial wrinkles compared to untreated skin. The product containing palmitoyl tripeptide-5 showed significant improvement in the visible appearance of facial wrinkles *in vivo* compared to the control treated skin and a significant reduction in

the size (24.78 ± 10.21 %) of facial wrinkles compared to untreated skin at the conclusion of the study.

The niacinamide clinical study results, with support from the niacinamide finite permeation study results of the formulation used i*n vivo*, suggest it is possible that niacinamide is penetrating the stratum corneum, reaching its site of action, and causing its anti-wrinkle effects. Based on the poor skin permeation of palmitoyl tripeptide-5 it is highly likely the visible improvement in wrinkle appearance was caused by superficial surface effects rather than the claimed collagen regeneration mechanism.

Statement of Originality

The Faculty Manager

Faculty of Pharmacy and Pharmaceutical Sciences

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I herby certify that the work presented has not been submitted by me or by any other person for the award of a degree at Monash University or any other institution. To the best of my knowledge the thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis. Results presented in Chapter Four are based on work performed in collaboration with Acrux Limited and the University of Melbourne. While the operation of the multiphoton microscope was performed by Dr Damian Bird, all sample preparation and the data analysis was performed by myself.

Anita Louise Schneider

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List of Abbreviations

~	approximately
$a_{1/}\, a_2$	relative contributions of the short / long fluorescence lifetime component
ACN	acetonitrile
ANOVA	one-way analysis of variance
ATR-FTIR	attenuated total reflectance fourier transform infrared
AWP	anti-wrinkle product
AWP-N	niacinamide anti-winkle product
AWP-P	palmitoyl tripeptide-5 anti-wrinkle product
Cm	centimeter
CV	co-efficient of variation
C_V	concentration of the drug in the vehicle
D	diffusion coefficient.
°C	degrees celsius
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
FDA	the food and drug administration
FLIM	fluorescence lifetime imaging
FWHM	full width at half maximum
g	grams
h	thickness of the stratum corneum
hrs	hours
Hz	hertz

IPB	isotonic phosphate buffer
IR	infrared
IRF	instrument response function
J	steady-state drug flux
$K_{SC/V}$	partition coefficient between the Stratum corneum and the vehicle
LOD	limit of detection
Log $K_{O/W}$	Log octanol-water partitioning coefficient
Log P	octanol-water partitioning coefficient
LOQ	limit of detection
Lys	Lysine
min	minute
mL	millilitre
µm	micrometre
MW	molecular weight
MP	melting point
nm	nanometre
NAD	nicotinamide adenine dinucleotide
N	number of samples
ns	nanosecond
OCT	optimal cutting temperature
Pal	palmitoyl
PEG	polyethylene glycol
PG	propylene glycol
pKa	aqueous ionisation constant

ps	picosecond
RNA	ribonucleic acid
ROS	reactive oxygen species
RP-HPLC	reverse phase high performance liquid chromatography
rpm	revolutions per minute
SC	stratum corneum
sec	second
SEM	standard error of the mean
SHG	second harmonic generation
SPC	single photon counting
STDEV	standard deviation
τ_1/ τ_2	short / long fluorescence lifetime component
3D	three dimensional
TCSPC	time-correlated single-photon counting
TEA	triethylamine
TEWL	trans epidermal water loss
TFA	trifluoroacetic acid
TGF	tissue growth factor
USA	United States of America
Val	Valine
UV	ultraviolet
V	Volts
Volpo/ Volpo N20	polyethylene glycol oleyl ether
W	Watts

Copyright Notices

Chapter One

Introduction

1.1 Introduction

As far back as 4,000 BC the ancient Egyptians used cosmetics, including cleansing creams and body scrubs [1]. The ancient Sumerians, Babylonians, and Hebrews also applied cosmetics. There is even mention in the Old Testament of face painting [2]. More recently in Europe a cosmetic known as Ceruse was used from the second century to the 19th century [3].

Over all these years women have been using whatever means possible to beautify themselves. More recently, the cosmetic industry has boomed especially in Europe, Asia and the USA. Now more than ever there is a growing demand from the public to "look younger for longer". Products sold under the banner of cosmeceuticals (a term referring to a cosmetic that directly combats aging through a biological effect) in the USA reached approximately $16 billion in 2007 and is estimated to exceed $21 billion in 2012 [4]. These products are claiming a beneficial effect at reducing aging, not just a way of masking the effects.

With this ever-growing market the cosmetic industry has focused on developing novel formulations that restore the skin's "youthful appearance" by reducing the signs of skin aging. However, on this quest a vital step has been neglected. Despite the availability of methodology, demonstrations of anti-wrinkle efficacy are few and far between. This has been a growing area for technological advancement but the justification for extrapolation between *in vitro* studies and the mechanisms *in vivo* is limited. Tests have been developed that can assess the mechanisms responsible for anti-wrinkle behaviour, such as increased collagen production, reduced cell muscle contraction and antioxidant behaviour. However, very few tests have been conducted to assess whether or not these anti-wrinkle actives actually penetrate through the skin to

their proposed site of action. It is important to remember that for an active ingredient to elicit its beneficial effects it must be stable in a formula, absorb into the skin, get to its site of action (generally the viable dermis) and be biologically active when it arrives at its target site. Penetration studies can indicate whether the ingredients are likely to reach their site of action in sufficient amounts to elicit an effect and / or cause harmful side effects. This information can be harnessed as a tool to predict a compound's anti-wrinkle potential before costly human trials are conducted.

The aim of the project is to examine the penetration of common anti-wrinkle compounds into and through the outer layers of the skin. The method could be used as a screening tool for cosmetic active ingredients, for only if an active penetrates the stratum corneum will it have the potential for anti-wrinkle activity. It could also be used as a regulatory technique because high concentrations of an ingredient permeating the skin can lead to toxicity. This could be important in formulations containing novel penetration enhancers which can propel even the smallest amount of active through the skin and accumulate greater amounts than a simple product with a large active concentration. Finally, the results from this thesis could be used to help elucidate the mechanism responsible for the anti-wrinkle effects caused by the compounds under examination, which in some cases is concentration dependent.

Once studies have been conducted to examine the penetration, *in vivo* clinical studies can be used to assess if the final product causes a reduction in the visible appearance of wrinkles and then whether this is further quantifiable as a reduction in the size and number of wrinkles. If an anti-wrinkle effect is seen it can then be determined whether this was likely to be caused by the 'active' ingredient or the vehicle (i.e. through moisturisation).

Furthermore, by coupling *in vitro* results with clinical studies *in vivo* an indication is given as to whether the anti-wrinkle effects are caused by more than superficial or surface effects, i.e. the claimed mechanism of action. The question may then arise, should the preparation be considered a cosmetic or a therapeutic product?

1.2 Skin Structure and Function

The skin is a multi-layered organ in which its outermost layer, the stratum corneum, acts as a barrier to the ingress of foreign substances [5]. The stratum corneum is a thin layer of keratinsed, epithelial cells creating a "dead" surface on the epidermis [6]. The stratum corneum is often described as a brick wall-like structure with protein filled corneocytes forming the bricks and an intercellular lipid matrix as the mortar, creating a highly impermeable barrier [7-8], as depicted below in Figure 1.

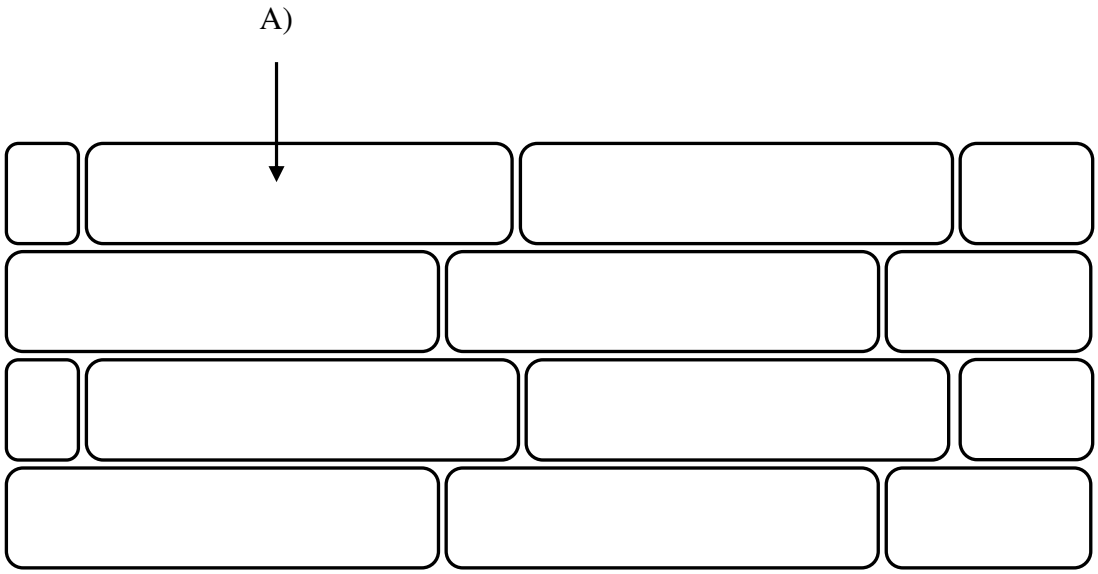

Figure 1. Schematic illustrations depicting the stratum corneum where, A), represents the corneocytes and the space between the corneocytes is the intracellular lipid matrix.

Due to the barrier properties of the stratum corneum, not all compounds are good candidates for topical administration. Therefore, the following considerations must be taken into account during the development and optimization of a topical formulation:

1. The compound(s) of interest must possess physico-chemical properties that are amenable to percutaneous penetration [9-12].
2. Poor inherent penetration of the stratum corneum can be overcome to some extent by the use of enhancement strategies. This could be achieved through the use of a penetration enhancer, which causes a modification of the skin's barrier function.

1.3 Aging in Skin

Skin aging results from a combination of both intrinsic and extrinsic factors. Aging due to intrinsic factors can essentially be attributed to the passage of time alone [13-14] and is a consequence of genetically-determined processes. Such processes include cell aging, which can lead to a decreased functionality of the skin through changes in collagen conformation, elastin degradation and alterations in the lipid matrix surrounding the corneocytes in the stratum corneum [15]. Intrinsic factors also include mechanical processes. For instance, the frequency of muscle contractions may increase with age in order to compensate for loss of muscle volume [16]. This excessive stimulation results in muscle fibres pulling the skin of the face "inward", which can ultimately lead to the formation of deep-set wrinkles, also known as expression lines.

Aging due to extrinsic factors results from external influences contributing to the skin aging, this is mainly caused by ultraviolet (UV) radiation, but can also be caused by environmental pollutants and even through smoking [16-18]. These factors can lead to the formation of free radicals and reactive oxygen species [13], which can damage

lipids, proteins, and DNA in the skin [14]. Both intrinsic and extrinsic aging of the skin can result in the formation of wrinkles.

1.4 Mechanisms of Anti-Wrinkle Compounds

The most common mechanisms proposed for anti-wrinkle compounds include: antioxidant behaviour, muscle relaxant effects and collagen regeneration. An agent can either act as an antioxidant directly or, in the case of niacinamide, increase the production of the endogenous antioxidants. Antioxidants mop up or neutralise reactive oxygen species (ROS) such as free radicals that cause tissue damage through oxidation reactions (i.e. collagen oxidation) [15, 19], and can also chelate metal ions. ROS can react with DNA, proteins and unsaturated fatty acids and they can also deplete endogenous skin antioxidants. It has also been shown that antioxidants such as vitamin C and E can have a synergistic effect when used in combination, providing greater than four times their effectiveness alone [20].

Stimulation of collagen production is the proposed mechanism for reduction of facial wrinkles for a number of compounds. Ascorbic acid [21] has shown longevity in the cosmetic industry as an anti-aging ingredient. It acts directly through the activation of the collagen gene (increasing the transcription rate and stabilising pro-collagen messenger RNA). Indirectly it acts by initiating lipid peroxidation, which produces a by-product (malondialdehyde) that stimulates collagen gene expression [21]. More recently marketed ingredients include: pal-KTTKS [22], estrogen and phytoestrogens i.e. genistein, which have been used to reduce skin aging in post-menopausal women. Collagen activation is one of the reported mechanisms of these ingredients [23], and is also another reported mechanisms of action of niacinamide [24].

Botulism Type A toxin (Botox®) injections have been proven to be very effective for reducing and even eliminating deep set wrinkles by the paralysis of facial muscles [25]. There has, therefore, been a search to find a topical alternative that utilises the same mechanism of action. At present, active ingredients claiming to exert a "Botox®-like" effect include acetyl hexapeptide-3 (Argireline®) and hydrolysed hibiscus esculentus extract (Myoxinol®) [26-27]. Although the mechanism by which hydrolysed hibiscus esculentus extract inhibits muscle contraction is not known, acetyl hexapeptide-3 has been found to cause a muscle relaxant effect by inhibiting neurotransmitter release [27].

As mentioned previously enhancement strategies can be incorporated into a product to increase the penetration of an active. In doing so this is likely to increase the effectiveness of the active ingredient. The vehicle, the concentration of the active in the product, the properties of the active and the mechanism of action all contribute to how effective the product is at reducing facial wrinkles.

1.5 Cosmeceutical

Anti-wrinkle ingredients utilising any of the above mentioned mechanisms of action are commonly characterized as "cosmeceuticals". The term "cosmeceutical" was coined by Dr Albert Kligman in 1993 [28]. It was developed to describe a group of products that are a cosmetic-pharmaceutical hybrid intended to enhance a person's beauty by the use of ingredients that provide an additional health-related function or benefit [3].

Although the term "cosmeceutical" has been extensively used by cosmetic companies, dermatologists, in literature and is even recognised by the general public,

the term has no legal standing with various regulatory authorities, including those in Australia, the United States of America, the United Kingdom, the European Union, Japan and New Zealand. In these countries a product is recognised as a drug, therapeutic good, medicine or a cosmetic. In fact, the FDA states it does not recognize any such category as cosmeceutical [29]. Only two countries, Japan and New Zealand, have developed a classification of products in this so called 'grey area' between cosmetic and therapeutic products, known as "quasi-drugs" and "related products" respectively [30].

Under the regulations of the above mentioned regulatory bodies, anti-wrinkle "cosmeceutical" products fall under the category of cosmetics. Under USA and UK regulations a therapeutic product is defined as: a substance "affecting the structure and function of the body" and "modifying physiological functions by exerting a metabolic action" [31]. Anti-wrinkle "cosmeceutical" products, therefore, should become classified as drugs. Under these regulations even moisturisers would be classified as a therapeutic product. Due to the limited regulations associated with the classification of cosmetics, the consumer can not be guaranteed a product containing a "cosmeceutical" ingredient will deliver its advertised claims. There is a driving force from cosmetic manufacturers in favour of classifying these so-called "cosmeceutical" anti-wrinkle products as cosmetics. This is because prior to market release therapeutic products are subjected to rigorous and expensive safety and efficacy requirements. The time and costs for product development would be so great cosmetic companies would simply avoid this area of ingredients, which is exemplified in the case of tretinoin.

1.6 Commonly Utilised Anti-Wrinkle Compounds

There are an incredibly large number of ingredients on the market claiming anti-wrinkle benefits and more are being developed and / or released every day. However, anti-wrinkle ingredients can generally be separated into the following groups: peptides, vitamins, antioxidants and hormones.

Retinols, also known as vitamin A and its derivatives, are the most well tested and documented compounds shown to produce anti-wrinkle activity. Tretinoin is the most effective form of vitamin A and was first shown to have anti-wrinkle benefits in 1986 [32]. Tretinoin is used as the "gold standard" in efficacy comparisons [15, 33]. However, the major problem with its use in products is it has a high incidence of irritation. Despite the effectiveness of tretinoin as an anti-wrinkle ingredient it has been classified as a prescription only medicine by both Australia and the USA [34-35].

Recently much research has focussed on the use of peptides to target some of the causes of skin aging. As the understanding of the biological processes of skin aging improves, a more targeted design of active ingredients will follow leading to an influx of peptide based products. New peptide cosmetic ingredients include, but are not limited to: hydrolysed hibiscus esculentus extract, acetyl hexapeptide-8 and pal-KTTKS [22, 26-27].

Table 1 provides information on: the mechanisms of action, clinical trials performed to demonstrate efficacy and penetration studies, for common anti-wrinkle compounds used commercially. The table shows few studies have been conducted assessing the penetration of common anti-wrinkle ingredients, with or without penetration enhancers. Penetration studies indicate whether the ingredients reach their sites of action, if there is enough to elicit an effect and / or cause harmful side affects.

Table 1: Commonly utilised anti-wrinkle compound

Group	Active	Mechanism of action	Clinical Efficacy	Penetration
Peptides	Hydrolysed Hibiscus esculentus extract	Muscle relaxant and antioxidant behaviour [26].	1% resulted in a 26% reduction of wrinkle depth after 3 weeks of treatment; analysed by image analysis with digital processing, n= 12 [26].	Not available
	Hibiscus Abelmoschus	Protection against degradation of FGF-2, increasing cell proliferation, increasing GAG synthesis and activation of collagen production [36].	3% resulted in 28% improvement in skin elasticity, 61%, 18% and 29% improvement in skin firmness, texture and density respectively over the placebo and a 1.8 fold reduction in wrinkle depth over the placebo, n=60 [36].	Not available
	Acetyl hexapeptide-8	Muscle relaxant, through inhibition of the SNARE complex [27].	10% concentration reduced wrinkle depth by ~30% upon 30 days of treatment; analysed by confocal laser scanning microscopy of silicon impressions, n=10 [27].	30% permeation in donor cell after 2hrs, through human stratum corneum using Franz diffusion cells [27].
	Palmitoyl pentapeptide, palmitoyl tripeptide-5	Stimulates collagen production [22, 37].	3ppm caused a significant improvement vs. placebo in the reduction of wrinkles/ fine lines by both quantitative computer image analysis and visual assessment over 2 and 3 months, n=93 [22]. 5ppm caused a significant improvement over the vehicle in surface roughness, wrinkle volume and wrinkle depth after 2 and 4 months; determined through quantitative image analysis of skin replica's, n=60 [38]. 3ppm significantly reduced fine lines / wrinkles; determined by quantitative analysis of facial images, and improved the appearance of fine lines/ wrinkles over the placebo; determined by visual assessment. seen after 2 and 3 months respectively, n=42 and 35 [39-40].	Not Available
	Cu-GHK	GHK is a fragment of dermal collagen and Cu is required for activity of lysyl oxidase in collagen synthesis [41-43].	The complex in a facial cream reported an improvement in fine lines and wrinkles and increases in skin density and thickness [44]. 2% resulted in an improvement in skin thickness, hydration, smoothness, and wrinkles over 12 weeks [45].	Not available
Vitamin	Vitamin A (retinol and derivatives)	Antioxidant [15, 46-47], stimulates proliferation and extracellular matrix production (increases collagen production) and	Tretinoin: 0.1% resulted in an 80% increase in collagen I formation from human biopsies, n=26 [51]. 0.05% caused a significant improvement in fine wrinkling, coarse wrinkling and roughness	Tretinoin: in a receptor solution of HEPES buffered with HHBSS resulted in a flux of 0.0014 ± 0.0010 $\mu g/cm^2/hr$ through rat dorsal skin, using Franz

		interferes with collagen breakdown [48-50].	over 3-12 months, n=15-349 [32-33, 52-57] this was confirmed by skin replica analysis, n=149 [58]. Although in one study 92% of participants recorded dermatitis, n=15 [52]. Tazarotene: 0.1% caused a significant improvement in fine wrinkling over 3- 6 months, n=10-563 [55-56, 59-62] with ~80% of participants showing at least 1 grade improvement over baseline, n=131 [56, 62]. This was confirmed by silicon mould digital analysis with a significant reduction in the no. of peaks and valleys, n=10 [60]. Retinol: 0.04% showed a significant reduction in roughness, mean depth of wrinkles and maximum wrinkle depth after 3 months; determined by replica optical shadowing analysis, n= 126 [63]. 0.1% caused a significant improvement in fine wrinkling; determined by visual assessment over 2-9 months, n=36 and 48 [64-65]. 0.4% significantly reduced visual appearance of photoaged skin after 6 months of treatment with 3 treatments per week, n=36 [66]. Isotretinoin: 0.05% caused a significant improvement in fine wrinkling; based on visual assessment, n=346, 776 [67-68]. This was confirmed as a significant improvement was found in the distance between highest peak and the lowest valley and the distance between all valleys and peaks from the mid-line using profilometry by replicas, n=346 [68]. 0.1% caused a significant improvement in fine wrinkles over the vehicle after 6-9 months, n=149 and 326 [58, 69]. Retinaldehyde: 0.05% significantly reduced skin wrinkling and roughness after 4.5 months and was well tolerated, n=125 [33]. Retinyl Propionate: 0.15% was found to be ineffective in treating photoaged skin after 12 months, n=60 [70].	diffusion cells occluded, ~0.02% cumulative of the applied dose [71]. 0.5% α-tocopherol acetate in a receptor solution of pH 7.4 with Brij® 98, recorded no permeability after 24 hours through full thickness pig skin using Franz diffusion cells, occluded. Tape stripping revealed 70% remained in the stratum corneum, 55% reached the epidermis and 35% the dermis. Pickering emulsion gave a 5 fold increase in absorption [72]. In a receptor solution of 50% hydroalcoholic a steady state flux ranging between 707.8 ± 115.5 ng/cm^2/hr to 7,685.79 ng/cm^2/hr was obtained with silicon nano particles and lecithin emulsion carriers respectively, through full thickness cellulose acetate membrane using Franz diffusion cells [73]. 0.025% w/w tretinoin in a receptor solution of pH 7.4 phosphate buffered physiological albumin resulted in ~30% of initial dose permeating after 48 hours through rat abdominal skin using Franz diffusion cells [74]. 0.3% tretinoin penetrated ~5µm into the skin without enhancers using Raman Spectrometer laser [75]. Retinol: in a receptor of phosphate buffered solution pH 7.0, recorded no permeability at 24 hours using Franz diffusion cells non occluded [76]. Percutaneous absorption of 1-2% of the applied dose was determined; the method was not stated [77]. Isotretinoin: Plasma levels were taken over the study, no trend was seen towards a

				continuing increase of the levels of isotretinoin or it's metabolites; determined through blood samples [47].
	Vitamin E/ Vitamin E esters	Antioxidant in the lipid phase of cells protects cells against lipid peroxidation caused by free radicals, however, only in form of α-tocopherol [15, 78-79].	Tocopherol: 5% significantly reduced skin wrinkling cause by UV exposure after 6 weeks, in studies on mice [80]. α-tocopherol and tocopherol sorbate: 5% of each significantly reduced skin wrinkling caused by UV exposure compared to the vehicle as early as 7 weeks over a 22.5 week study on the dorsal skin of hairless mice [81]. tocopherol acetate: 5% significantly reduced skin wrinkling compared to the vehicle at weeks 7-12 of a 22.5 week study on the dorsal skin of hairless mice [81].	α-tocopherol: 5 mg/cm^2 applied to hairless mice for 24 hours resulted in a 62 fold increase in the epidermis and 22 fold increase in the dermis of α-tocopherol determined by extraction after sacrifice [82]. 0.5% [^{14}C]-D-α-tocopherol, resulted in a recovery from the receptor of ≤0.02% of the dose after 24 hours for o/w and w/o emulsion, liposome dispersion and hydrogel as vehicle through human skin *ex vivo* flow through finite dose diffusion studies. 2.5-10.4% [^{14}C]-D-α-tocopherol was recovered in the epidermis, the majority in the SC [83]. α-tocopherol acetate: 5% was absorbed and retained in the skin, after application on hairless mice for 2 weeks. The skin concentration of α-tocopherol acetate increased from 0 to 154.1 µg/ml and α-tocopherol increased 7 fold [84].
	Vitamin C (ascorbic acid)	Biological active form is L-ascorbic acid antioxidant activity protects water parts of cells, regenerates vitamin E and stimulates collagen synthesist [15, 78-79, 85-86].	5% vitamin C resulted in a significant improvement in fine and coarse wrinkling; determined by visual assessment, confirmed by a 6.7% reduction in arithmetic mean of roughness and a 5% reduction in the peak to valley mean roughness after 6 months over the placebo; analysed via laser profilometry n=20 [87]. 10% ascorbic acid, 7% tetrahexylated ascorbate in an anhydrous polysilicone gel base showed a significant reduction in wrinkling compared to baseline after 3 months, this was not significantly different compared to the vehicle, n=10 [88]. 3% ascorbic acid o/w emulsion resulted in a significant anti-wrinkle effect over the placebo;	L-ascorbic acid levels measured through punch biopsies of pig skin resulted in a maximum penetration from 20% L-ascorbic acid pH 3.2 of ~1100 pmol/mg [90]. 8% ascorbic acid in a novel formula after 2 hours of application to human skin, ascorbic acid levels rose 8.5 times the normal tissue value, this dropped off after 8 hours; determined by microdialysis [91]. Hairless mice studies using a Franz type diffusion cell system with receptor solution of 50 % glycerine

			determined from silicon mould data measured using PRIMOS fringe projection and 3D topography as early as 1 month, this was confirmed by visual assessment showing a significant improvement in fine lines and wrinkles after 3 months n=23 [89].	aqueous, 12 mg/ml vitamin C, resulted in a steady state flux of 3.43 ± 0.74 µg/cm^2/hr [92]. Infinite study of vitamin C in a receptor of glycerine : water pH 4 resulted in a flux ranging between 0.077 to 0.108µg/cm^2/min depending on the o/w/o vehicle type study over 4 hrs through cellulose acetate membrane using a Franz dissolution cell dialysis [85].
	Vitamin B_3 (niacinamide)	Procurer to NAD(P)H a potent antioxidant. Improves lipid barrier function by increasing; ceramide, free fatty, keratin, filaggrin and involurin, which leads to a reduction in TEWL. Also stimulates collagen synthesis through fibroblast activation [19, 93-94].	5 % after 3 months caused a reduction in fine lines and wrinkles by 5.5% versus the control; analysed by image analysis with computer processing and is confirmed by visual assessment, n=50 [19, 93].	Nicotinic acid: absorption across human skin was found to be 0.7 ± 0.2% of the applied dose at 72 hr; determined with flow through diffusion cells [95]. Niacinamide: absorption into the receptor fluid at 24 hours was found to be 28.8% of the starting dose; analysed using flow through diffusion cells [96]. At 24 h ~1-3% of the starting dose was measured in the receptor fluid after application of an o/w emulsion containing niacinamide determined with Franz diffusion cells [97]. 200mg/ml in a receptor solution of 0.9% NaCl using Franz type diffusion cells resulted in a flux of 18.08 ± 5.49 µg/cm^2/hr through rabbit ear skin and 77.06 ± 13.58 µg/cm^2/hr through pig ear skin [98].
Antioxidant	Co enzyme Q_{10} (ubiquinone, idebenone)	An endogenous cellular antioxidant present in almost all tissues of the body. Some research has shown that tissue levels of Co enzyme Q_{10} decrease with age [15, 78-79].	Udebenone: 0.5% resulted in a 23% reduction in skin roughness and 27% reduction in fine lines / wrinkles via visual assessment over 6 weeks, n=21. 1% resulted in a 26% reduction in skin roughness and 29% reduction in fine lines / wrinkles via visual assessment over 6 weeks, n=20 [99].	Not available

	Flavonoids (e.g. quercetin, hesperetin, naringenin, genistein and diadzein, grape seed extract, green tea extract)	Antioxidant activity [15, 78, 100-102]. Soy isoflavones stimulate hyaluronic acid which leads to water retention. Also processes estrogenic activity [103-105].	Green Tea extract: 10% showed no significant effect on the visual assessment of wrinkles after 8 weeks, n=40 [106].	Quercetin: 1% emulsions; percutaneous absorption studies did not detect quercetin in the receptor medium after 12 hours using pig ear skin determined via Franz diffusion cells [107]. Cumulative amount permeating after 24 hour through excised human skin was found to be 4.63-10.28 ± 0.95-1.96 % of the dose. Hesperetin was found to have 3.75-8.13 ± 0.99-1.70 % permeating of the applied dose. Naringenin and Quercetin was found to have 0.59-0.91 ± 0.07-0.15 % permeating of the dose. Assessed via Franz diffusion cells [108-109]. Genistein: human skin tested via Franz type diffusion cells with a receptor solution of 0.9% NaCl soln/TR (80/20 v/v) resulted in 7.1 ± 0.8 µg/cm^2/hr in PEG 400 for pure Genistein and a flux of 0.5 ± 0.1 and 1.3 ± 0.4 µg/cm^2/hr for a saturated solution of dry extract from propylene glycol (PG) and PEG 400 respectively. Daidzein: under the same conditions resulted in a flux of 1.7 ± 0.8 µg/cm^2/hr for pure daidzein from PEG 400 and a flux of 0.4 ± 0.2 and 1.0 ± 0.5 µg/cm^2/hr for a saturated solution of dry extract from PG and PEG 400 respectively [110].
	Fucose	Antioxidant, causes a down regulation of skin matrix degrading enzymes, increases skin elastin biosynthesis and improves collagen fibrillogenesis [111-113].	1% fucose-containing cream resulted in an average improvement of 28% of the WI (effect on cumulative length of wrinkles and average wrinkle width) after 4 weeks of treatment; analysed by image analysis with computer processing n= [111].	1% radio active labelled L-fucose had an average of 0.046 nCi/ mg (radioactive units) over 6 hours in the epidermis (5-50µm depth) of rat skin while having an average of 0.272 nCi/ mg in the dermis (55-800 µm) over 6 hours; determined through skin biopsies. Results indicate L-fucose is penetrating rapidly into the epidermis [114].

	Alpha-lipoic acid	Lipid and water soluble antioxidant that can chelate a number of transition metals and regenerates other antioxidants such as vitamin E and C, through an indirect route [115].	5% after 3 months resulted in a statistically significant improvement in fine lines though visual assessment, which was verified in a significant average reduction in the skin roughness over the placebo; determined via laser profilometry, n=33 [116]. 5% with 3% dimethylaminoethanol (penetration enhancer) twice daily applications for 12 weeks resulted in an average of; 83% improvement in fine lines, 46% improvement of medium depth wrinkles and 29% improvement in deep lines; analysed by image analysis with visual assessment n=14 [117].	At 4 hours, the α-lipoic acid recovered was found to a distribution of: ~95% in the stratum corneum, ~1% in the residual epidermis and ~4% in the dermis and subcutaneous fat in hairless mice determined through tape stripping [118].
Hormones	Estrogens (estriol, estradiol, progesterone and oestrogen)	Thickens elastic fibres and stimulates collagen metabolism [15, 119].	Estriol: 0.3% treatment resulted in a 12.5 µm reduction in the overall skin roughness after 6 months; determined through mechanical profilometry, and an 80% visual improvement in wrinkle depth after 4 months; determined by image analysis with visual assessment, n=8. Estradiol: 0.01% treatment resulted in a 13.8 µm reduction in the overall skin roughness after 6 months; determined through mechanical profilometry, and an 80% improvement in wrinkle depth after 4 months; determined via image analysis with visual assessment, the study was not controlled, n= 10 [119]. Progesterone: 2% did not result in a significant reduction in wrinkle size compared to the control over 4 months; determined via image analysis with visual assessment, n=40 [120]. Oestrogen: 0.0625% over 6 months did not show any significant difference between the product and the placebo; determined through mechanical profilometry, n=54 [121].	Estradiol: 0.1% and 0.6% after 24 hrs resulted in 18.2 ± 3.5% and 17.4 ± 4.8% recovery of the applied dose through human epidermal membrane in Franz diffusion [172] 100 µg in gel had a recovery of 41.7% of the applied dose after 6 hrs through human epidermis and 22.9% through porcine skin in flow through diffusion [173] 1% in PG through hairless mice skin resulted in a flux of 0.2±0.02 µg/cm^2/hr in Franz diffusion [174] 5µg/cm^2 after 16 hrs resulted in a recovery of 18.00 ± 1.15% of the applied dose through full thickness mice skin in flow through diffusion [175] 4µg/cm^2 in an acetone solution resulted in 0.056%/hr recovery in urine between 12-24 hrs in vivo human assay [176] Oestradiol: saturated solution aqueous and 90% PG/water resulted in a flux of 8.34-11.5 and 72.8-286 ng/cm^2 respectively in flow through diffusion with human epidermal membrane [177] Progersterone: 4µg/cm^2 in a acetone solution resulted in 0.264%/hr recovery in urine between 12-24 hrs in vivo human assay [176]

Table 1 demonstrates the difficulty for consumers in comparing the capabilities of anti-wrinkle compounds as the methods used to measure efficacy vary immensely. This makes direct comparisons regarding product effectiveness difficult, if not impossible, raising the question: is this a conscious decision on the part of manufactures?

1.7 Techniques used to Study the Efficacy of Anti-Aging Compounds

1.7.1 *In Vitro* Methods

In vitro methods have been used to assess the effectiveness of potential anti-wrinkle compounds before conducting studies on human subjects. Human studies can be time consuming, costly and highly variable. *In vitro* methods include: assessment of antioxidant behaviour, muscle cell activity, collagen production and testing the diffusivity of a compound through the skin to determine whether it will reach the site required to carry out the above mentioned anti-wrinkle effects.

Antioxidant capacity can be assessed via a number of tests both chemical and biochemical. The Fenton reaction is a test to detect non-enzymatic autoxidation. If an antioxidant reacts with the $HO^{\cdot}$ radical it reduces the overall optical density at 523 nm, the degree of reduction indicates the effectiveness of the antioxidant [26, 122]. The anti $DPPH^{\cdot}$ test involves measuring the optical density of a solution of $DPPH^{\cdot}$ with the suspected antioxidant. $DPPH^{\cdot}$ forms a purple solution, which becomes transparent when a free radical scavenger is present. The absorbance at 517 nm is measured and the difference between the test sample and the control is calculated [26, 123]. The Luminol method assesses the antioxidants effectiveness at reacting with O_2^{-} radicals [26, 124].

Glutathione, in its reduced state (GSH), protects cell membranes and the stratum corneum from oxidation. Therefore, a compound that increases the concentration of GSH will support the cells natural defence against oxidation. This test is commonly conducted using MRC_5 human fibroblasts. Once the suspected antioxidant is applied to the fibroblasts the fluorescence can be determined to assess relative glutathione concentrations [26, 100].

The effects of a compound on collagen production can be tested *in vitro*. This can be done using skin specimens or cell cultures. Pro-collagen protein levels are assessed via western blot analysis and / or immunohistology (i.e. mouse monoclonal IgG1 antibody) [48, 51] before and after the compound under investigation has been applied [48-49, 125-126]. Alternatively, a staining agent such as picro-Sirius red can be used, where the degree of staining is assessed either by optical density determination or based on a 6-point scale (i.e. 0 = no staining 3 = moderate staining and 5 = maximal staining) [51, 113]. Collagen synthesis could also be determined by evaluating the ratio of ^{3}H-hydroxyproline : ^{3}H-proline through HPLC coupled with fluorescence detection [113].

The ability of an anti-wrinkle compound at causing muscle relaxant effects can also be studied *in vitro*. This can be done through cell culture experimentation, performed on chromaffin or muscle cells cultures [27]. The frequency of spontaneous muscle contractions can be measured over a specific time interval i.e. 30 secs, with muscle cells seeded with motor neurons. The frequency is measured immediately before treatment with the test product, then again at a specific time interval preceding treatment, i.e. 1 min [26]. Alternatively, products efficacy at modulating neurotransmitter release, namely by vesicular fusion known as the SNARE complex or catecholamine release, can be assessed on chromaffin cell cultures. Inhibition of these

factors indicates inhibition of neurotransmitter release, therefore, demonstrating a products ability to emulate the mechanism of action utilised by Botox® injections [27].

Another important *in vitro* method is determining the penetration of the anti-wrinkle compound through the skin. Penetration studies can indicate the ability of a compound to cause its anti-wrinkle mechanism of action without time consuming and expensive *in vivo* studies. Without penetration it is unlikely the compound can illicit the anti-wrinkle effect. There are a number of techniques which can assess compounds percutaneous absorption characteristics. Surface recovery is a method where the loss of material from the surface is determined as it penetrates into the skin over a certain time. Penetration, however, has generally been assessed by either diffusion cells (flow through or static) or through the tape stripping method [127-129]. Flow through diffusion cells consist of an upper half (donor cell) and an open receptor cell where the solution can be collected and analysed for active concentration at varying time points. The skin, be it human or similar to mimic human, is placed between the two halves [138]. Permeation can also be assessed via tape stripping, where a compound is applied to the skin of a subject or skin sample for a certain amount of time, and then adhesive tape strips are applied to the area removing stratum corneum layers. This method assesses the distribution of the compound into the layers of stratum corneum [139-140]. Little data has been published concerning the penetration of anti-wrinkle compounds via any of the penetration analysis methods, as mentioned previously and evident in Table 1.

The method estimates absorption based on a biological / pharmacological response *in vivo* i.e. inflammation, narrowing of the blood vessels. Total absolute bioavailability measures the amount of compound in blood and urine after topical administration [127-128]. Attenuated total reflectance fourier transform infrared (ATR-

FTIR) spectroscopy involves the diffusion of a permeant being monitored by the appearance and increase of a compound-specific IR absorbance, recorded as either a function of time or depth into the skin [129]. Suction blisters involve applying a controlled suction onto the skin, which raises the skin causing the separation of the stratum corneum from the underlying dermis. The blister fills with interstitial fluid, which can be tested for concentration of the compound [130-131]. Microdialysis is a technique, which introduces an ultra thin, semipermeable tube, in the dermis. The tube has a steady flow of a tissue-compatible fluid through it and the compound is able to passively diffuse into the tube. The fluid is sampled at regular intervals and analysed [132-133]. Punch biopsy analysis involves removing a small section of the skin and calculating the concentration of the compound within the removed section [134-135]. Recently new microscopic techniques have been developed that can measure the amount of a compound within human skin, these methods rely on confocal or multiphoton microscopy [136-137].

As of yet intensive toxicology studies are generally not performed on cosmetic products, this is because it is commonly assumed the product is to be applied to the skin surface where the ingredients will remain to elicit their effects. A cosmetic is generally regarded as safe if its application does not result in skin irritation. However, with the introduction of “cosmeceutical” products (a cosmetic that contains biologically active ingredients) more serious health risks could become prevalent. The issue arises since the biologically active ingredients must penetrate the skin to generate the desired effect. Ultimately, the ingredient may reach the blood stream and has the potential to cause toxicity not encompassed by a skin irritation response.

1.7.2 *In Vivo* Methods

For a long time simple procedures have been employed to assess changes in skin topography. A method know as Image Analysis can be used, which involves capturing an image of either the skin or a silicone replica. The image can then either be visually assessed for change in wrinkle appearance over time [22, 47, 59, 87, 116, 141-145] or assessed via a digital / computer processing method [19, 22, 26, 47, 111, 145-147]. Alternatively, a silicone impression can be taken of the skin surface and analysed under a microscope, referred to as Silicone Skin Replication. This can be done either in the horizontal plane, where a cross section is analysed [148] or in the vertical plane, where the base of a wrinkle and the peak of the wrinkle are focused separately and the reading difference is equivalent to wrinkle depth [149]. Finally, skin biopsies can be taken and then analysed [150], although the test area is limited to reduce scaring and the technique is invasive.

More sophisticated methods of quantitatively measuring skin topography have been developed recently, and commonly referred to as profilometry. These methods are applied either directly on the skin or indirectly, analysing silicone replicas. The limitation with silicon moulds is the possible lack of homogeneity between and within replicas and also the replica underestimating wrinkle depth [150]. If the surface is too smooth or the furrows are too numerous and/or deep, it can become difficult to detect differences in these parameters. Profilometry can be measured using the following techniques: optical, mechanical, laser, transmission, interference fringe and electrical analysis methods. The information presented in Table 2 allows for comparisons to be made between the sensitivity, cost (based on quotes from 2008 / 2010), accuracy, advantages and limitations of the techniques.

Table 2: Common *in vivo* wrinkle analysis techniques

Technique	Sensitivity (mm)	Cost (Australian dollars)	Accuracy (mm)	Advantages	Limitations
Visual Image Analysis	At least average of three assessments, commonly based on a 6 point scale.	Camera $500-$1,500, Tripod $50 and lighting ~ $50 - $500 [151] and computer ~$3,000 **Total** ~$5,050	Qualitative. 62 % reproducibility [152]	An inexpensive, rapid method that directly assesses improvement of the entire face. Real time and objective technique.	Based on perception
Digital/ Computer processed Image Analysis	1-30 [22]	Same as Visual Image Analysis but with computer program ~$500-1,000 [153] **Total** ~$6,050	~0.1 % uncertainty [146]	A relatively inexpensive, rapid, quantitative method that directly analyses the skin and the entire face.	Although the method is more accurate than Visual Image Analysis and provides an objective assessment of wrinkle appearance, the method can not assess wrinkle depth. 2D assessments only.
Silicone Skin Replication (microscopic assessment)	0.01 [148-149]	Impression material ~$200 Microscope ~ $14, 000 computer software stand and camera ~ $13,000 **Total** ~$27,200	10^{-2} [148-149]	A simple and reasonably accurate method that produces quantitative results with easily accessible, relatively inexpensive equipment. Measures the change in depth of wrinkles not only visual changes at the surface as with digital image processing.	The method is restricted to analysing silicone moulds, which add errors associated with replication of skin surface. Underestimates wrinkle depth, as the replica material can not penetrate fine parts of wrinkles. The type of replica material used can accentuate or diminish the actual surface topography [154]. Bubbles may form in the silicone which can affect the depth accuracy. The direct contact to the skin surface can change the surface being measured [155].
Optical Profilometry	0.0125-0.8 [154, 156] large heights may be detected depending on the system.	~$42,000 for the Primos 3D pico [156].	10^{-3} [154, 157-158]	Immediate analysis, rapid data acquisition time and can assess the skin directly. The method can be used directly on	Issues may arise if the skin is directly analysed, such as the diffusion of light within the skin can effect wrinkle morphology, the

				the skin or with silicon moulds. 3D images can be created rapidly.	projection of light at a constant angle on facial skin is not easily attainable and epidermal pigmentation, such as hair and the red colour of blood generates noise that reduces the accuracy of measurements [154]. The method is limited to the field of vision (30x24mm). A common problem with triangulation is the existence of shaded (hidden) areas that go uninspected [159].
Laser Profilometry	0.0025-1 [150, 160]	~$163,000 for the HEW250HS [160]	10^{-2}-10^{-5} [160-161] depending on the system.	Rapid analysis time and 3D images produced.	Expensive method which is susceptible to measuring errors [150]. If assessing the skin directly it can be dangerous around the eye area, therefore, limited to mould analysis. Due to the shadow effects caused by the incident light not being perpendicular to the surface, it is impossible to measure high aspect ratio surfaces. Lateral resolution is limited to 30 µm, sometimes to 10 µm, therefore, it makes it difficult to accurately measure skin roughness.
Mechanical Profilometry	0.08-0.32 [162]	Not available at time of publication	$<10^{-3}$ [161]	High precision and reproducibility, a relatively inexpensive method. Can test steep angles (depending on the device). Originally developed for the metal industry.	The probe can not be in direct contact with the skin because the pressure of the stylus creates plastic deformation of the skin surface [163]. This could also deform soft silicone moulds over time. Another mould would need to be taken with a harder substance;

					this will add larger errors with regard to underestimating wrinkle size. Images normally taken in 2D, the acquisition time is slow, especially if 3D imaging is performed.
Electrical Profilometry	0.001-0.01 [165]	~$3,500 [164]	10^{-2} [165]	A relatively inexpensive method with a rapid generation of results. This method was originally developed for fingerprint analysis.	A relatively new device with little published data available. Vellus hair can hinder analysis as these appear white. Measures variations in the skin at a micrometer level, this method is not suitable for measuring large wrinkles at this stage.
Interference Fringe Profilometry	0.004-0.1 [157]	~$84,000-100,000 for the Xi100Plus and the OPTOSURF[166-167]	5.10^{-3} [157]	Good compromise between acquisition time (1 min), ease of repositioning and accuracy [157]. A stitching technique, which allows for large areas to be analysed.	As the skin is transparent to light, problems may arise *in vivo* for smaller wrinkles. Difficulty on high angular surfaces or surfaces where the interference pattern line is difficult to see. Limited in examination area and the method measures at the micrometer level, which is not suitable to analyse large wrinkles.
Transmission Profilometry	0.01-0.02 best [168], max furrow depth of 0.5 mm [150]	~$33,000 for SV600 [169]	10.10^{-3} [150, 157]	Rapid measurement and analysis.	The method uses fragile moulds (thin print) and the replication process is multi step which is susceptible to error [150].

Optical Profilometry is based on projecting light onto the skin / replica and detecting variations in the reflected light. The method is dependent on the depths and angles of the wrinkles [21, 154-155, 163].

Laser Profilometry is an optical method based on the amplification and reflection of light from the replica, which is dependent on the surface of the skin. The technique can involve dynamic focusing, where the laser beam is permanently focused on the specimen and the height of the surface at that point is determined from the position of the mobile lens. Alternatively, the technique can involve triangulation (either single or double) as the height of the surface varies, the position of the light spot reflected onto the detector varies and, therefore, allows for the determination of height. In both methods the surface is scanned by mechanically moving the light over the replica to create a 3D map of the replica surface [27, 87, 142, 170].

Mechanical Profilometry consists of running a stylus (needle like appendage) across the surface of a skin replica. Vertical movements of the stylus alter the voltage of the output signal, this is converted into a digital signal, which can allow for the development of a 2D and eventually a 3D map of the replica surface [157, 171].

Electrical Profilometry involves micro-sensors that contain active capacitance feedback circuits that record capacitance based on contact with the skin surface. A large capacitance is recorded when the skin is close to the sensor and then a smaller capacitance the further away the skin is to the sensor i.e. a wrinkle (vice-versa for a replica) [165].

Interference Fringe Profilometry consists of calculating a phase image, which gives information on the height of the projections at each point, from interference fringe projections [157, 161].

Transmission Profilometry measures the variation of absorbance, associated with transparency, which relates to the thickness of the replica, the thicker the replica the larger the wrinkle depth [157, 168].

1.8 Conclusion

The anti-wrinkle cosmetic industry is growing rapidly as evident by cosmeceuticals sales reaching roughly $16 billion in 2007 in the USA alone [4]. Therefore, cosmetic companies will continue to develop new compounds, with innovative mechanisms of action, however the question arises, will these mechanisms be responsible for any anti-wrinkle effects?

From the information collected in this chapter it is clear, in general, cosmetic companies assess the effectiveness of an anti-wrinkle ingredient at producing a desired mechanism of action i.e. collagen regeneration, antioxidant capacitance or muscle relaxation. Tests for a specific mechanism of action are generally performed directly on the target i.e. specific cell cultures, skin specimens or specific chemicals such as free radicals. The entire product is then tested on human subjects for anti-wrinkle efficacy and generally compared against untreated skin. These experiments, firstly, ignore the largest barrier a compound has to over come before reaching its site of action, the stratum corneum. Secondly, the tests only show whether the product as a whole reduces wrinkles not the specific anti-wrinkle ingredient itself. Although superficial anti-wrinkle effects, such as moisturisation, do provide the desired consumer effect, the product is only providing a temporary solution, masking the effects of aging. The consumer will be required to continue using these types of products, which is desirable for the cosmetic company but not the consumer. As a mechanism of action, hydration has a limited efficacy potential for a product. Alternatively, if the product provides a "cosmeceutical" anti-wrinkle effect, such as collagen regeneration, the ingredient treats the cause of wrinkle formation, therefore, providing a much larger efficacy potential. A company spending money on product development and efficacy testing are not

distinguishable to those companies that questionably test their products. Therefore, it becomes a gamble for the consumer which product will deliver on its claims, if any.

The aim of this project is to examine the penetration and *in vivo* efficacy of some anti-wrinkle compounds commonly used commercially. Three ingredients will be selected to be examined. Selection will aim at choosing common anti-wrinkle compounds that represent the four groups shown in Table 1: hormones, peptides, vitamins and antioxidants. Penetration studies will be conducted on the test compounds from simple solutions to examine the maximum penetration potential, and from products i.e. creams, to indicate the compounds penetration potential from consumer products. Compounds will be selected, based on *in vitro* penetration studies for further investigation *in vivo*.

In vivo clinical studies will be conducted as contra-lateral split face clinical studies. These clinical studies will be used to assess whether the "Anti-Wrinkle Product" containing the ingredient under investigation markedly reduces or eliminates the visible appearance, size and number of wrinkles when compared to either untreated skin or skin treated with the identical formulation without the active ingredient, a control.

These experiments will investigate whether the anti-wrinkle compounds are likely to enter the skin and reach their site of action. Finally, a correlation between *in vitro* penetration and *in vivo* anti-wrinkle effects will indicate whether the anti-wrinkle effect could have been caused by the claimed mechanism of action.

1.9 Research Objectives

- To evaluate the penetration of a number of commonly used anti-wrinkle ingredient.

- From penetration studies select commercially used products containing one or more of the tested compounds to investigate the *in vivo* efficacy of the selected anti-wrinkle ingredients.

- Evaluate the correlation between *in vitro* permeation studies and effects seen *in vivo.*

1.10 References

1. Lucas, A., (1930) Cosmetics, Perfumes and Incense in Ancient Egypt. *J Egypt Archaeol*, **16**: 41-53.
2. Ezekiel, *The Old Testament*: **23**:40.
3. Dureja, H., et al., (2005) Cosmeceuticals: An Emerging Concept. *Indian J Pharmacol*, **37**: 155-158.
4. Market Trends: The U.S. Cosmeceuticals Market, June 1, 2008. Packaged Facts: A Division of Marketresearch.Com Available at http://www.packagedfacts.com/. Accessed 18 Jan 2010.
5. Idson, B., (1975) Percutaneous Absorption. *J Pharm Sci*, **64**: 901-924.
6. Scheuplein, R.J. et al., (1971) Permeability of the Skin. *Physiol Rev*, **81**: 702-747.
7. Barry, B.W., (1991) Lipid-Protein-Partitioning Theory of Skin Penetration Enhancement. *J Control Release*, **15**: 231-248.
8. Elias, P.M., (1983) Epidermal Lipids, Barrier Function, and Desquamation. *J Invest Derm*, **80**: 44-49.
9. Guy, R.H., et al., (1989) Selection of Drug Candidates for Transdermal Drug Delivery. Editors: Guy, R.H. and Hadgraff, J. Transdermal Drug Delivery: Development Issues and Research Initiatives, Marcel Dekker Inc., New York, USA, **35**, 59-81.
10. Guy, R.H., (1996) Current Status and Future Prospects of Transdermal Drug Delivery. *Pharm Res*, **13**: 1765-1769.
11. Flynn, G.L., et al., (1988) Percutaneous Drug Penetration: Choosing Candidates for Transdermal Development. *Drug Dev Res*, **13**: 169-185.

12. Oakley, D.M., et al., (1987) Effects of Ionization on the Percutaneous Absorption of Drugs: Partitioning of Nicotine into Organic Liquids and Hydrated Stratum Corneum. *J Pharm Sci*, **76**: 866-871.

13. Kosmadaki, M.G., et al., (2004) The Role of Telomeres in Skin Aging/Photoaging. *Micron*, **35**: 155-159.

14. Gilchrest, B.A. (Editor), (1995) Photodamage. Blackwell Science Inc., Cambridge Massachusetts, USA, 295.

15. Kerscher, M. (2003) Dermocosmetics: Cosmetics and Skin Care from Dermatological View- Current Trends in Dermocosmetics. Skin Care Forum **35**. Available at http://www.scf-online.com Internet Access Jan 2010

16. Rocquet, C., et al., (2002) Molecular Aspects of Skin Ageing-Recent Data. *Acta Derm Venerol*, **11**: 1-35.

17. Taylor, C.R., et al., (1990) Photoaging/Photodamage and Photoprotection. *J Am Acad Derm*, **22**: 1-15.

18. Davis, B.E., et al., (1993) Faces Going up in Smoke. A Dermatologic Opportunity for Cancer Prevention. *Arch Derm*, **129**: 907.

19. Bissett, D.L., et al., (2004) Topical Niacinamide Reduces Yellowing, Wrinkling, Red Blotchiness, and Hyperpigmented Spots in Aging Facial Skin. *Int J Cosmet Sci*, **26**: 231-238.

20. Lin, J-Y., et al., (2003) UV Photoprotection by Combination Topical Antioxidants Vitamin C and Vitamin E. *J Am Acad Derm*, **48**: 866-874.

21. Traikovich, S., (1999) Use of Topical Ascorbic Acid and it's Effects on Photodamaged Skin Topography. *Arch Otolaryngol-Head Neck Surg*, **125**: 1091-1098.

22. Robinson, L.R., et al., (2005) Topical Palmitoyl Pentapeptide Provides Improvement in Photoaged Human Facial Skin. *Int J Cosmet Sci*, **27**: 155-160.

23. Verdier-Sevrain, S., (2007) Effect of Estrogen on Skin Aging and the Potential Role of Selective Estrogen Receptor Modulators. *Climateric*, **10**: 289-297.

24. Gehring, W., (2004) Nicotinic Acid / Niacinamide and the Skin. *J Cosmet Derm*, **3**: 88-93.

25. Niamtu, J., (2003) Botulinum Toxin A: A Review of 1,085 Oral and Maxillofacial Patient Treatments. *J Oral Maxillofacial Surg*, **61**: 317-324.

26. Benoit, I., et al., (2004) Oligopeptides to Smooth from Hibiscus Esculentus Seeds Expression Lines. *SOFW Journal*, **130**: 52-59.

27. Blanes-Mira, C., et al., (2002) A Synthetic Hexapeptide (Argireline) with Antiwrinkle Activity. *Int J Cosmet Sci*, **24**: 303-310.

28. Kligman, A.M., (1993) Why Cosmecuticals? *Cosmet Toiletries*, **108**: 37-38.

29. FDA/ CFSAN. "Cosmeceutical." U.S. Food and Drug Administration, Centre for Food Safety & Applied Nutrition, Office of Cosmetics and Colours Product Information Sheet, 2002. Available at http://www.fda.gov/ Cosmetics/ProductandIngredientSafety/ProductInformation/ Internet Accessed Jan 2010.

30. Cosmetic Japan Ltd Information Regarding the Japanese Cosmetics and Quasi-Drug Market – For Companies Looking to Enter the Cosmetics Market using OEM products. Available at http://www.cosmetecjapan.com/ Internet Accessed Jan 2010.

31. FDA / CFSAN. Is it a Cosmetic, a Drug or Both (or is it Soap?). U.S. Food and Drug Administration, Centre for Food Safety & Applied Nutrition, Office of Cosmetics and Colours Fact Sheet, 2002. Available at

http://www.fda.gov/cosmetics/guidancecomplianceregulatoryinformation/. Internet. Accessed Nov 2009.

32. Kligman, A.M., et al., (1986) Topical Tretinoin for Photoaged Skin. *J Am Acad Derm*, **15**: 836-859.

33. Creidi, P., et al., (1998) Profilometric Evaluation of Photodamage after Topical Retinaldehyde and Retinoic Acid Treatment. *J Am Acad Derm*, **39**: 960-965.

34. U.S. Food and Drug Administration. Available at http://www.fda.gov/. Internet. Accessed Feb 2010

35. Therapeutic Goods Administration. EBS Australian Register of Therapeutic Goods Medicines. Available At: http://www.ebs.tga.gov.au/ Internet. Accessed Feb 2010.

36. Rival, D., et al., (2009) A *Hibiscus Abelmoschus* Seed Extract as a Protective Active Ingredient to Favour FGF-2 Activity in Skin. *Int J Cosmet Sci*, **31**: 419-426.

37. Foldvari, M., et al., (1998) Palmitoyl Derivatives of Interferon A: Potential for Cutaneous Delivery. *J Pharm Sci*, **87**: 1203-1208.

38. Mas-Chamberlin C., et al., (2002) Relevance of Antiwrinkle Treatment of a Peptide 4 Month Clinical Double Blind Study Vs Excipient. *Ann Derm Venerol*, **129**: 1S456.

39. Bisset, D., et al., (2003) Topical Palmitoyl Pentapeptide Effective Agent to Reduce the Appearance of Facial Aging. *Am Acad Derm Annul Meet* San Francisco CA, USA.

40. Robinson, L., et al., (2003) Pentapeptide Offers Improvement in Human Photoaged Facial Skin. *Am Acad Derm Annul Meet* San Francisco CA, US.

41. Smith-Mungo, L.I., et al., (1998) Lysyl Oxidase: Properties, Regulation and Multiple Functions in Biology. *Matrix Biology*, **16**: 387-398.

42. Wegrowski, Y., et al., (1992) Stimulation of Sulfated Glycosaminoglycan Synthesis by the Tripeptide-Copper Complex Glycyl-L-Histidyl-L-Lysine-Cu^{2+}. *Life Sciences*, **51**: 1049-1056.

43. Pickart, L. (2002) Copper-Peptides for Tissue Regeneration. *Spec Chem Mag,* **1347**.

44. Lupo, M.P., (2005) Cosmeceutical peptides. *Derm Surg*, **31**: 832-836.

45. Bissett, D.L., (2009) Common Cosmeceuticals. *Clin Derm*, **27**: 435-445.

46. Ellis, C.N., et al., (2001) Uses and Complications of Isotretinoin Therapy. *J Am Acad Derm*, **45**: S150-S157.

47. Maddin, S., et al., (2000) Isotretinoin Improves the Appearance of Photodamaged Skin: Results 36-Week, Multicenter, Double-Blind, Placebo-Controlled Trial. *J Am Acad Derm*, **42**: 56-63.

48. Varani, J., et al., (1990) All-Trans Retinoic Acid Stimulates Growth and Extracellular Matrix Production in Growth-Inhibited Cultured Human Skin Fibroblasts. *J Invest Derm*, **94**: 717-723.

49. Varani, J., et al., (2000) Vitamin A Antagonizes Decreased Cell Growth and Elevated Collagen-Degrading Matrix Metalloproteinases and Stimulates Collagen Accumulation in Naturally Aged Human Skin. *J Invest Derm*, **114**: 480-486.

50. Draelos, Z.D., (2005) Retinoids in Cosmetics. *Cosmet Derm*, **18S**: 3-5.

51. Griffiths, C., et al., (1993) Restoration of Collagen Formation in Photodamaged Human Skin by Tretinoin (Retinoic Acid). *N Engl J Med*, **329**: 530-535.

52. Weiss, J.S., et al., (1988) Topical Tretinoin Improves Photoaged Skin: A Double-Blind Vehicle-Controlled Study. *JAMA*, **259**: 527-532.

53. Olsen, E.A., et al., (1992) Tretinoin Emollient Cream: A New Therapy for Photodamaged Skin. *J Am Acad Derm*, **26**: 215-224.

54. Weinstein, G.D., et al., (1991) Topical Tretinoin for Treatment of Photodamaged Skin. A Multicenter Study. *Arch Derm*, **127**: 659-665.

55. Kang, S., et al., (2001) Tazarotene Cream for the Treatment of Facial Photodamage: A Multicenter, Investigator-Masked, Randomized, Vehicle-Controlled, Parallel Comparison of 0.01 %, 0.025 %, 0.05 %, and 0.1 % Tazarotene Creams with 0.05 % Tretinoin Emollient Cream Applied Once Daily for 24 Weeks. *Arch Derm*, **137**: 1597-1604.

56. Lowe, N.J., et al., (2004) Tazarotene 0.1 % Cream Versus Tretinoin 0.05 % Emollient Cream in the Treatment of Photodamaged Facial Skin: A Multicenter, Double-Blind, Randomized, Parallel-Group Study. *J Cosmet Laser Ther*, **6**: 79-85.

57. Leyden, J.J., et al., (1989) Treatment of Photodamaged Facial Skin with Topical Tretinoin. *J Am Acad Derm*, **21**: 638-644.

58. Olsen, E.A., et al., (1997) Tretinoin Emollient Cream for Photodamaged Skin: Results of 48-Week, Multicenter, Double-Blind Studies. *J Am Acad Derm*, **37**: 217-226.

59. Kang, S., et al., (2005) A Multicenter, Randomized, Double-Blind Trial of Tazarotene 0.1 % Cream in the Treatment of Photodamage. *J Am Acad Derm*, **52**: 268-274.

60. Sefton, J., et al., (2000) Photodamage Pilot Study: A Double-Blind, Vehicle-Controlled Study to Assess the Efficacy and Safety of Tazarotene 0.1 % Gel. *J Am Acad Derm*, **43**: 656-663.

61. Phillips, T.J., et al., (2002) Efficacy of 0.1 % Tazarotene Cream for the Treatment of Photodamage: A 12-Month Multicenter, Randomized Trial. *Arch Derm*, **138**: 1486-1493.

62. Lowe, N., et al., (2006) Tazarotene Versus Tazarotene Plus Hydroquinone in the Treatment of Photodamaged Facial Skin: A Multicenter, Double□Blind, Randomized Study. *J Cosmet Laser Ther*, **8**: 121-127.

63. Piérard-Franchimont, C., et al., (1998) Tensile Properties and Contours of Aging Facial Skin. A Controlled Double-Blind Comparative Study of the Effects of Retinol, Melibiose-Lactose and their Association. *Skin Res Technol*, **4**: 237-243.

64. Tucker-Samaras, S., et al., (2009) A Stabilized 0.1 % Retinol Facial Moisturizer Improves the Appearance of Photodamaged Skin in an Eight-Week, Double-Blind, Vehicle-Controlled Study.(Report). *J Drugs Derm*, **8**: 932-935.

65. Bellemère, G., et al., (2009) Antiaging Action of Retinol: From Molecular to Clinical. *Skin Pharmacol and Phys*, **22**: 200-209.

66. Robinson, D.L., (2002) Results of a Randomised, Double-Blind Trial Suggest Palmitoyl Pentapeptide (Pal-KTTKS) Improves the Appearance of Photodamaged Facial Skin. *20th World Congress of Derm*. Paris, France.

67. Sendagorta, E., et al., (1992) Topical Isotretinoin for Photodamaged Skin. *J Am Acad Derm*, **27**: S15-S18.

68. Griffiths, C.E.M., et al., (2005) Treatment of Photoaged Skin with a Cream Containing 0.05 % Isotretinoin and Sunscreens. *J Derm Treat*, **16**: 79-86.

69. Armstrong, R.B., et al., (1992) Clinical Panel Assessment of Photodamaged Skin Treated with Isotretinoin Using Photographs. *Arch Derm*, **128**: 352-356.

70. Green, O., et al., (1998) A Clinicopathological Study of the Effects of Topical Retinyl Propionate Cream in Skin Photoageing *Clin Exp Derm*, **23**: 162-167.

71. Singla, R., et al., (2003) Effects of Glycerol on the *In Vitro* Percutaneous Absorption of All-Trans Retinoic Acid. *Pharm Devel Technol*, **8**: 229-237.

72. Frelichowska, J., et al., (2009) Topical Delivery of Lipophilic Drugs from O/W Pickering Emulsions. *Int J Pharm*, **371**: 56-63.

73. Eskandar, N.G., et al., (2009) Nanoparticle Coating Submicron Emulsions: Sustained *In-Vitro* Release and Improved Dermal Delivery of All-Trans-Retinol. *Pharm Res*, **26**: 1764-1775.

74. Fresno Contreras, M.J., et al., (2005) *In Vitro* Percutaneous Absorption of All-Trans Retinoic Acid Applied in Free Form or Encapsulated in Stratum Corneum Lipid Liposomes. *Int J Pharm*, **297**: 134-145.

75. Mélot, M., et al., (2009) Studying the Effectiveness of Penetration Enhancers to Deliver Retinol through the Stratum Cornum by *In Vivo* Confocal Raman Spectroscopy. *J Control Release*, **138**: 32-39.

76. Oh, Y-K., et al., (2006) Skin Permeation of Retinol in Tween 20-Based Deformable Liposomes: *In-Vitro* Evaluation in Human Skin and Keratinocyte Models. *J Pharm Pharmacol*, **58**: 161-166.

77. Kockaert, M., et al., (2003) Systemic and Topical Drugs for Aging Skin. (Focus On: Skin Rejuvenation). *J Drugs Derm*, **2**: 435-437.

78. Baumann, L., (2005) How to Prevent Photoaging? *J Invest Derm*, **125**: xii-xiii.

79. Chiu, A., et al., (2003) Topical Vitamins, Minerals and Botanical Ingredients as Modulators of Environmental and Chronological Skin Damage. *Br J Derm*, **149**: 681-691.

80. Bisset, D., et al., (1989) The Hairless Mouse as a Model of Skin Photoaging: Its use to Evaluate Photoprotective Materials. *Photoderm*, **6**: 228-233.

81. Jurkiewicz, B.A., et al., (1995) Effect of Topically Applied Tocopherol on Ultraviolet Radiation-Mediated Free Radical Damage in Skin. *J Inv Derm*, **104**: 484-488.

82. Lopez-Torres, et al., (1998) Topical Application of α-Tocopherol Modulates the Antioxidant Network and Diminishes Ultraviolet-Induced Oxidative Damage in Murine Skin. *Br J Derm*, **138**: 207-215.

83. Dreher, F., et al., (2002) Comparison of Cutaneous Bioavailability of Cosmetic Preparations Containing Caffeine or α-Tocopherol Applied on Human Skin Models or Human Skin *Ex Vivo* at Finite Doses. *Skin Pharmacol Physiol*, **15**: 40-58.

84. Norkus, E.P., et al., (1993) Uptake and Bioconversion of α-Tocopheryl Acetate to α-Tocopherol in Skin of Hairless Mice. *Photochem Photobiol*, **57**: 613-615.

85. Farahmand, S., et al., (2006) Formulation and Evaluation of a Vitamin C Multiple Emulsion. *Pharm Devel Technol*, **11**: 255-261.

86. Davidson, J.M., et al., (1997) Ascorbate Differentially Regulates Elastin and Collagen Biosynthesis in Vascular Smooth Muscle Cells and Skin Fibroblasts by Pretranslational Mechanisms. *J Biol Chem*, **272**: 345-352.

87. Humbert, P.G., et al., (2003) Topical Ascorbic Acid on Photoaged Skin. Clinical, Topographical and Ultrastructural Evaluation: Double-Blind Study Vs. Placebo. *Exp Derm*, **12**: 237-244.

88. Fitzpatrick, R.E., et al., (2002) Double-Blind, Half-Face Study Comparing Topical Vitamin C and Vehicle for Rejuvenation of Photodamage. *Derm Surg*, **28**: 231-236.

89. Raschke, T., et al., (2004) Topical Activity of Ascorbic Acid: From *In Vitro* Optimization to *In Vivo* Efficacy. *Skin Pharmacol Phys*, **17**: 200-206.

90. Pinnell, S.R., et al., (2001) Topical L-Ascorbic Acid: Percutaneous Absorption Studies *Derm Surg*, **27**: 137-142.

91. Leveque, N., et al., (2004) *Ex Vivo* Cutaneous Absorption Assessment of a Stabilized Ascorbic Acid Formulation using a Microdialysis System. *Skin Pharmacol Physiol*, **17**: 298-303.

92. Lee, A.R.C., et al., (1998) Characterization of Skin Permeation of Vitamin C: Theoretical Analysis of Penetration Profiles and Differential Scanning Calorimetry Study. *Chem Pharm Bull*, **46**: 174-177.

93. Bissett, D.L., et al., (2005) Niacinamide: A B Vitamin that Improves Aging Facial Skin Appearance. *Derm Surg*, **31**: 860-865.

94. Tanno, O., et al., (2000) Nicotinamide Increases Biosynthesis of Ceramides as well as Other Stratum Corneum Lipids to Improve the Epidermal Permeability Barrier. *Br J Derm*, **143**: 524-531.

95. Hotchkiss, S.A.M., et al., (1992) Percutaneous Absorption of Nicotinic Acid, Phenol, Benzoic Acid and Triclopyr Butoxyethyl Ester through Rat and Human Skin *In Vitro*: Further Validation of an *In Vitro* Model by Comparison with *In Vivo* Data. *Food Chem Tox*, **30**: 891-899.

96. Franz, T., (1975) Percutaneous Absorption on the Relevance of *In Vitro* Data. *J Invest Derm*, **64**: 190-195.

97. Andersen, F., (2005) Final Report of the Safety Assessment of Niacinamide and Niacin. *Int J Tox*, **24**: 1-31.

98. Nicoli, S., et al., (2008) Characterization of Rabbit Ear Skin as a Skin Model for *In Vitro* Transdermal Permeation Experiments: Histology, Lipid Composition and Permeability. *Skin Pharm Phys*, **21**: 218-226.

99. McDaniel, D., et al., (2005) Clinical Efficacy Assessment in Photodamaged Skin of 0.5 % and 1.0 % Idebenone. *J Cosmet Derm*, **4**: 167-173.

100. Casagrande, R., et al., (2006) Protective Effect of Topical Formulations Containing Quercetin against UV B-Induced Oxidative Stress in Hairless Mice. *J Photochem Photobio B: Bio*, **84**: 21-27.

101. Widyarini, S., et al., (2006) Isoflavonoid Photoprotection in Mouse and Human Skin is Dependent on Metallothionein. *J Invest Derm*, **126**: 198-204.

102. Wei, H., et al., (2002) Inhibition of Ultraviolet Light-Induced Oxidative Events in the Skin and Internal Organs of Hairless Mice by Isoflavone Genistein. *Cancer Letters*, **185**: 21-29.

103. Cheng, E., et al., (1953) Estrogenic Activity of Isoflavone Derivatives Extracted and Prepared from Soybean Oil Meal. *Science*, **118**: 164-165.

104. Miyazaki, K., et al., (2002) Genistein and Daidzein Stimulate Hyaluronic Acid Production in Transformed Human Keratinocyte Culture and Hairless Mouse Skin. *Skin Pharmacol Appl Skin Physiol*, **15**: 175-183.

105. Wei, H., et al., (2003) Isoflavone Genistein: Photoprotection and Clinical Implications in Dermatology. *J Nutrition*, **133**: 3811S-3819S.

106. Chiu, A.E., et al., (2005) Double-Blinded, Placebo-Controlled Trial of Green Tea Extracts in the Clinical and Histologic Appearance of Photoaging Skin. *Derm Surg*, **31**: 855-860.

107. Casagrande, R., et al., (2007) *In Vitro* Evaluation of Quercetin Cutaneous Absorption from Topical Formulations and its Functional Stability by Antioxidant Activity. *Int J Pharm*, **328**: 183-190.

108. Saija, A., et al., (1998) Influence of Different Penetration Enhancers on *In Vitro* Skin Permeation and *In Vivo* Photoprotective Effect of Flavonoids. *Int J Pharm*, **175**: 85-94.

109. Bonina, F., et al., (1996) Flavonoids as Potential Protective Agents against Photo-Oxidative Skin Damage. *Int J Pharm*, **145**.

110. Minghetti, P., et al., (2006) Evaluation of *Ex Vivo* Human Skin Permeation of Genistein and Daidzein. *Drug Del*, **13**: 411-415.

111. Robert, C., et al., (2005) Effect of a Preparation Containing a Fucose-Rich Polysaccharide on Periorbital Wrinkles of Human Voluntaries. *Skin Res Technol*, **11**: 47-52.

112. Isnard, N., et al., (2004) Pharmacology of Skin Aging. Stimulation of Glycosaminoglycan Biosynthesis by L-Fucose and Fucose-Rich Polysaccharides, Effect of *In Vitro* Aging of Fibroblasts. *Biomed Pharmacother*, **58**: 202-204.

113. Robert, L., et al., (2004) Effect of L-Fucose and Fucose-Rich Polysaccharides on Elastin Biosynthesis, *In Vivo* and *In Vitro*. *Biomed Pharmacother*, **58**: 123-128.

114. Fodil-Bourahla, I., et al., (2003) Effect of L-Fucose and Fucose-Rich Oligo- and Polysaccharides (FROP-s) on Skin Aging: Penetration, Skin Tissue Production and Fibrillogenesis. *Biomed Pharmacoth Aging Age Related Diseases*, **57**: 209-215.

115. Choi, C.M., et al., (2006) Cosmeceuticals. *Semin Cutan Med Surg*, **25**: 163-168.

116. Beitner, H., (2003) Randomized, Placebo-Controlled, Double Blind Study on the Clinical Efficacy of a Cream Containing 5 % α-Lipoic Acid Related to Photoageing of Facial Skin. *Br J Derm*, **149**: 841-849.

117. Perricone, N.V., (2000) Topical 5 % Alpha Lipoic Acid Cream in the Treatment of Cutaneous Rhytids. *Aesthetic Surg J*, **20**: 218-222.

118. Podda, M., et al., (1996) Kinetic Study of Cutaneous and Subcutaneous Distribution Following Topical Application of [7,8-^{14}c]Rac-a-Lipoic Acid onto Hairless Mice. *Biochem Pharmacol*, **52**: 627-633.

119. Schmidt, J.B., et al., (1994) Treatment of Skin Ageing Symptoms in Perimenopausal Females with Estrogen Compounds. A Pilot Study. *Maturitas*, **20**: 25-30.

120. Holzer, G., et al., (2005) Effects and Side-Effects of 2 % Progesterone Cream on the Skin of Pre- and Postmenopausal Women: Results from a Double-Blind, Vehicle-Controlled, Randomized Study *Br J Derm*, **153**: 626-634.

121. Creidi, P., et al., (1994) Effect of a Conjugated Oestrogen (Premarin(R)) Cream on Ageing Facial Skin. A Comparative Study with a Placebo Cream. *Maturitas*, **19**: 211-223.

122. Caillet, S., et al., (2007) Fenton Reaction Applied for Screening Natural Antioxidants. *Food Chem*, **100**: 542-552.

123. Ahmad, R., et al., (2005) Antioxidant, Radical-Scavenging, Anti-Inflammatory, Cytotoxic and Antibacterial Activities of Methanolic Extracts of Some Hedyotis Species. *Life Sci*, **76**: 1953-1964.

124. Marquele, F.D., et al., (2005) Assessment of the Antioxidant Activities of Brazilian Extracts of Propolis Alone and in Topical Pharmaceutical Formulations. *J Pharm Biomed Anal*, **39**: 455-462.

125. Katayama, K., et al., (1993) A Pentapeptide from Type I Procollagen Promotes Extracellular Matrix Production. *J Biol Chem*, **268**: 9941-9944.

126. Han, B., et al., (2005) Transdermal Delivery of Amino Acids and Antioxidants Enhance Collagen Synthesis: *In-Vivo* and *In-Vitro* Studies. *Connect Tissue Res*, **46**: 251-257.

127. Zesch, A., (1982) Methods for Evaluation of Drug Concentration in Human Skin, in Dermal and Trandermal Absorption, Editors: Brandau, R amd Lippold, B.H., Wissenschaftliche Veragsgesellschaft mbH: Stuttgart, Germany. 116-132.

128. Wester, R.C., et al., (1999) *In Vivo* Methods for Percutaneous Absorption Measurements, in Percutaneous Absorption: Drugs-Cosmetics-Mechanisms-Methodology, Editors: Bronaugh, R.L. and Maibach, H.I., Marcel Dekker Inc.: New York, USA. 215-227.

129. Moser, K., et al., (2001) Passive Skin Penetration Enhancement and its Quantification *In Vitro. Euro J Pharm Biopharm*, **52**: 103-112.

130. Fetterly, G.J., et al., (2005) Pharmacokinetics of Oritavancin in Plasma and Skin Blister Fluid Following Administration of a 200-Milligram Dose for 3 Days or a Single 800-milligram Dose. *Antimicrob Agents Chemother*, **49**: 148-152.

131. Laethem, T., et al., (2003) Tissue Penetration by Ertapenem, a Parenteral Carbapenem Administered Once Daily, in Suction-Induced Skin Blister Fluid in Healthy Young Volunteers. *Antimicrob Agents Chemother*, **47**: 1439-1442.

132. Benfeldt, E., (1999) *In Vivo* Microdialysis for the Investigation of Drug Levels in the Dermis and the Effect of Barrier Perturbation on Cutaneous Drug Penetration. Studies in Hairless Rats and Human Subjects. *Acta Derm Venereol Suppl (Stockh)*, **206**: 1-59.

133. Benfeldt, E., et al., (1999) Effect of Barrier Perturbation on Cutaneous Salicylic Acid Penetration in Human Skin: *In Vivo* Pharmacokinetics Using Microdialysis and Non-Invasive Quantification of Barrier Function. *Br J Derm*, **140**: 739-748.

134. Surber, C., et al., (1993) *In Vivo* Skin Penetration of Acitretin in Volunteers Using Three Sampling Techniques. *Pharm Res*, **10**: 1291-1294.

135. Mansbridge, J.N., et al., (1993) Penetration of Lucifer Yellow into Human Skin: A Lateral Diffusion Channel in the Stratum Corneum. *J Histochem Cytochem*, **41**: 909-914.

136. Stracke, F., et al., (2006) Multiphoton Microscopy for the Investigation of Dermal Penetration of Nanoparticle-Borne Drugs. *J Invest Derm*, **126**: 2224-2233.

137. Kushner, J., et al., (2007) Dual-Channel Two-Photon Microscopy Study of Transdermal Transport in Skin Treated with Low-Frequency Ultrasound and a Chemical Enhancer. *J Invest Derm*, **127**: 2832-2846.

138. Akhter, S.A., et al., (1984) An Automated Diffusion Apparatus for Studying Skin Penetration. *Int J Pharm*, **21**: 17-26.

139. Loffler, H., et al., (2004) Stratum Corneum Adhesive Tape Stripping: Influence of Anatomical Site, Application Pressure, Duration and Removal. *Br J Derm*, **151**: 746-752.

140. Jacobi, U., et al., (2005) Estimation of the Relative Stratum Corneum Amount Removed by Tape Stripping. *Skin Res Technol*, **11**: 91-96.

141. Gupta, A.K., et al., (2003) Critical Review of the Manner in which the Efficacy of Therapies for Rosacea are Evaluated. *Int J Derm*, **42**: 909-916.

142. Tsukahara, K., et al., (2000) Determination of Age-Related Changes in the Morphological Structure (Sagging) of the Human Cheek using a Photonumeric Scale and Three-Dimensioal Surface Parameters. *Int J Cosmet Sci*, **22**: 247-258.

143. Beer, K.R., (2006) Comparative Evaluation of the Safety and Efficacy of Botulinum Toxin Type A and Topical Creams for Treating Moderate-to-Severe Glabellar Rhytids. *Derm Surg*, **32**: 184-192.

144. Perin, F., et al., (2000) Assessment of Skin Improvement Treatment Efficacy Using the Photograding of Mechanically-Accentuated Macrorelief of Thigh Skin. *Int J Cosmet Sci*, **22**: 147-156

145. Miyamoto, K., et al., (2002) Utilization of a High-Resolution Digital Imaging System for the Objective and Quantitative Assessment of Hyperpigmented Spots on the Face. *Skin Res Technol*, **8**: 73-77.

146. Setaro, M. et al., (2001) Irregularity Skin Index (ISI): A Tool to Evaluate Skin Surface Texture. *Skin Res Technol*, **7**: 159-163.

147. Miyamoto, K., et al., (2002) Development of a Digital Imaging System for Objective Measurement of Hyperpigmented Spots on the Face. *Skin Res Technol*, **8**: 227-235.

148. Hatzis, J., (2004) The Wrinkle and its Measurement: A Skin Surface Profilometric Method. *Micron*, **35**: 201-219.

149. Grover, R., et al., (1998) A Quantitative Method for the Assessment of Facial Rejuvenation: A Prospective Study Investigating the Carbon Dioxide Laser. *Br J Plastic Surg*, **51**: 8-13.

150. Fischer, T.W., et al., (1999) Direct and Non-Direct Measurement Techniques for Analysis of Skin Surface Topography. *Skin Pharm App Skin Phys*, **12**: 1.

151. Adorama. Photoflex Starlite Medium Kit. Available at

http://www.adorama.com. Internet Accessed Sep 2006

152. Honeck, P., et al., (2003) Reproducibility of a Four-Point Clinical Severity Score for Glabellar Frown Lines. *Br J Derm*, **149**: 306-310.

153. Accusoft Pegasus. Tampa, US. Available at: http://www.accusoft.com/. Accessed Sep 2006

154. Akazaki, S., et al., (2002) Age-Related Changes in Skin Wrinkles Assessed by a Novel Three-Dimensional Morphometric Analysis. *Br J Derm*, **147**: 689-695.

155. Jacobi, U., et al., (2004) *In Vivo* Determination of Skin Surface Topography using an Optical 3D Device. *Skin Res Technol*, **10**: 207-214.

156. GFMesstechnik GmbH, Berlin. Primos 3D. Available at: http://www.gfm3d.com. Accessed Sep 2006.

157. Lagarde, J.M., et al., (2001) Skin Topography Measurement by Interference Fringe Projection: A Technical Validation. *Skin Res Technol*, **7**: 112-121.

158. Friedman, P.M., et al., (2002) 3D *In-Vivo* Optical Skin Imaging for Topographical Quantitative Assessment of Non-Ablative Laser Technology. *Derm Surg*, **28**: 199-204.

159. Costa, M.F.M. et al., (1993) System of Optical Noncontact Microtopography. *Appl Opt*, **32**: 4860-4863.

160. Voxelan, HEW-50 and HEW250HS. HAMANO Engineering Co. Ltd., Japan. Available at: http://www.voxelan.co.jp/index-en.html. Accessed Sep 2006.

161. Lagarde, J.M., et al., (2005) Topography and Anisotropy of the Skin Surface with Ageing. *Skin Res Technol*, **11**: 110-119.

162. Hommel-Etamic GmgH, T1000 and T500. Jepoptik. Germany. Available at: www.hommel-etamic.com. Accessed Sep 2006/Feb 2010.

163. Nardin, P., et al., (2002) Automation of a Series of Cutaneous Topography Measurements from Silicon Rubber Replicas. *Skin Res Technol*, **8**: 112-117.

164. Upek Inc., California. Available at: http://www.upek.com. Accessed Oct 2006.

165. Leveque, J.L. et al., (2003) Skinchip®; a New Tool for Investigating the Skin Surface *In Vivo. Skin Res Technol*, **9**: 343-347.

166. Eotech Partnered with Isis-Sentronics, France. Optosurf Available At: http://www.eotech.fr/. Accessed Oct 2006.

167. Ambios Technology Inc., California. Xi-100 Plus. Available at: http://www.ambiostech.com/index.html. Accessed: Sep 2006/ Feb 2010.

168. Paepe, K.D., et al., (2000) Microrelief of the Skin Using a Light Transmission Method. *Arch Derm Res*, **292**: 500-510.

169. Popteam. SV600. Popteam International Management Consultation Co. Ltd., Guangzhou Popteam Development Co. Ltd., China. Available at: www.naupro.com. Accessed Sep 2006.

170. Sauermann, K., et al., (2002) Age Related Changes of Human Skin Investigated with Histometric Measurements by Confocal Laser Scanning Microscopy. *In Vivo. Skin Res Technol*, **8**: 52-56.

171. Tanaka, M., et al., (2003) The "Haptic Finger" A New Device for Monitoring Skin Conditions. *Skin Res Technol*, **9**: 131-136.

172. Walters, K.A., et al., (1998) Comparison of the Transdermal Delivery of Estradiol from Two Gel Formulations. *Maturitas,* **29**: 189-195.

173. Mahmoud, A., et al., (2005) Cutaneous Estradiol Permeation, Penetration and Metabolism in Pig and Man. *Skin Pharmacol Physiol,* **18**: 27-35.

174. Monti, D., et al., (2002) Effect of Different Terpene-Containing Essential Oils on Permeation of Estradiol through Hairless Mouse Skin. *Int J Pharm*, **237**: 209-214.

175. Kao, J. et al., (1987), Skin Absorption and Cutaneous First Pass Metabolism of Topical Steroids: *In Vitro* Studies with Mouse Skin in Organ Culture. *J Pharmacol Exp Ther,* **241**: 482-487.

176. Feldmann, R.J., et al., (1969) Percutaneous Penetration of Steroids in Man. *J Invest Derm,* **52**: 89-94.

177. El Maghraby, G.M., et al., (2000) Skin Delivery of Oestradiol from Lipid Vesicles: Importance of Liposome Structure. *Int J Pharm,* **204**: 159-169.

Chapter Two

Compound Characterisation

2.1 Introduction

The vast numbers of techniques that can be utilised for examining the potential efficacy of a compound claiming anti-wrinkle benefits have been reviewed in Chapter One. This research aims to utilise both *in vitro* and *in vivo* methods mentioned in Chapter One to examine the potential efficacy of a few, commonly used, anti-wrinkle compounds.

Compound selection will aim at reflecting ingredients with: different proposed mechanisms of action i.e. collagen regeneration, muscle relaxation and antioxidant capacitance; different ingredient types i.e. hormones, peptides, vitamins, and antioxidants; a presence in commercially available products; and available analytical techniques or the capability of developing techniques. Three compounds will be chosen to examine the link between penetration and efficacy of common anti-wrinkle ingredients.

In vitro tests can be employed to examine the permeation of compounds through human skin, demonstrating the potential of a compound under investigation to reach its site of action and therefore indicate its ability to cause its proposed mechanism of action, in this case an anti-wrinkle effect. This will be accomplished by measuring the diffusion of a compound across human skin using flow through diffusion cells and by calculating the diffusivity of the permeant within the stratum corneum over a specific time through tape stripping. A novel method known as Multiphoton Fluorescence Lifetime Imaging will also be investigated for monitoring the transdermal diffusion pathway and diffusion rate of topically applied compounds in human skin.

Infinite dose permeation studies indicate the maximum permeation potential of a compound through human skin. Finite dose permeation studies allow for the

examination of the effects caused by a formulation on the permeability of a compound under investigation. Both permeation and tape stripping experiments require the use of an accurate analytical method to assess the amount of compound undergoing percutaneous permeation or distribution.

2.1.1 Percutaneous Absorption

Transdermal absorption of compounds is generally accepted to occur by passive diffusion. While numerous mechanisms have been reported for this process, including diffusion through skin appendages (i.e. hair follicles and sweat glands known as shunt pathways) skin appendages only occupy 0.1 % of the total human skin surface, therefore, this pathway is considered negligible [1]. Compound permeation through the skin is generally regarded as limited by the stratum corneum [1-3]. There are two pathways compounds can take through this intact barrier: the transcellular route crossing the corneocytes and the intercellular lipid matrix between corneocytes and the intercellular lipid route between the corneocytes, both routes are shown in Figure 1.

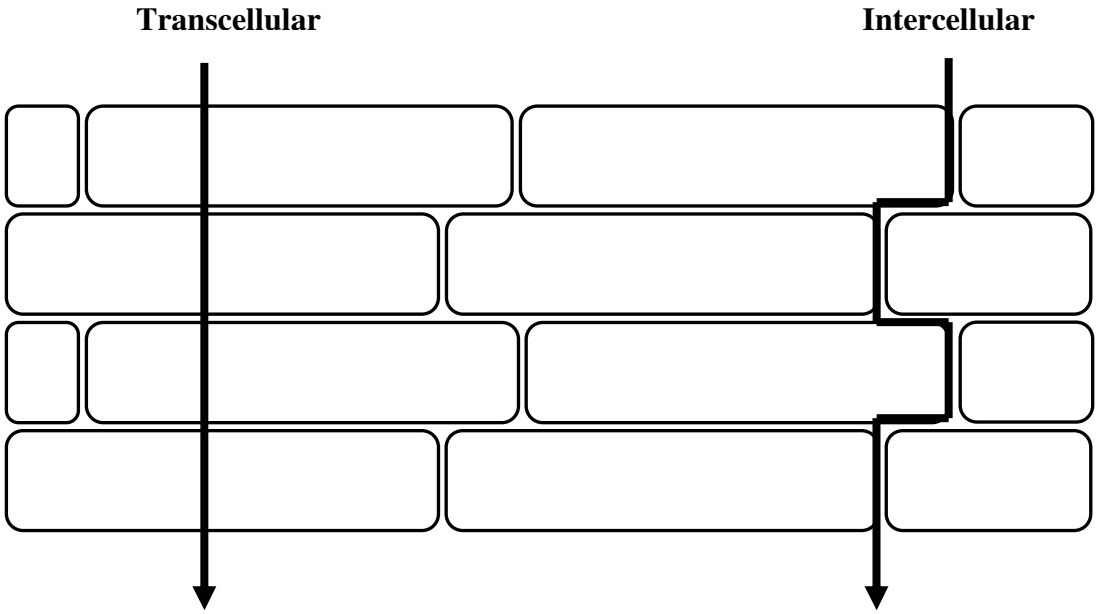

Figure 1. Schematic illustration: depicting the possible diffusion pathways of a compound through the stratum corneum.

As with both pathways through the stratum corneum, the intercellular and transcellular pathways, the permeant must at some point diffuse through the intercellular lipid matrix, which is recognised as the major factor responsible for the percutaneous diffusion rate [4]. It is generally believed that diffusion through the spaces between the corneocytes within the stratum corneum is the main mechanism responsible for the permeation of compounds through the skin [5].

2.1.2 Physico-chemical Properties

The most important factor affecting whether a permeant reaches its site of action within the skin is its diffusion through the stratum corneum, which is affected primarily by diffusion through the intercellular lipid matrix. Prediction of permeation can be made based on the physico-chemical properties of the compound.

Important physico-chemical properties include: molecular weight, melting point, lipid and water solubility (log $K_{O/W}$), hydrogen bonding groups, aqueous ionisation constant (pK_a) and the chemical structure. These can give insight into how a chemical might perform under various conditions. The information is also useful in experimental design, helping with the identification of possible compatibility issues that may arise and developing methods for analysis. Physico-chemical properties that are important for prediction of percutaneous penetration and method development compatibility issues arising from these properties include:

i. Molecular Weight - Percutaneous penetration is reduced with increasing molecular weight. Penetration is best at MW less than 500 Da [6].

ii. Partition coefficient - A partition coefficient (log P) between 1 - 3 provides the optimum permeation [7], slightly lipophilic in nature [6]. A compound possessing a high log value will require a larger concentration of organic solvent for RP-HPLC separation [8].

iii. Melting Point - Melting point is indicative of solubility. The higher the melting point the lower the solubility [5].

iv. Hydrogen bonding - Less than three hydrogen bonding groups is preferable for maximum skin penetration [7]. While compounds with multiple hydrogen bonding groups react strongly with water, increasing their solubility in this solvent, they do not dissolve easily in organic phases, and therefore have difficulty diffusing through the space between coenocytes [5]. A process known as endcapping can be employed to reduce tailing associated with hydrogen bonding interactions in RP-HPLC separation through the addition of a competing base i.e. triethylamine (TEA) into the mobile phase [9].

v. Ionisation - The pK_a of a compound predicts its degree of ionisation at a particular pH value. This in turn affects the ability of a chemical to pass through the skin, since the unionised form of an organic substance is generally more likely to enter and passively diffuse through lipid cell membranes [10].

2.2 Aim

To select three commonly used anti-wrinkle ingredients to investigate their ability to enter the human skin and cause an anti-wrinkle effect. Analyse the compounds pysico-chemical properties to make prediction of their percutaneous penetration capabilities and possible compatibilities issues that may arise under experimental conditions. Develop and validate sensitive analytical methods to accurately assess the concentration of each compound throughout experimentation, critical for tape stripping and permeation studies.

2.3 Analytical Method Selection

Reverse phase HPLC / UV was the preferred method of analysis due to: the availability of the equipment, available knowledge of the equipment within the research group and therefore ease of method development, and the ability of this method to separate and quantify a large variety of compounds with a variety of difference functionalities. The physical characteristics of each compound were assessed for expected interactions that may occur within a RP-HPLC system. The

expected interactions, along with methods previously reported in literature were used for the development of specific assays for each ingredient under investigation.

2.4 Compound Selection

The compounds selected for further investigation were niacinamide, genistein and palmitoyl tripeptide-5. Preliminary investigation was conducted on Co Enzyme Q_{10} and hydrolysed hibiscus esculentus extract, although difficulties in developing the RP-HPLC method prevented further analysis. The three selected ingredients represent three of the four groups of common anti-wrinkle compounds listed in Chapter One: a hormone, a peptide and a vitamin. Niacinamide also acts as an antioxidant, which is the final group. All three ingredients are used extensively in cosmetic products currently on the market claiming anti-wrinkle benefits. Genistein is not generally incorporated into commercially available products in pure form but as a component of soy and its derivatives. Using an internet search engine from NewDerm.com [11] a search of over 2000 American cosmetic products resulted in 153, 200 and 45 commercially available products containing niacinamide, genistein and palmitoyl tripeptide-5 respectively.

2.4.1 Niacinamide

Niacinamide (also known as vitamin $B_{3,}$ nicotinamide, nicotinic acid amide and 3-Pyridinecarboxamide) was selected as a test compound. This is a multifunctional ingredient. Niacinamide acts as a precursor to nicotinamide adenine dinucleotide (NAD) and its phosphate derivatives. The activation of NAD and its derivatives can lead to the production of the endogenous antioxidants. Antioxidants mop up or neutralise reactive

oxygen species (ROS) such as free radicals that cause tissue damage through oxidation reactions (i.e. collagen oxidation) [12-13] and also chelate metal ions. ROS can react with DNA, proteins and unsaturated fatty acids and they can also deplete endogenous skin antioxidants. NAD activation can also lead to epidermal lipid production which can improve the skin barrier function and reduce skin water loss, which results in an increase in the moisturisation of the skin [14]. Finally, it can also enable dermal and epidermal cell growth and stimulate collagen synthesis [15].

The anti-aging benefits reported with the application of niacinamide include: moisturisation, improved skin texture, reduced hyperpigmentation, reduced skin yellowing and reduced red blotchiness [12, 16-17]. Clinical investigations have revealed 5 % niacinamide application after 3 months caused a reduction in fine lines and wrinkles by 5.5 % versus the control, which was determined by image analysis coupled with computer processing and confirmed by visual assessment, n = 50 [12, 16].

There are three reported studies examining the permeation of niacinamide to date. The absorption of niacinamide into the receptor fluid at 24 hrs was 28.8 % of the starting dose analysed with flow through diffusion cells [18]. At 24 hrs 13 % of the starting dose was measured in the receptor fluid after application of an o/w emulsion containing niacinamide determined using Franz diffusion cells [19]. Finally, the application of 200 mg/ml niacinamide using Franz type diffusion cells with a receptor solution of 0.9 % NaCl resulted in a flux of 18.08 ± 5.49 $\mu g/cm^2$/hr through rabbit ear skin and 77.06 ± 13.58 $\mu g/cm^2$/hr through pig ear skin, equivalent to ~ 13 and 50 % of the amount applied respectively [20].

One of the most important factors is that niacinamide has been widely used in cosmetic anti-wrinkle products from some of the worlds most well known cosmetic brands. An example of such a brand includes, Olay by Procter and Gamble (which has

29 products containing niacinamide i.e. Regenerist Regenerating Serum, and the Total Effects Instant Smoothing Serum [21]) to name just a few. Other anti-wrinkling products containing niacinamide include: Skin Rejuvenation Lotion I, II and III by M.D Forte®, 'Agent" Anti Aging Serum for Blemished Skin by Sircuit Cosmeceuticals Inc., Pure Night by Dermalogica® and Niacinamide Serum by Rationale®[22-25]. The chemical structure of niacinamide is shown below in Figure 2.

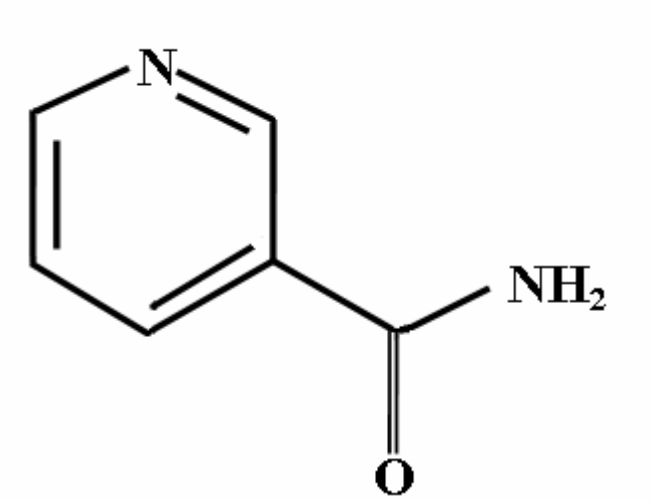

Figure 2. Chemical structure of niacinamide [26]

Known physico-chemical properties of Niacinamide include:

i. Molecular Weight (MW) 122.12 [26]

ii. Melting Point (MP) 128 - 131 ºC [19, 26]

iii. Log P 0.37 [19]

iv. pKa 3.328 ± 0.01, 3.35, heteroaromatic base [26-27]

v. Hydrogen bonding groups 3

Niacinamide used for these studies was obtained from DSM Nutritional Products Pty Ltd (Sisseln Switzerland). Its physico-chemical properties indicate it has the potential to penetrate the skin due to its low MW and reasonably low melting point, indicating high solubility. Niacinamide has slightly lower log P than is desirable for optimal permeation.

2.4.2 Genistein

Genistein is also known as 4′, 5, 7-trihydroxyisoflavone, prunetol and genisteol. It is a soybean isoflavone with diverse biological activities. These include being a potent antioxidant, a phytoestrogen and a specific inhibitor of protein tyrosine kinase [28]. As an antioxidant, genistein has been shown to scavenge peroxyl radicals and protect against: lipid peroxidation, UV-induced oxidation and cellular DNA oxidation [29-30]. As a phytoestrogen, genistein activates estrogen receptors in the dermal fibroblasts and epidermal keratinocytes [31]. Once activated the estrogen receptors can initiate collagen production, increase water retention and increase skin elasticity [32-33]. In studies using skin fibroblasts, genistein was found to increase collagen gene expression [34]. It also has anticancer properties through the inhibition of tyrosine protein kinase (TPK) [35]. Although the potential for genistein to act as an anti-wrinkle agent has been shown through *in vitro* studies, and it is available in many commercial products, its efficacy *in vivo* has not been reported.

Only one *in vitro* permeation study has been reported on genistein. Human skin tested via Franz type diffusion cells with a receptor solution of 0.9 % NaCl soln/Transcutol® (80/20 v/v) resulted in 7.1 ± 0.8 $\mu g/cm^2/hr$ in PEG 400 for pure genistein and a flux of 0.5 ± 0.1 and 1.3 ± 0.4 $\mu g/cm^2/hr$ for a saturated solution of dry extract from propylene glycol and PEG 400 respectively [36].

In commercial cosmetic products genistein is generally included as a component of soy peptide, soy bean oil, soybean protein, soy protein, glyceryl stearate (soy) or simply as soy, rather than genistein itself. Under these identities genistein is extensively used world wide as an anti-wrinkle ingredient in cosmetic products. Nivea, the worlds largest skin and beauty care brand [37], sells multiple products containing genistein,

which include the Vital Triple Action Soy + range [38]. Other products containing genistein include: LifeCell All In One Anti-Aging Treatment by LifeCell [39], Eye Balm by SkinCeuticals [40], Age Defyer Daily Moisturizer from CURES by Avance® [41] and BioActive Care Eye Serum by Mineral Care [42], to name a few. The chemical structure of genistein, shown in Figure 3, and its known physico-chemical properties are shown below.

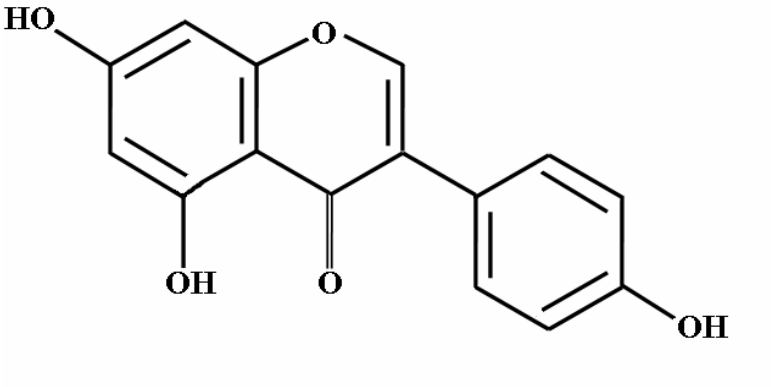

Figure 3. Chemical structure of genistein [26]

The known physico-chemical properties of genistein include [26, 43]:

i. MW 270.24

ii. MP 297 - 298 ºC

iii. Log P 2.82

iv. Hydrogen bonding groups 5

v. pKa 7.2, 10.0 and 13.1 [44]

Genistein used for the following studies was obtained from A&E Connock (Hampshire, England). The physico-chemical properties of genistein indicate the potential for it to penetrate the stratum corneum. The molecular weight of genistein (less than 500) is favourable to skin permeation. Genistein also has a log P between 1 and 3 indicating an optimal lipophilic and hydrophilic balance for permeation. Hydrogen bonding groups and ionisable groups may inhibit permeation to some extent.

2.4.3 Palmitoyl tripeptide-5

Palmitoyl tripeptide-5, also referred to as PTri-5, was previously named palmitoyl tripeptide-3. Palmitoyl tripeptide-5 mimics thrombospondin I (TSP), a multifunctional natural protein that activates the biologically inactive form of the tissue growth factor TGF-β. TGF-β is the key element in the synthesis of collagen. Hence, palmitoyl tripeptide-5 can lead to the production of collagen [45]. *In vitro* studies showed 25 μM of palmitoyl tripeptide-5 increased collagen synthesis by 119 % compared to a positive control of 0.4 nM TGF-β [45]. The only reported clinical study was conducted by the manufacturer. The study was conducted over 84 days with twice daily applications of 2.5 % palmitoyl tripeptide on 60 participants. A significant reduction in the average and maximum skin roughness was shown over the placebo, for both 1 and 2.5 % palmitoyl tripeptide-5, using the Primos® system an interference fringe profilometry technique [45].

Palmitoyl tripeptide-5 has been used as an anti-wrinkle ingredient in a large number of cosmetic products. Products containing palmitoyl tripeptide-5 include: RevivaLift by RevivaDerm [46], Mega Rich Intensive Anti-Aging Cellular Eye Crème by Peter Thomas Roth (multiple products from this company include this ingredient) [47], Kinerase Ultimate Moisturizer line i.e. Pro+ Therapy Ultra Rich Night Repair by Kinerase [48] and Age Braker Line Breaker™ Serum by Guthy-Renker® [49]. The chemical structure of palmitoyl tripeptide-5 is shown in Figure 4.

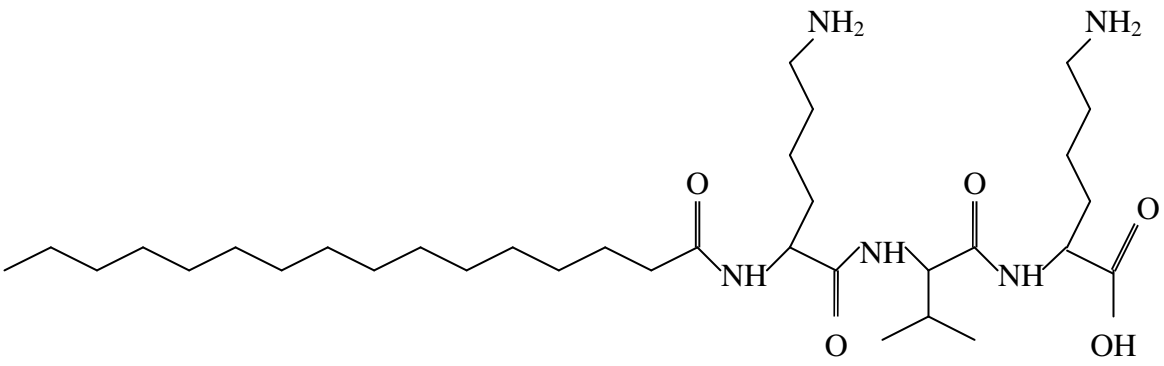

Figure 4. Chemical structure of palmitoyl tripeptide-5, molecular formula $C_{33}H_{65}N_5O_5$ and the peptide sequence Pal-Lys-Val-Lys-OH [50]

Little information has been reported on the physico-chemical properties of palmitoyl tripeptide-5. Reported information includes:

i. MW 611.90 [50]

ii. Hydrogen bonding groups 10

Palmitoyl tripeptide-5 used in this research was obtained from Pentapharm Ltd, (Basel, Switzerland). It was supplied as a 1000 µg/ml solution in 10 – 25 % water, made to volume with glycerine. Although it is unlikely, based on the relatively high molecular weight, there is a possibility that palmitoyl tripeptide-5 will penetrate the skin. While optimal stratum corneum permeation is found when a compound possesses a MW less than 500 Da, this value is only indicative as some compounds up to MW of 700 are known to penetrate the skin [51]. The large number of hydrogen bonding groups present result in the peptide sequence being hydrophilic which is also likely to hinder percutaneous permeation. The peptide sequence allows for the activation of collagen synthesis however in order to increase the lipophilic properties of the peptide sequence it is transformed with palmitoyl to aid delivery across the skin.

2.5 Methods

2.5.1 Analytical Analysis

Quantification of the three selected compounds within permeation, tape stripping and / or solubility studies was determined using reverse phase HPLC, coupled with UV detection. The HPLC system consisted of an Alliance 2690 separation system coupled to a 2487 Dual Absorbance Detector with data processing completed using Empower Pro, all supplied by Water (Massachusetts, USA).

2.5.1.1 Method Development

The chromatographic parameters were initially evaluated for a standard solution of the three test compounds (the analytes): 50 µg/ml niacinamide and palmitoyl tripeptide-5 and 1 µg/ml genistein, where concentration selection was based on concentrations used for separation methods reported in literature. Retention time, tailing factor and separation from other species in the matrix were optimised for different proportions of acetonitrile (ACN) and the aqueous solvents. The final assay methods were novel. The methods were, therefore, validated for their ability to gain reproducible results. Intra- and inter-day reproducibility was assessed.

2.5.1.2 Method Validation

2.5.1.2.1 Linearity

Linearity of each method was assessed through calibration curves. Standard solutions were prepared. Aliquots of the standard solution were then diluted to $n < 6$ concentration intervals. A linear relationship between peak area and concentration was confirmed by the correlation coefficient generated by the linear regression of the curve, using a least squares method. The concentration range of each calibration curve was a function of both the expected permeation concentration and also the limit of detection of the method.

Standard solutions were made up containing 1 g/ml niacinamide, 50 µg/ml genistein and 1 g/ml palmitoyl tripeptide-5. The linear range for niacinamide was 0.5 - 50 µg/ml niacinamide in isotonic phosphate buffer, consisting of 0.1829 % $NaH_2PO_4.2H_2O$, 1.9797 % $Na_2HPO_4.12H_2O$, 0.4375 % sodium chloride obtained from Ajax Finechem (New South Wales, Australia), 0.1 % sodium azide from Sigma-Aldrich (Missouri, USA) made to 100 % with water collected from a Milli-Q water purification system from Millipore (Massachusetts, USA)). The linear range for genistein was, 0.01 - 1 µg/ml genistein in 0.5 % polyoxyethylene 20 oleyl ether (Volpo N20) obtained from Sigma-Aldrich (Missouri, USA) and 0.1 % sodium azide in water. The linear range for palmitoyl tripeptide-5 was 10 - 50 µg/ml palmitoyl tripeptide-5 in 0.1 % sodium azide in water. Aliquots of each concentration were injected onto the HPLC equipment and a calibration curve for each compound over each concentration range was obtained in relation to peak area versus concentration.

2.5.1.2.2 Precision

Precision of the analytical assays was assessed to determine the degree of variation in the results associated with random errors of the assays. Variations could be associated with the intra-day changes in injection separation and inter-day changes caused by varoation in the environment between days. Precision was obtained through injecting the same homogeneous sample, under the same conditions, multiple times.

The intra-day precision was evaluated by analysing standard solutions (n = 5) at three different concentrations: low, medium and high, dependent on the sensitivity and calibration curve of the assay, giving a total of n = 15 samples analysed for each assay. The precision was then expressed as a co-efficient of variation (CV) at each concentration level, shown in Equation 1.

$$CV = \frac{\text{Standard Deviation}}{\text{Mean}} \times 100 \qquad 1)$$

Similarly, inter-day precision of the assay was determined over three different days using standard solution freshly prepared each day of the analysis. Calibration curves were constructed on the three days. The precision was determined as the co-efficient of variation (CV) of the resultant slopes.

The mean value at each different concentration and slope was not to exceed ± 15 % CV and was to be within ± 20 % CV at the LOQ (limit of quantitation, the lowest amount of the analyte in a sample which can be measured with defined precision and accuracy under the experimental conditions) to be considered acceptable.

2.5.1.2.3 Accuracy

Accuracy was determined in conjunction with precision analysis. It was expressed as the deviation of the measured concentration from the theoretical concentration (bias), shown below in Equation 2.

$$\text{Accuracy} = \frac{\text{Calculated Concentration - Theoretical Concentration}}{\text{Calculated Concentration}} \times 100 \qquad 2)$$

The intra-day accuracy was evaluated by determining the calculated concentration of the standard solutions (n = 5), at the three concentrations: low, medium and high. A calibration curve was run before and after injecting the standard solutions. From the calibration curves a regression line was determined for each and the mean intercept and slope was used to calculate the concentration of the standard solutions based on their resultant peak areas, as shown in the Equation 3 below.

$$\text{Calculated Concentration} = \frac{\text{Peak Area - Mean Intercept}}{\text{Mean Slope}} \qquad 3)$$

Inter-day accuracy of the assays involved the analysis, through calibration curved, of freshly prepared standard solution analysed on three different days. The resultant regression line for the curves was used to determine a mean intercept and slope. The mean slope and intercept was used to determine the calculated concentration at each value in the standard curve.

Accuracy was to be within ± 15 % of the theoretical concentration, although within ± 20 % was considered acceptable at the LOQ.

2.5.1.2.4 Sensitivity

Blank samples consisted of: IPB for niacinamide, 0.5 % Volpo and 0.1 % sodium azide in water for genistein and 0.1 % sodium azide in water for palmitoyl tripeptide-5. To determine both, the LOQ (limit of quantification) and the LOD (limit of detection, the lowest concentration of the compound under examination that will yield an assay response significantly different from that of a blank sample), blank samples were injected onto the HPLC under the assay conditions (n = 5). The mean peak areas for the blank samples were determined and the calculated concentrations were generated from the mean intercept and slope of the calibration curves. The LOD is expressed as three times the calculated concentration, corresponding to a signal to noise ratio of 3:1. The LOQ is the lowest concentration at which the acceptable limits for precision and accuracy are met (± 20 %) which can be the LOD.

2.5.1.2.5 Storage Stability

Test compounds loss by degradation or absorption onto the containers may occur over time. Therefore, the stability of the compounds under storage was determined. Stock solutions for the standard curves were stored in a refrigerator. The standard curve was run after 1, 2, 3, 7 and 14 days to assess stability to determine the maximum storage time for the samples.

2.5.2 Solubility Studies

Solubility studies determine the maximum concentration of solute dissolvable in a solvent at a specific temperature. The method involved an excess amount of the solid ingredient being added to a volume of a solvent system. For organic systems, 2 ml and for aqueous systems, 6 ml of solvent was used. This was performed in 10 ml centrifuge tubes. Each tube was vortexed for 30 secs, then placed in a shaking water bath for 24 hrs and maintained at 32 °C (this temperature was chosen to mimic the temperature at the surface of the skin). It was ensured that prior to sampling the ingredient had not dissolved completely; if all was dissolved more was added until this was achieved. At 24 hrs the solutions were centrifuged for 15 mins at 3500 rpm. The supernatant was then diluted and assayed by HPLC. The remaining solutions were then vortexted and returned to the shaking water bath for a further 24 hrs. At 48 hrs the solutions were again centrifuged and assayed via HPLC. To ensure accuracy of the solubility measurement equilibrium must be reached, which generally occurs between 24 and 48 hrs. Equilibrium was considered to be reached if consecutive solubility readings were ± 5 % within 24 hrs. If this was not attained after 48 hrs the solutions were returned to the water bath and assayed at 24 hr intervals until equilibrium was reached. Solubility experiments were performed in triplicate and the mean, standard deviation and coefficient of variation was calculated for each experiment. For aqueous based solvent systems the pH was also recorded at the beginning and end of the experiment.

2.5.3 Tape Stripping

A strong correlation has been found to exist between the total amount of a compound penetrating the skin within 96 hrs and the amount of compound found within the stratum corneum (SC) after a 30 min exposure time ($r^2 = 0.96$, $p < 0.001$) [52]. This correlation was independent of the contact time, dose applied, vehicle used, anatomical site and animal species used. A technique referred to as "tape stripping" can be used to investigate the localization and distribution of substances within the SC. The method involves layers of the SC being removed by an adhesive tape. A number of adhesive tapes are applied to the same area and SC layers along with the compound under investigation are sequentially removed. The adhesive tapes are weighed before and after stripping and the weight difference is indicative of the number of SC layers removed. The ingredient is extracted from the sequential adhesive tape strips and its concentration within the extracts are determined using a suitable analytical method [53].

2.5.3.1 Skin Preparation

Human female abdominal tissue was obtained following abdominoplasty. The skin collection method was approved by *The Standing Committee on Ethics in Research Involving Humans*, Monash University, Victoria, Australia. Each donor was provided with an Explanatory Statement, which briefly outlined the nature of the study and the donor was required to sign an Informed Consent form. Full-thickness skin samples were prepared by removing subcutaneous tissue and fat from the underside of the dermal membrane using a stainless steel surgical blade. Excess fat residue was removed from the skin surface by gentle blotting with low-lint, absorbent tissue (Kimwipes®,

Kimberly-Clark Australia). The skin was stored in aluminium foil at –20 °C for up to 12 months prior to use [54]. Before the commencement of each study, the skin was defrosted under ambient conditions for approximately 2 to 4 hr. The SC surface was rinsed with water and gently wiped with Kimwipes® four times, in order to remove any surface contamination.

The skin was then laid flat, SC side up, on a teflon board and dissected into 1 x 1 cm (width x length) sections using a surgical blade. Each section was laid flat, dermal side up, and quick-dry super glue (UHU® Supa Glue, UHU GmbH & Co, Malaysia) was blotted onto the dermis. The skin sections were mounted, SC side up, on to 1 x 3 cm pieces of cardboard. Gentle pressure was then applied to the skin for ~10 secs to ensure contact with the cardboard. The SC surface of each sample was quickly (< 20 secs) wiped with a Kimwipes® (pre-moistened with ethanol) to ensure complete removal of sebaceous lipids and the skin samples were then left untouched for 5 min to allow the glue to completely dry.

2.5.3.2 Validation Procedure

2.5.3.2.1 Surface Wipe Samples

A solution containing 0.1 % and 0.5 % of genistein or niacinamide in 100 % propylene glycol was applied as a "finite dose" (5 µl/cm^2) to 1 x 1 cm sections of skin (n = 5) such that the amount of genistein or niacinamide deposited at the SC surface was 1.67 and 8.33 µg/ml in the extraction solution, applied for 20 secs. The surface was wiped with 2 cotton buds that were then placed in glass vials. The extraction procedure was then performed.

2.5.3.2.2 Surface Wipe Standards

For surface wipe standards, 5 µl of propylene glycol was applied to the 1 x 1 cm sections of skin. Blank cotton buds were placed in vials and 5 µl of 0.1 % and 0.5 % of genistein or niacinamide in 100 % propylene glycol were added to 10 glass vials, the extraction procedure was then performed.

2.5.3.2.3 Tape Strip Samples

For tape strip samples, 5 µl of propylene glycol was applied to the 1 x 1 cm sections of skin for 20 secs. After the surface wipe was performed 5 tape strips per skin sample were taken. The tape strips were laid flat, adhesive side up. Propylene glycol solutions containing 40, 400 and 2000 µg/ml of niacinamide or genistein were applied to the samples, in order to deposit 0.1, 1 and 5 µg of compounds. The samples were then placed onto filter paper and placed in 5 ml plastic centrifuge vials. The extraction procedure was then performed.

2.5.3.2.4 Tape Stripping Standards

The skin was prepared and tape stripped using the method described above. However, rather than dosing the tape strips with the analyte, tape stripping standards were left blank and placed onto filter paper adhesive side down, then transferred to 5 ml plastic centrifuge vials. Next 5 µl aliquots of propylene glycol solutions containing 40, 400, or 2,000 µg/ml of niacinamide or genistein were added to the vials. The extraction procedure was then performed.

2.5.3.2.5 Extraction Procedure

The extraction procedure consisted of 2 ml, HPLC grade water (100 %) for niacinamide or ethanol (95 %) for genistein, being added to centrifuge vials. All the vials were vortexted for 30 secs. Surface swab vials were then placed in the roller mixer for 24 hrs at 32 °C. Tape strip vials were placed in the shaking water bath for 24 hrs at 32 °C. All vials were then vortexted for 30 secs. The tape strips were removed from the tape strip vials and the vials were centrifuged for 15 mins at 3500 rpm and 32 °C. The supernatant was analysed via HPLC, undiluted. The cotton buds were removed from the surface swab vials and placed in an empty vial and 1 ml of either water or 95 % ethanol was added. The samples from the initial vial and the new vial were added together and vortexted for 1 min. One ml of the extract was centrifuged at 3500 rpm for 15 mins at 32 °C. The supernatant was analysed via HPLC undiluted.

2.5.3.2.6 Recovery, Accuracy and Precision

2.5.3.2.6.1 Surface Wipe Procedure

Recovery of the surface wipe procedure was determined by comparing the peak areas of test compounds extracted from the surface wipe samples to the peak areas generated from surface wipe standards (i.e. blank surface wipes spiked with analyte). Intra-day precision and accuracy of the procedure was determined from the surface wipe samples (n = 5) at the two different concentrations (1.67 and 8.33 μg/ml in the extraction solution). Inter-day precision was determined by repeating the surface wipe sample procedure for these different concentrations on three different days.

2.5.3.2.6.2 Tape Stripping Procedure

Recovery of the tape stripping procedure was determined by comparing the peak areas of test compound extracted from the tape strip samples to the peak areas of blank tape strips to which the compound was added. Intra-day precision and accuracy of the procedure were determined from the tape strip standards (n = 5) prepared at the different concentrations (0.1, 1 or 5 μg/cm^2 of skin surface area). Inter-day precision was determined from the tape strip standards at the known concentrations prepared on three different days.

2.6 Results

2.6.1 Niacinamide

2.6.1.1 RP-HPLC Method Validation

The amount of niacinamide was assessed by the use of reverse phase HPLC using a Symmetry® C_{18} column (3.9 x 150 mm, 5 μm). An isocratic method was employed with a mobile phase of 0.5 % acetonitrile, 0.1 % triethylamine (TEA) from Sigma-Aldrich (Missouri, USA), 99.5 % (0.02 M KH_2PO_4 from Ajax Finechem (New South Wales, Australia) to volume with water and adjusted to pH 5 with orthophosphoric acid from Merck (Darmstadt, Germany)). The flow rate was 0.9 ml/min, injection volume was 30 μl, and UV absorbance was monitored at 261 nm. The retention time was ~3.8 mins. A representative chromatogram is shown in Figure 5.

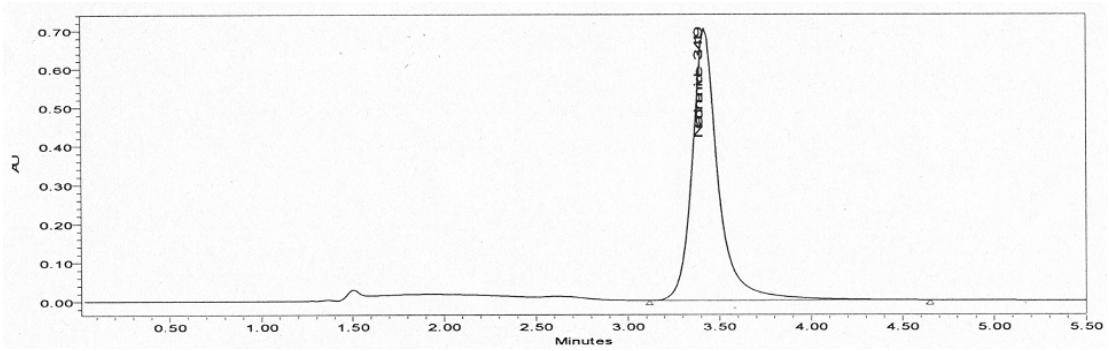

Figure 5. A representative chromatogram of niacinamide, a 10 µg/ml solution of niacinamide in IPB.

Linearity of the assay method was assessed through calibration curves. Standard solution of 1 g/ml niacinamide in IPB were prepared and diluted to make 6 concentration intervals, 0.5, 1, 2, 5, 10, 20 and 50 µg/ml niacinamide in IPB. Aliquots of each concentration were injected onto the HPLC equipment and a calibration curve for each compound over each concentration range was obtained in relation to peak area versus concentration shown in Figure 6.

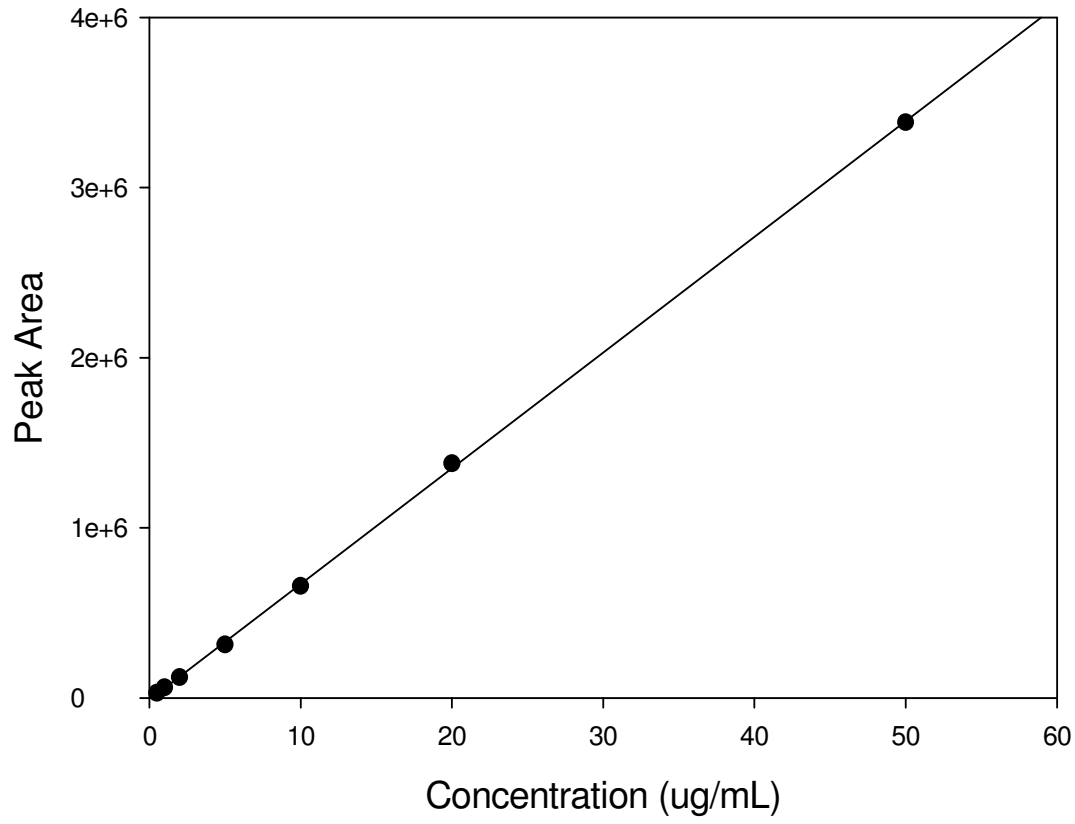

Figure 6. A calibration curve was developed using standard solutions of concentrations 0.5, 1, 2, 5, 10, 20 and 50 µg/ml niacinamide in IPB. A linear relationship between peak area and concentration was confirmed by the correlation coefficient generated by linear regression of the curve. The linearity of the calibration curve was excellent with an r^2 = 0.9998.

Intra-day precision % CV was found to be 4.58, 0.39 and 0.56 % at concentrations 0.05, 5 and 50 µg/ml respectively. Mean percent accuracy was 17.47 ± 0.19, -1.53 ± 0.38 and 0.84 ± 0.55 % ± STDEV for 0.05, 5 and 50 µg/ml respectively.

The method was found to be highly reproducible with an inter-day precision % CV of 1.26 %. Inter-day accuracy was ranging from 8.54 at 0.5 µg/ml to -0.05 at 50 µg/ml.

The LOD was determined to be 0.27 µg/ml. The LOQ, based on precision and accuracy results, was found to be 0.5 µg/ml.

There was no detectable loss of niacinamide after 14 days of storage in the refrigerator. It was decided samples would be stored for a maximum of 14 days prior to analysis under these storage conditions.

2.6.1.2 Niacinamide Solubility Studies

In order to select a suitable receptor fluid for permeation studies the solubility of niacinamide in propylene glycol was determined. Saturated solubility of niacinamide was performed in triplicate. The saturated solubility of niacinamide was not determined in IPB because its log P value of 0.37 [19] indicated a very high water solubility. Therefore, it was unlikely the solubility of niacinamide in IPB (consisting largely of water) would be an issue throughout experimentation.

The saturated solubility of niacinamide in 100 % propylene glycol was found to be 246 mg/ml.

2.6.1.3 Tape Stripping Extraction Validation

Validation of the tape stripping extraction method was performed by extracting specific concentrations of niacinamide in propylene glycol (PG) from swabs and tape strips using water. Tape strip concentrations of 40, 400 and 2000 µg/ml of niacinamide in PG were applied to the tape strips. This equates to 100 % recovery, equivalents of 0.1, 1 and 5 µg/ml in the extraction solution. Swabs applied in doses of 0.1 % and 0.5 %

niacinamide in PG as 100 % recovery would be equivalent to 1.67 and 8.33 µg/ml in the extraction solution. Results are presented in Tables 1 and 2.

Table 1: Validation of niacinamide extraction from swabs for tape stripping

Amount (µg)	% Recovery (Mean ± SD)	Intra-day precision (% CV)	Inter-day precision (% CV)	Accuracy (%)
1.67	99.6 ± 0.05	2.7	3.0	7.9
8.33	100.0 ± 0.12	7.2	1.4	6.6

Table 2: Validation of niacinamide extraction from tape strips

Amount (µg)	% Recovery (Mean ± SD)	Intra-day precision (% CV)	Inter-day precision (% CV)	Accuracy (%)
0.1	99.9 ± 0.15	11.4	40.1	- 38.0
1	100.0 ± 0.98	6.6	9.5	- 26.6
5	100.0 ± 0.06	5.41	12.3	- 4.4

2.6.2 Genistein

2.6.2.1 RP-HPLC Method Validation

The amount of Genistein was assessed by the use of reverse phase HPLC using a Symmetry® C_{18} column (3.9 mm x 150 mm, 5 µm). A gradient method was employed starting with 100 % of Mobile Phase A (10 % Acetonitrile, 90 % water and 0.1 % glacial acetic acid obtained from Ajax Finechem (New South Wales, Australia)). The initial conditions were held isocratic for 3 mins, then increased to 50 % Mobile A and 50 % Mobile B (90 % acetonitrile, 10 % water and 0.1 % glacial acetic acid) over 1 min. The conditions were increased again to 30 % Mobile A and 70 % Mobile B over 3

mins and finally to 100 % Mobile Phase B over 0.5 mins. Over 1.5 mins the conditions were reverted back to the initial state ready for the following injection. A flow rate of 1.2 ml/min was used, with an injection volume of 20 µl and the UV absorbance was monitored at 262 nm. The retention time was ~6.5 mins. A representative chromatogram is shown in Figure 7.

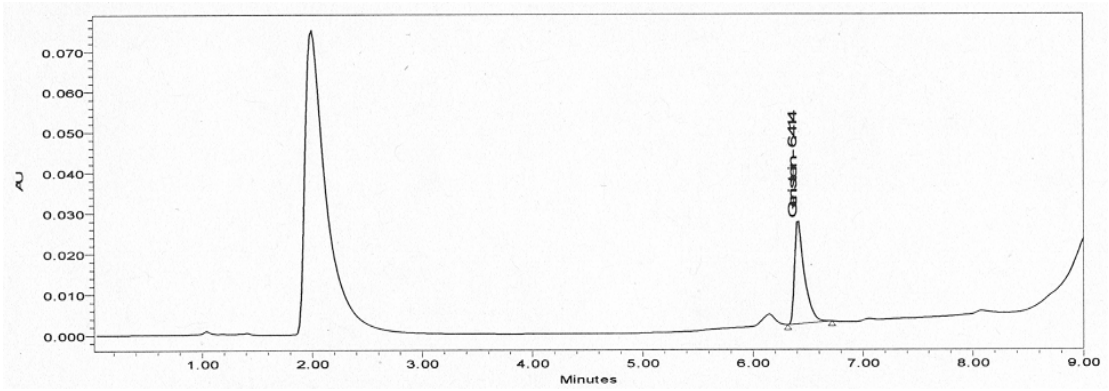

Figure 7. A representative chromatogram of genistein, a 1 µg/ml solution of genistein in 0.5 % Volpo N20, 0.1 % sodium azide in water

Linearity of the assay method was assessed through calibration curves. Standard solution of 50 µg/ml genistein in 0.5 % Volpo N20, 0.1 % sodium azide in water were prepared and diluted to make 6 concentration intervals, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5 and 1 µg/ml. Volpo N20 was included in the genistein standard solutions to increase its water solubility, this decision was based on solubility studies the results are shown in Section 2.6.2.2. Aliquots of each concentration were injected onto the HPLC equipment and a calibration curve for each compound over each concentration range was obtained in relation to peak area versus concentration, shown in Figure 8.

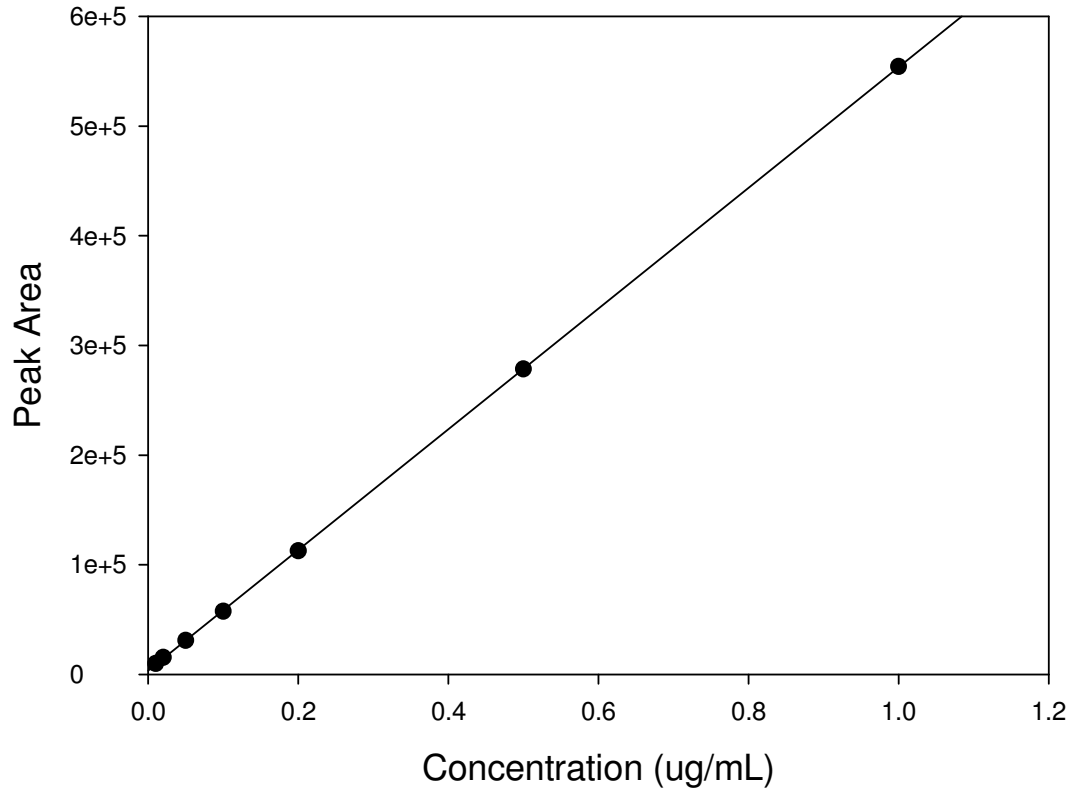

Figure 8. A calibration curve was developed using standard solutions of concentrations 0.01, 0.02, 0.05, 0.1, 0.2, 0.5 and 1 µg/ml Genistein in 0.5 % Volpo, 0.1 % sodium azide and water. The linearity of the calibration curve was excellent with an $r^2 = 1$

The assay showed intra-day precision with a % CV of 5.08, 3.02 and 4.00 % for concentrations 0.01, 0.05 and 1 µg/ml respectively. The accuracy was determined to be 15.32 ± 6.67, -2.04 ± 3.53 and -1.73 ± 4.04 % for 0.01, 0.05 and 1 µg/ml respectively.

Inter-day precision was found to be 1.81 % and inter-day accuracy ranged from 9.75 at 0.01 µg/ml to 0.11 at 1 µg/ml.

The LOD was determined to be 0.0017 µg/ml. The LOQ, based on precision and accuracy results, was found to be 0.01 µg/ml.

There was no detectable loss of genistein after 14 days of storage in the refrigerator, therefore, sampled were stored for a maximum of 14 days prior to analysis under these storage conditions.

2.6.2.2 Genistein Solubility Studies

The results from solubility studies on genistein indicated that this compound had poor solubility in IPB and water. Therefore, to maintain sink conditions throughout the permeability study it was decided to add a surfactant, Volpo N20, which has been used when a compound has poor solubility in water. Consequently the solubility study conducted with genistein included varying concentrations of Volpo N20.

Saturated solubility of genistein in various medium was performed in triplicate and the results are shown below in Table 3.

Table 3: Saturated solubility of genistein in various mediums.

Solvent Medium	Saturated Solubility (µg/ml)
100 % water	1.17
Isotonic Phosphate Buffer	2.76
0.5 % Volpo N20, 0.1 % sodium azide in water	125.23
1 % Volpo, 0.1 % sodium azide in water	247.33
2 % Volpo, 0.1 % sodium azide in water	447.35
3 % Volpo, 0.1 % sodium azide in water	642.33
100 % propylene glycol	8925.12

From these experiments a 0.5 % Volpo solution in water was selected as the receptor medium for all genistein permeability studies.

2.6.2.3 Tape Stripping Extraction Validation

Genistein was extracted from the swabs and adhesive tape strips using 95 % ethanol. Concentrations of 40, 400 and 2000 µg/ml genistein in PG were applied to the

tape strips. Considering a 100 % recovery and dilution the applied concentrations are equivalent to 0.1, 1 and 5 µg/ ml genistein in the extraction solution. The applied dose for the swabs was 0.1 % and 0.5 % genistein in PG, which is equivalent to 1.67 and 8.33 µg/ml in the extraction solution, taking into account 100 % recovery. The validation results are shown below in Tables 4 and 5.

Table 4: Validation of genistein extraction from swabs for tape stripping

Amount (µg)	% Recovery (Mean ± SD)	Intra-day precision (% CV)	Inter-day precision (% CV)	Accuracy (%)
1.67	99.98 ± 0.07	5.14	2.9	0.08
8.33	99.98 ± 0.06	5.74	3.6	0.47

Table 5: Validation of genistein extraction from tape strips

Amount (µg)	% Recovery (Mean ± SD)	Intra-day precision (% CV)	Inter-day precision (% CV)	Accuracy (%)
0.1	100.1 ± 0.05	5.1	15.5	19.9
1	100.0 ± 0.08	6.6	7.4	5.8
5	100.0 ± 0.21	4.9	6.8	2.2

2.6.3 Palmitoyl tripeptide-5

2.6.3.1 RP-HPLC Method Development

HPLC method development revealed compatibility issues preventing the separation of palmitoyl tripeptide-5 and the receptor solution isotonic phosphate buffer (IPB commonly employed in permeation experiments) and therefore the quantification of the peptide.

To overcome this incompatibility a simple receptor medium was selected which consisted of 0.1 % sodium azide in water.

2.6.3.2 RP-HPLC Method Validation

The amount of palmitoyl tripeptide-5 collected over the study was analysed by the use of reverse phase HPLC using a Symmetry® C_{18} column (3.9 mm x 150 mm, 5 µm). A linear gradient method was employed with the initial conditions of 70 % (v/v) Mobile A, itself consisting of 5 % acetonitrile from Merk (Darmstadt, Germany), 95 % water and 0.1 % trifluoroacetic acid, (TFA) from BDH Laboratory Supplies (Poole, England) and 30 % (v/v) of Mobile B comprising of 85 % acetonitrile, 15 % water and 0.08 % TFA this was graduated to 100 % Mobile B over 7 mins. The initial conditions were then re-established and equilibrated over 3 mins in preparation for subsequent injections. A flow rate of 1 ml/min and an injection volume of 100 µl were used, and the UV absorbance was monitored at 220 nm. The retention time was ~5.8 mins. A representative chromatogram is shown below in Figure 9.

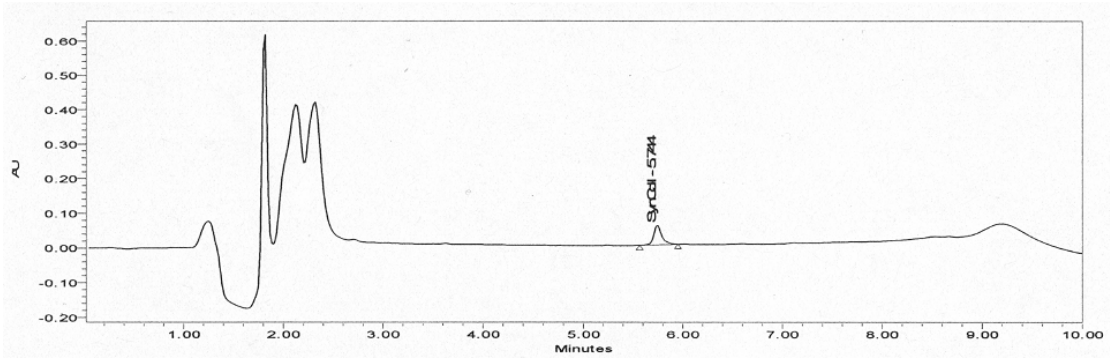

Figure 9. A representative chromatogram of palmitoyl tripeptide-5, a 50 µg/ml solution of palmitoyl tripeptide-5 in 0.1 % sodium azide and water.

Linearity of the assay method was assessed through calibration curves. Standard solutions of 1 g/ml palmitoyl tripeptide-5 in 0.1 % sodium azide in water were prepared and diluted to make 6 concentration intervals, 10, 15, 20, 25, 40 and 50 µg/ml. Calibration curves were obtained in relation to peak area versus concentration shown in Figure 10.

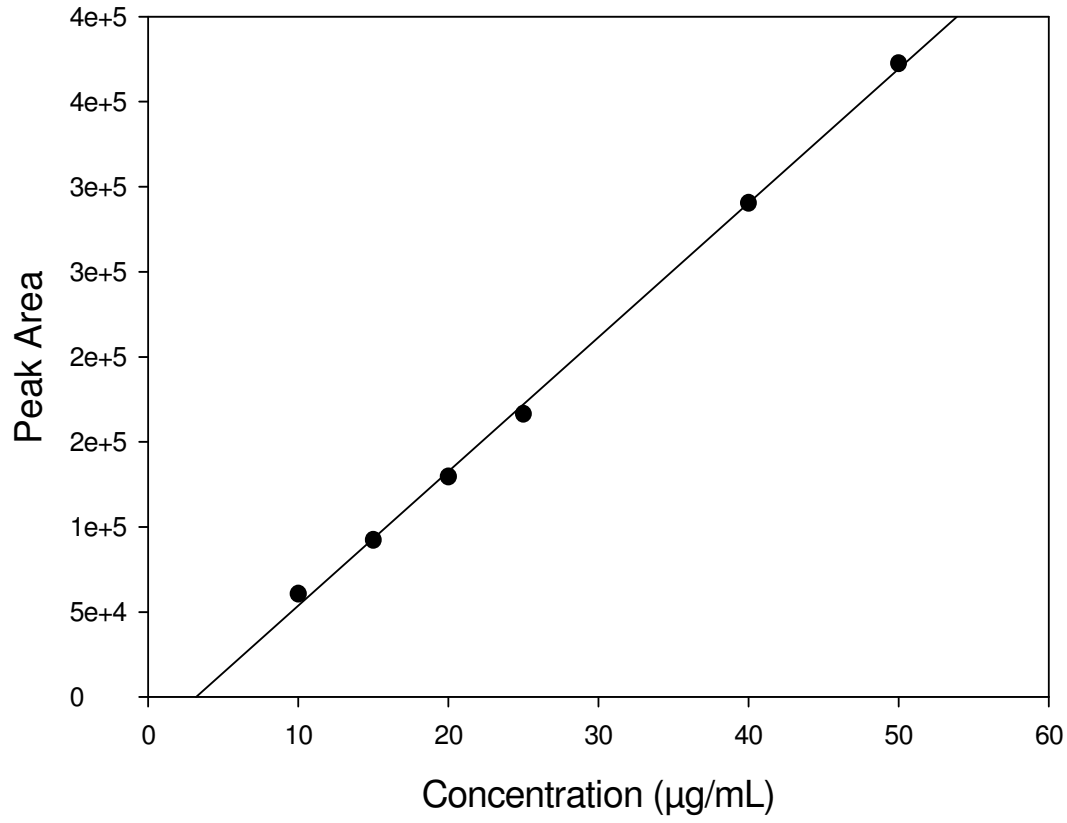

Figure 10. A calibration curve was developed using standard solutions of concentrations 10, 15, 20, 25, 40 and 50 µg/ml palmitoyl tripeptide-5 in 0.1 % sodium azide in water. The linearity of the calibration curve was highly satisfactory with an r^2 = 0.9986.

Intra-day precision (%CV) of the assay was found to be 2.37, 5.07 and 1.41 % at concentrations 10, 25 and 50 µg/ml respectively. Accuracy was determined to be 15.89 ± 1.31, -7.92 ± 4.62 and 7.98 ± 1.20 (% ± STDEV) for 10, 25 and 50 µg/ml respectively.

Inter-day precision (%CV) was found to be 11.97 %, and inter-day accuracy ranged from 9.07 at 10 µg/ml to 2.68 at 50 µg/ml.

From the LOD of the assay was determined to be 6.58 µg/ml. The LOQ based, on precision and accuracy results, was found to be 10 µg/ml.

No loss of palmitoyl tripeptide-5 was observed after 14 days storage in the refrigerator. Samples were stored for a maximum of 7 days prior to analysis.

2.6.3.3 Palmitoyl tripeptide-5 Solubility Studies

As the palmitoyl tripeptide-5 is provided in solution the solubility could not be determined.

2.7 Conclusion

Three commonly used anti-wrinkle compounds were selected for investigation of their percutaneous permeation and anti-wrinkle efficacy. The compounds included: niacinamide, genistein and palmitoyl tripeptide-5.

Reverse phase HPLC coupled with UV detection methods were developed and validated for the three selected compounds. The methods can be utilised for quantifiably determining the percutaneous permeation of the compounds. Intra- and inter-day reproducibility was found to be acceptable for all the methods.

Preliminary method validation for tape stripping, where by the penetration depth of an ingredient over time is investigated, was conducted for both niacinamide and genistein. Tape stripping methods were, however, not conducted for these compounds due to time constraints. Rather than investigating the distribution of the ingredients

within the stratum corneum over time it was considered more relevant to investigate the total amount permeating the stratum corneum through permeation studies.

Niacinamide's physico-chemical properties indicate it has the potential to penetrate the skin. The saturated solubility of niacinamide in 100 % propylene glycol was found to be 246 mg/ml. The receptor medium for niacinamide permeation studies was selected to be isotonic phosphate buffer (IPB).

Hydrogen bonding and ionisable groups may inhibit the permeation of genistein to some extent. Genistein was found to have poor solubility in IPB and water. Therefore, 0.5 % Volpo N20 in water was selected as the receptor medium for permeation studies.

It was found that although it is possible, it's unlikely palmitoyl tripeptide-5 will undergo percutaneous penetration based on its high molecular weight. Due to compatibility issues between palmitoyl tripeptide-5 and IPB, 0.1 % sodium azide in water was selected as the receptor medium for palmitoyl tripeptide-5 permeation studies.

2.8 References

1. Moser, K., et al., (2001) Passive Skin Penetration Enhancement and its Quantification *In Vitro. Euro J Pharm Biopharm*, **52**: 103-112.
2. Guy, R.H., (1996) Current Status and Future Prospects of Transdermal Drug Delivery. *Pharm Res*, **13**: 1765-1769.
3. Potts, R.O., et al., (1991) The Influence of Stratum Corneum Morphology on Water Permeability. *J Invest Derm*, **96**: 495-499.
4. Potts, R.O., et al., (1992) Predicting Skin Permeability. *Pharm Res*, **9**: 663-669.
5. Flynn, G.L., et al., (1988) Percutaneous Drug Penetration: Choosing Candidates for Transdermal Development. *Drug Dev Res*, **13**: 169-185.
6. Lipinski, C.A., et al., (1997) Experimental and Computational Approaches to Estimate Solubility and Permeability in Drug Discovery and Development Settings. *Adv Drug Del Reviews*, **23**: 3-25.
7. Guy, R.H. et al., (1989) Selection of Drug Candidates for Transdermal Drug Delivery. Transdermal Drug Delivery: Development Issues and Research Initiatives. New York: Marcel Dekker Inc.: 59-81.
8. Skoog, D.A., et al., (2004) High-Performance Liquid Chromatography, in Fundamentals of Analytical Chemistry. Thomson Brooks/ Cole: California. 982-985.
9. Nwuw, U.D., (2001) HPLC Troubleshooting Guide, Waters Corportation: Massachusetts.
10. Oakley, D.M. et al., (1987) Effects of Ionization on the Percutaneous Absorption of Drugs: Partitioning of Nicotine into Organic Liquids and Hydrated Stratum Corneum. *J Pharm Sci*, **76**: 866-871.

11. Ingredient Search, Newderm.Com., New York. Available at www.4derm.com. Internet Accessed, Feb 2010.

12. Bissett, D.L., et al., (2004) Topical Niacinamide Reduces Yellowing, Wrinkling, Red Blotchiness, and Hyperpigmented Spots in Aging Facial Skin. *Int J Cosmet Sci*, **26**: 231-238.

13. Kerscher, M. (2003) Dermocosmetics: Cosmetics and Skin Care from Dermatological View- Current Trends in Dermocosmetics. Skin Care Forum **35**. Available at http://www.scf-online.com Internet Access Jan 2010

14. Tanno, O., et al., (2000) Nicotinamide Increases Biosynthesis of Ceramides as well as other Stratum Corneum Lipids to Improve the Epidermal Permeability Barrier. *Br J Derm*, **143**: 524-531.

15. Gehring, W., (2004) Nicotinic Acid/ Niacinamide and the Skin. *J Cosmet Derm*, **3**: 88-93.

16. Bissett, D.L., et al., (2005) Niacinamide: A B Vitamin that Improves Aging Facial Skin Appearance. *Derm Surg*, **31**: 860-865.

17. Otte, N., et al., (2005) Nicotinamide - Biologic Actions of an Emerging Cosmetic Ingredient. *Int J Cosmet Sci*, **27**: 255-261.

18. Franz, T., (1975) Percutaneous Absorption on the Relevance of *In Vitro* Data. *J Invest Derm*, **64**: 190-195.

19. Andersen, F., (2005) Final Report of the Safety Assessment of Niacinamide and Niacin. *Int J Tox*, **24**: 1-31.

20. Nicoli, S., et al., (2008) Characterization of Rabbit Ear Skin as a Skin Model for *In Vitro* Transdermal Permeation Experiments: Histology, Lipid Composition and Permeability. *Skin Pharmacol Physiol*, **21**: 218-226.

21. Olay Ingredient Analyzer, Olay. Available at http://www.olay.com. Internet Accessed. Feb 2010.

22. M. D. Forte under Allergan, California, Skin Rejuvenation Lotion. Available At: www.mdforte.com Accessed Feb 2010.

23. Sircuit Cosmeceuticals Inc., California. Agent Anti-Aging Serum. Available At: http://www.sircuitskin.com. Accessed Feb 2010.

24. Dermalogica® St Lenards Australia, Pure Night. Available At: http://www.dermalogica.com.au. Accessed Feb 2010.

25. Rationale® Skincare, Melbourne Australia. Available At: http://rationaleskincare.com/. Accessed Feb 2010.

26. The Merck Index. 14 Ed. New Jersy: Merck Research Laboratories div of Merck & Co. Inc.

27. Prankerd, R.J., (2007) Appendix A, in Profiles of Drug Substances, Excipients and Related Methodology, Editor: Harry, G.B.. Academic Press. 282.

28. Wei, H., et al., (2003) Isoflavone Genistein: Photoprotection and Clinical Implications in Dermatology. *J Nutrition*, **133**: 3811S-3819S.

29. Pinnell, S.R., (2003) Cutaneous Photodamage, Oxidative Stress, and Topical Antioxidant Protection. *J Am Acad Derm*, **48**: 1-19.

30. Kang, s., et al., (2003) Topical N-Acetyl Cysteine and Genistein Prevent Ultraviolet-Light-Induced Signaling that Leads to Photoaging in Human Skin *In Vivo*. *J Invest Derm*, **120**: 835-841.

31. Cheng, E., et al., (1953) Estrogenic Activity of Isoflavone Derivatives Extracted and Prepared from Soybean Oil Meal. *Science*, **118**: 164-165.

32. Verdier-Sevrain, S., (2007) Effect of Estrogen on Skin Aging and the Potential Role of Selective Estrogen Receptor Modulators. *Climateric*, **10**: 289-297.

33. Brincat, M.P., et al., (2005) Estrogens and the Skin. *Climacteric*, **8**: 110-123.

34. Greenwel, P., et al., (1995) Tyrosine Dephosphorylation of Nuclear Proteins Mimics Transforming Growth Factor Beta-1 Stimulation of Alpha-2 (I) Collagen Gene Expression. *Mol Cell Biol*, **15**: 6813-6819.

35. Barnes, S., (1995) Effect of Genistein on *In Vitro* and *In Vivo* Models of Cancer. *J Nutr*, **125 (suppl)**: 7779-7839.

36. Minghetti, P., et al., (2006) Evaluation of *Ex Vivo* Human Skin Permeation of Genistein and Daidzein. *Drug Delivery*, **13**: 411-415.

37. Euromonitor International Pty Ltd, Singapore. Cosmetic and Toiletries Excluding Fragrances and Haircolourants Retail Sales Values 2008. Available At: www.euromonitor.com. Accessed Feb 2010.

38. Nivea, Vital Triple Action Soy + under Beiersdorf, North Ryde Australia. Available At: www.nivea.com. Accessed: Feb 2010.

39. Lifecell All in One Anti-Aging Treatment, South Beach Skincare Inc., Florida. Available At: http://www.lifecellskin.com. Accessed Feb 2010.

40. Skin Ceuticals, Eye Balm. Available at http://www.skinceuticals.com. Accessed Feb 2010.

41. Cures Avance®, New Jersey, Age Defyer Daily Moisturizer. Available At: http://curebyavance.com. Accessed Feb 2010.

42. DSP Cosmetics Pty Ltd, Victoria, Bioactive Care Eye Serum by Mineral Care. Available At: http://www.deadseaproducts.com.au/. Accessed Feb 2010.

43. Lee, S. et al., (2003) Estimation for Retention Factors of Isoflavones in Physico-Chemical Properties. *Bull Korean Chem Soc*, **24**: 1265-1268.

44. Zielonka, J., et al., (2003) Radical Scavenging Properties of Genistein. *Free Radical Bio Med*, **35**: 958-965.

45. DSM Nutritional Products Ltd a Branch of Pentapharm Syn®Coll Boost and Protect Collagen to Iron out Wrinkles. Available At: www.pentapharm.com. Accessed Dec 2008.

46. Revivaderm™ Trademark of Netderma Inc., California, Reviva Lift. Available At: http://revivaderm.com. Accessed Feb 2010.

47. Peter Thomas Roth Labs, Llc. Available At: http://www.peterthomasroth.com. Accessed: Feb 2010.

48. Kinerase® a Trademark of Valeant Pharmaceuticals International, California. Available At: http://www.kinerase.com. Accessed: Feb 2010.

49. Principle Secret by Guthy Renker®, New South Wales, Age Breaker Line Breaker Serum. Available At: www.principlesecret.com.au. Accessed Feb 2010.

50. Xiamen Bj Chem. Technology Co. Ltd., China. Cosmetic Ingredients. Available At: http://www.bjchemicals.com. Accessed: Feb 2010.

51. Guy, R.H. et al., (1988) Physicochemical Aspects of Percutaneous Penetration and Its Enhancement. *Pharm Res*, **5**: 753-758.

52. Rougier, A., et al., (1987) An Original Predictive Method for *In Vivo* Percutaneous Absorption Studies. *J Soc Cosmet Chem,* **28**: 397-417.

53. Surber, C., et al., (1999) Tape-Stripping Technique, in Percutaneous Absorption Drugs, Cosmetics, Mechanisms, Methodology. Editors: Bronaugh, R.L., Maibach, H.I., Marcel Dekker, Inc.: New York. 395-409.

54. Harrison, S.M., et al., (1984) Effects of Freezing on Human Skin Permeability. *J Pharm Pharmacol*, **36**: 261-262.

Chapter Three

In Vitro Permeation Study through Human Skin

3.1 Introduction

A large number of "cosmeceutical" anti-aging products have recently been released onto consumer shelves. The main mechanisms being claimed include: increasing collagen production, reducing cell muscle contraction and exhibiting antioxidant behaviour [1-3]. All of these mechanisms require action within the skin, not on the skin surface. While the mechanisms by which these "active" ingredients act have often been demonstrated by chemical and / or biological experimentation, a vital step has been overlooked. Few tests have been conducted to assess whether these anti-wrinkle actives actually penetrate the skin to reach the proposed sites of action. Permeation studies performed with human skin *in vitro* can be used to determine whether the ingredients reach their site of action in sufficient quantities to elicit an effect and/ or indicate potential safety concerns prior to costly human trials. Because of the barrier properties of the stratum corneum [4-5] not all compounds are good candidates for topical administration. During the development and optimisation of a topical formulation, the compound(s) of interest must possess physicochemical properties that are amenable to percutaneous penetration, as mentioned in Chapter Two.

Also, the delivery system in which the compound is formulated must be considered when examining the penetration potential of that candidate. The delivery system can dramatically alter the percutaneous penetration characteristics of the compound. For example poor inherent penetration of the stratum corneum can be overcome to some extent by the use of enhancement strategies. This could be achieved through the use of a penetration enhancer, which causes a modification of the skin's barrier function. Penetration enhancers generally elicit their effect on the skin's barrier functions either through mechanical disruption such as with the use of ultrasound [6],

electrical disruption by the application of electrical pulses (> 30 V for about 1 ms) to the skin [7] or chemical modification of the barrier function of the skin (i.e. fatty acids are commonly used) [8-9]. Penetration enhancement can also be achieved through occlusion. Occlusion prevents the evaporation of water from the surface of the skin causing the membrane to swell from increased water content, which in turn compromises the skins' barrier function [10]. Supersaturation is another method employed to increase the skin penetration of a compound. Supersaturated systems can increase the thermodynamic activity of a compound; this can be achieved by developing a volatile: non-volatile vehicle. Once the formulation is applied the volatile portion evaporates, increasing the concentration of the compound and subsequently increasing the driving force for partitioning into the skin [8].

Despite these well established criteria for optimising skin penetration, these principles have only recently been applied to anti-wrinkle actives. Because of all these possible variables affecting penetration, it is important to show the extent of penetration of active ingredients from a particular product both for efficacy and safety reasons.

The information from permeation studies can be used to give an indication of the possible effectiveness of an enhancement strategy before costly human trials.

3.1.1 Infinite Dose Technique

The infinite dose approach involves the application of a sufficient concentration of the test compound in which changes to that concentration caused by diffusion or evaporation throughout the experiment is negligible. This method is used for the evaluation of a compound's diffusion parameters (i.e. its permeability coefficient), to evaluate penetration enhancement techniques or to gain an initial assessment of a

compound's penetration potential for comparison. This technique makes data analysis and interpretation simpler. Data collection can be obtained for compounds with a limited permeation, where, under finite experimental conditions, the permeation concentrations may fall below the limit of detection of the analysis method. Infinite dose studies also allow for the measurement of the inherent permeability characteristics of a compound, with the influence of the vehicle on the leaving tendency from the applied solution. Therefore, the thermodynamic activity of the compound should be constant.

The expected penetration profile of an infinite dose experiment is shown in Figure 1. The "Steady State Diffusion" phase will continue over the length of the study period because the change in concentration of the test compound in the donor chamber is insignificant.

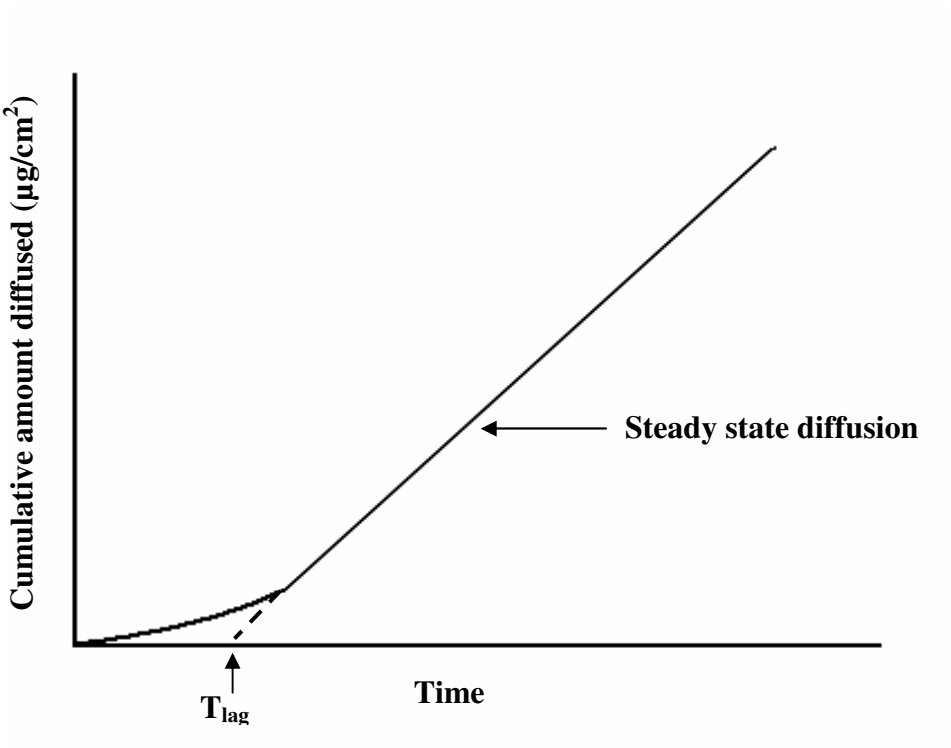

Figure 1. Penetration time profile for an idealised test compound diffusing through a membrane, applied as an infinite dose.

3.1.2 Finite Dose Technique

A finite dose application study involves the application of a set concentration of the permeant. In this case depletion of the test compound in the donor chamber occurs where the proportion of the compound entering the skin is larger than the amount applied. As the depletion of the compound in donor chamber occurs this becomes evident in the permeation profile seen as a plateau, shown in Figure 2.

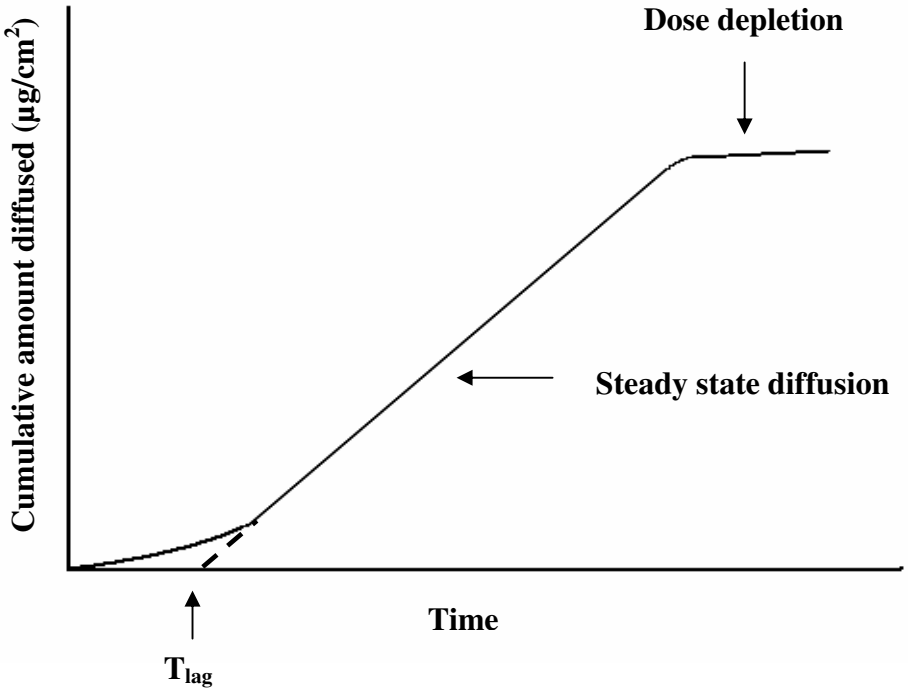

Figure 2. Penetration time profile for an idealised test compound diffusing through a membrane, applied as a finite dose.

3.2 Aim

To examine the permeation characteristics of niacinamide, genistein and a palmitoyl tripeptide-5 through infinite and finite dose permeation studies. From these

permeation results the potential efficacy of these well documented anti-aging ingredients at reaching their site of action can be demonstrated. The information gathered from these experiments will then be used to select products to conduct *in vivo* clinical trials.

3.3 Materials and Methods

3.3.1 Skin Preparation and Handling

Human abdominal skin samples were obtained from patients who had undergone abdominal plastic surgery. Full thickness skin, complete with subcutaneous fat, was stored at -20 °C prior to use. It was essential to freeze the skin with the skin surface as flat as possible to aid in the dermatoming process. To achieve this, the skin samples were placed stratum corneum down onto a glass plate, sealed to avoid damage to the samples and then frozen. Prior to separation the skin was removed from the freezer and left at ambient laboratory conditions (~22 - 24 °C and 40 - 60 % relative humidity) to thaw for ~3 hrs (until the skin was slightly malleable but could maintain a solid structure). The skin was moulded into a flat surface, while any hollow sections were propped up. Using the Padgett Dermatome from Padgett Instruments Inc. (Missouri, USA) 500 µm thick skin slices were obtained. The skin slices were rinsed in water, (all water was collected from a Milli-Q water purification system from Millipore (Massachusetts, USA)), to remove any subcutaneous fat, blotted to remove excess moisture, placed stratum corneum up onto a glass plate, sealed in aluminium foil and frozen at -20 °C. Frozen dermatomed skin was stored for up to 12 months prior to use

[11]. Prior to permeation studies, the frozen dermatomed skin was floated on 0.1 % sodium azide in water, thawed and hydrated for ~3 hrs.

3.3.2 Skin Permeation Study

3.3.2.1 Donor Solutions

The application method for which a test compound is applied to the skin surface is paramount to its subsequent permeation. A number of factors must be considered which include: the type of vehicle, the compound concentration and the exposure time. There are two basic approaches to applying substances in the case of permeation studies: infinite and finite dosage.

3.3.2.1.1 Infinite Dose Studies

Infinite dose studies involved the use of saturated solutions where excess compound was added to 10 ml of the solvent, 100 % propylene glycol from Sigma-Aldrich (Missouri, USA) (for niacinamide and genistein studies). Solutions were then stirred with a magnetic stirrer for 48 hrs (to attain equilibrium) at ambient laboratory conditions prior to experimentation.

3.3.2.1.2 Finite Dose Studies

Finite dose studies involved the use of the dose medium at set concentrations of the test compound. Doses were prepared immediately prior to use and stirred at ambient

temperature. Only finite dose studies were conducted on palmitoyl tripeptide-5 because it was only available from the manufacturer as a 1000 µg/ml solution in 10 - 25 % water made to volume with glycerine. Permeation studies were initially conducted on simple solutions where palmitoyl tripeptide-5 was diluted with water at various concentrations, much higher than that recommended for cosmetic application.

After the initial assessment of the compounds permeation characteristics was conducted, finite permeation studies were then performed on the compounds from a simple base cream. The Simple Cream used for these studies consisted of the following ingredients: water, rice bran oil, caprylic triglycerides, cetearyl alcohol, dicetyl phosphate, ceteth-10 phosphate, avocado oil, glycerin stearate citrate, cyclomethicone, caprylyl glycol, phenoxyethanol, hexylene glycol, phenoxyethanol, hexylene glycol, tocopheryl acetate, rice starch, xanthan gum and disodium EDTA.

Once a selection was made as to which compounds would be further tested for *in vivo* clinical trials, permeation studies were conducted on the compound from the cream used in the commercially available product containing our test compound. The cream under investigation for niacinamide included the following ingredients: water, glycerin, almond oil, crambe oil, ethyl macadamate, octyl salicylate, xanthan gum, EDTA, cetearyl alcohol, hydrogenated olive oil, olive oil, olive oil unsaponifiable, cetearyl olivate, sorbitan olivate, dimethicone, bisabolol, lecithin, borage oil, caprylic / capric triglyceride, retinyl palmitate, tocopheryl acetate, ascorbyl palmitate, alcohol, N-acetyl glucosamine, mica, titanium dioxide, tin dioxide, pea extract, pineapple extract and carrot oil.

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3.3.2.2 Receptor Solutions

For determining the permeation of a compound under investigation the receptor solution must not influence its permeation rate. The receptor solution must maintain sink conditions beneath the skin, i.e. the concentration of the permeating substance should not exceed 10 % of its saturated solubility in the receptor solution [12]. If the concentration was to exceed 10 % it could lead to a decrease in the rate of absorption, which may result in an underestimation of the bioavailability of the test compound. The choice of the receptor solution was, therefore, determined by the saturated solubility of the compound in the solution at 32 °C (temperature of skin surface) (results shown in Chapter Two) and the amount of test substance predicted to permeate the skin. The most commonly employed receptor solution is isotonic phosphate buffer (IPB) pH 7.4, although this is not always appropriate. If a compound has an aqueous solubility of less than ~10 µg/ml the addition of solubilisers (e.g. polyethylene glycol oleyl ether (Volpo N20)) becomes necessary [13]. Preservatives are generally included to inhibit microbial growth in the receptor fluid (e.g. sodium azide). Microbial growth may cause problems because it can cause degradation of the membrane, also, less significantly, the test compound may partition into and / or be metabolised by the bacteria.

3.3.2.3 Permeation Method

The receptor solution was degassed then placed in the ultrasonic bath for ~20 mins to ensure no bubbles were present. Collection vials were weighed prior to, and on completion of permeation studies, weights were used to accurately determine the amount of compound collected at each time point. The receptor solution was flushed

through the tubing and all air bubbles were removed. Receptor solution was pumped through the diffusion cells at a flow rate of 1 ml/cm^2/hr ± 10 % using a microcassette peristaltic pump from Watson-Marlow Pumps Group (Cornwall, United Kingdom). Stainless steel, flow-through permeation cells (see Figure 3) with a permeation area of 1.0 cm^2 were placed on heated bars and heated to a temperature of ~32 °C (±1 °C). A stainless steel wire mesh sat at the bottom of the permeation area, which maintained a turbulent flow of the receptor solution below the skin. A stainless steel T-piece was attached to the tubing leading out of the permeation cell. The tubing, which had receptor fluid pumping through, was attached to the permeation cells. Dermatomed skin samples were sliced into sections (minimum size of 1.5 x 1.5 cm^2), thawed and hydrated by floating on 0.5 % sodium azide from Sigma-Aldrich (Missouri, USA) in water for ~2 hrs prior to conducting the permeation studies. Skin sections were placed on the permeation cells and secured in place by bolting the donor and receptor chambers of the cell together, as shown in Figure 3. The skin was left to equilibrate for ~1 hr, after which, the skin was examined for holes by applying ~500 µl of water to the donor chamber (on the skin surface) for ~1 hr. Disappearance of the water indicated the presence of a hole in the skin, in these cases the skin samples were replaced.

For infinite dose and palmitoyl tripeptide-5 finite dose experiments the donor chambers were dosed with 800 µl of the test solution. For niacinamide and genistein finite dose experiments, involving the application of the test compound in a cream product, an amount of cream was applied into each donor chamber, to the skin sample, with a cotton bud. The cream was rubbed onto the skin to resemble *in vivo* application. The amount of cream applied onto each skin sample was determined by weight, as the amount of cream on the cotton bud lost on application. After the test solution was dosed into the donor chamber, the collection vial was moved into position under the T-piece.

The samples were collected on an automated fraction collector from Isco Retriever II, (Nebraska, USA), usually at four hour intervals. In some cases, for niacinamide and genistein, a two hour sample was collected and then four hourly samples were taken following this. The initial time point, at 2 hrs, was taken to gain information of the lag time, the time taken for the compound to reach equilibrium for its permeation. All permeation experiments were run over 24 hrs.

At the end of the experiment, samples were refrigerated until analysed. As shown in Chapter Two, niacinamide and genistein, exhibited stability on refrigeration for a period of 14 days, therefore, sample analysis was performed within 14 days of sample collection. Palmitoyl tripeptide-5 was shown to exhibit stability on refrigeration for a period of 14 days, the samples were, however, analysed within 7 days of collection. Prior to analysis each vial was vortexted for 30 secs. Samples were then analysed via reverse phase HPLC, methods detailed in Chapter Two.

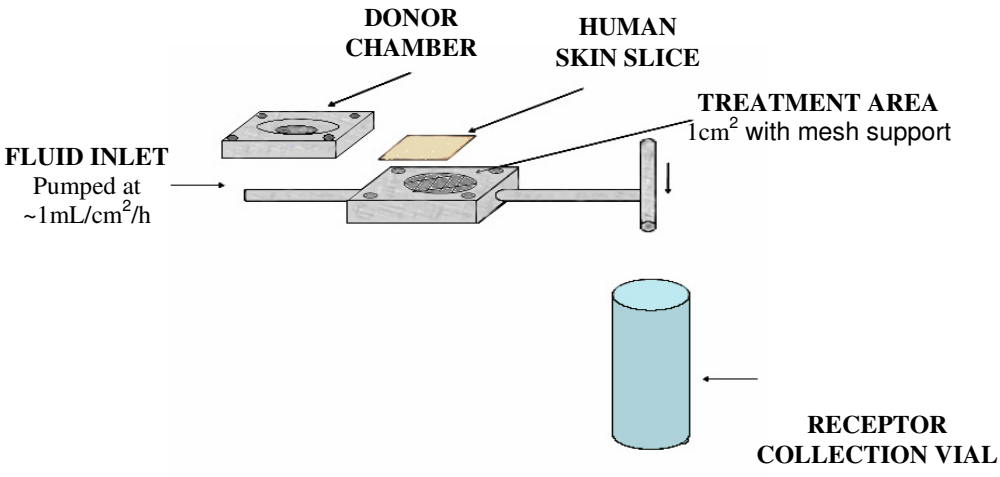

Figure 3. Schematic illustrations depicting the Permeation Cell set up. The set up consists of: a treatment area, a circle of mesh to support a 1 cm^2 area where the skin sample sits; a donor chamber where the anti-aging compound is placed as a saturated solution for infinite dose or as a finite dose; a fluid inlet where receptor solution is pumped through, and collection vial where the receptor solution is collected and is automatically changed at regular time intervals.

3.3.2.4 Skin Extraction

Additional steps were conducted for palmitoyl tripeptide-5 permeation studies. The concentration of compound left in the donor chamber was analysed and also any drug that had permeated into the skin was extracted. This method consisted of the removal of the fluid in the donor chamber at the end of the experiment, which was transferred to a 10 ml volumetric flask. The donor chamber was washed with the Mobile Phase A for palmitoyl tripeptide-5 separation, as was the cover glass slip used to occlude the donor chamber and the syringe used to remove the fluid. The volumetric flask was made up to volume, stirred vigorously, then the solution analysed via RP-HPLC. The skin samples were removed from the permeation cell and frozen with liquid

nitrogen and maintained on dry ice. The skin samples were then crushed in a stainless steel mortar and pestle type chamber. The pulverized skin samples were then placed into 2 ml centrifuge vials and 0.75 ml of Mobile A for palmitoyl tripeptide-5 separation was added. The samples were held in a shaking water bath for 24 hrs maintained at 32 °C to extract the compound from the skin. Samples were then centrifuged for 15 mins, at 32 °C and 3500 rpm. The supernatant was then analysed via RP-HPLC.

3.3.2.5 Mathematical Analysis

Compound permeation through the skin in its simplest form can be regarded as a thermodynamically favoured, passive diffusion process that can be described in terms of Fick's first law of diffusion, shown below in Equation 1 [14]:

$$J = \frac{DK_{SC/V}\Delta C_V}{h} \qquad 1)$$

J is the steady-state compound flux across the membrane (the stratum corneum), *D* is the diffusion coefficient of the compound within the stratum corneum, $K_{SC/V}$ is the partition coefficient of the compound between the stratum corneum and the vehicle, C_V is the concentration of the compound within the vehicle and *h* is the thickness of the stratum corneum. It is evident from this equation the parameters that can be modified in order to enhance the compound flux from the skin are: *D,* $K_{SC/V}$ and C_V. As C_V represents the *effective* concentration of the compound within the applied vehicle (i.e. the concentration of the compound that is available for partitioning into the stratum corneum), it is evident that optimising the compound flux across the skin by increasing

the C_V is limited by the fact that the compound will have a finite solubility within a given formulation / vehicle.

Compound flux (J) across the skin was calculated from the permeability coefficient, where:

$$J = K_P \times C_V \qquad 2)$$

Hence, the flux can be determined by calculating the slope of the graph in steady state diffusion. Therefore, points on the curve are taken which are responsible for the straight portion of the cumulative amount versus time graph.

3.4 Results and Discussion

3.4.1 Niacinamide

For all permeation studies conducted on niacinamide, a receptor solution of IPB with 0.1 % sodium azide was employed. All studies were conducted over 24 hrs and samples of the receptor solution were collected at 4 hr intervals, a sample was also collected at 2 hrs into the study to give a better view of the lag phase of the profile.

3.4.1.1 Infinite Study

Permeation studies were conducted on a saturated solution of niacinamide in 100 % propylene glycol, the donor solutions were made up as per the Donor Solution Method Section 3.3.2.1. Over these permeation experiments all the cells were occluded.

This study was performed to gain an understanding of the permeation of niacinamide under favourable conditions, providing an indication of the maximum permeation. Studies were conducted as described earlier in Section 3.3.2.3. An average of seventeen permeation studies were performed on dermatomed skin prepared as per method Section 3.3.1 on three different donors. A plot of the cumulative amount of niacinamide penetrating the skin versus time is shown below in Figure 4.

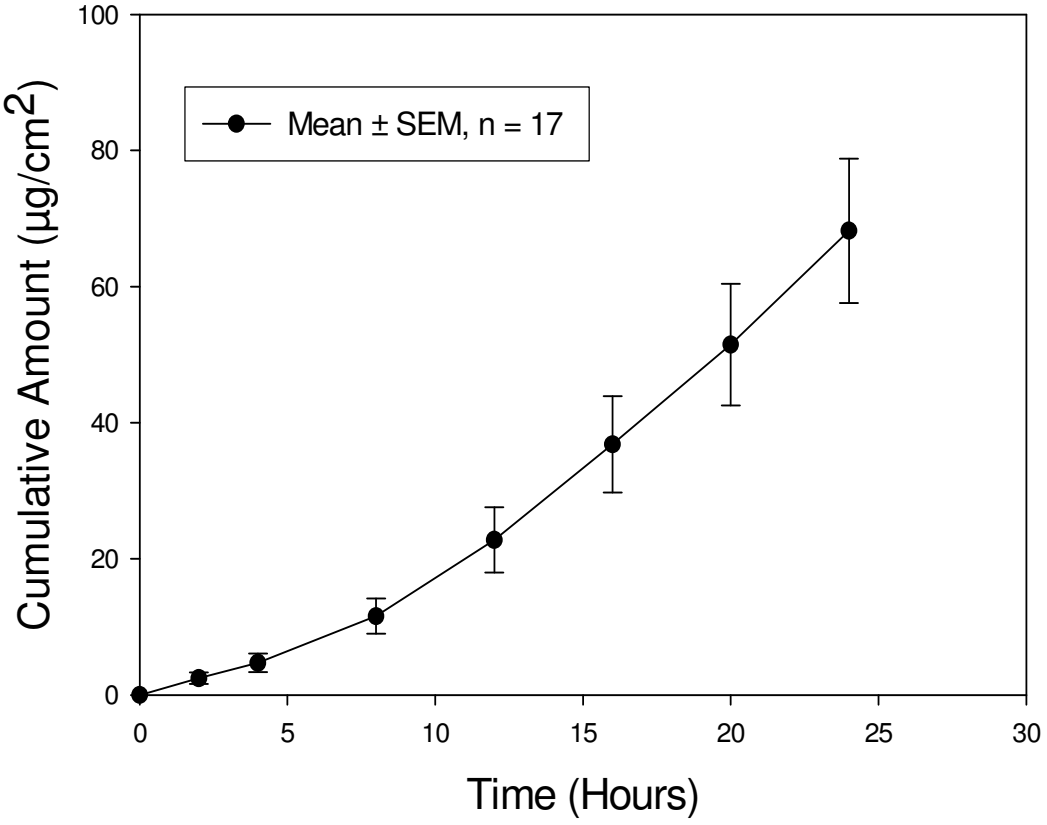

Figure 4. Mean niacinamide permeation over 24 hours. Results are expressed as mean ± SEM, n = 17, n = 3 skin donors age range = 39 - 57 years

After 12 hrs niacinamide permeation has reached a steady state. The slope of the graph after this time was given as the average flux, which was found to be 3.773 ± 0.5138 µg/cm^2/hr.

Niacinamide infinite dose studies were conducted in 100 % propylene glycol, which is itself a penetration enhancer and the cells were also occluded. These conditions

should favour the penetration of the test compound and thus these results are likely to reflect the highest penetration rates that might be expected from products containing this ingredient.

Infinite dose study results indicated niacinamide penetrates the skin rapidly under the test conditions. Therefore, further finite studies were conducted on niacinamide. Finite application techniques aimed at mimicking *in vivo* application. This was done because it is not only the compound that's important when determining its permeation potential but also the application system in which the compound is applied to the skin that can significantly affect the results.

3.4.1.2 Finite Study: From Simple Cream

Due to the ability of niacinamide to permeate through the skin under ideal circumstances, permeation studies were conducted on a simple cream containing 5 % niacinamide (a concentration commonly employed for products containing niacinamide on the market) [15]. Permeation cells were not occluded. Results consisted of an average of 24 permeation studies on three different skin donors. A plot of the cumulative amount of niacinamide penetrating the skin versus time from the cream is shown in Figure 5.

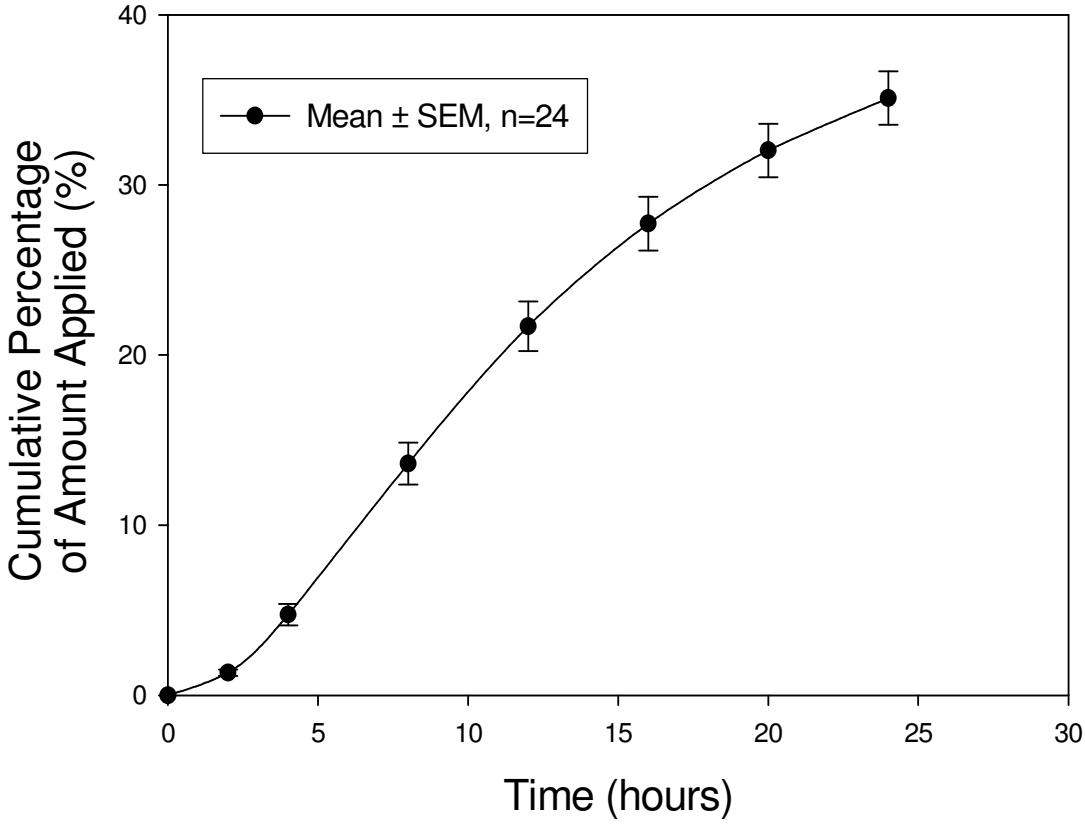

Figure 5. Permeation results for 5 % niacinamide out of a simple cream over 24 hours, 3 donors age range = 37 - 54 years

The permeation profile of niacinamide from the Simple Cream follows the expected finite dose profile. The average results were fitted to a sigmoidal, logistic, 3 parameter curve, using Equation 3, shown below. From this curve the maximum permeation of niacinamide from the Simple Cream was found to be 45.66 ± 0.210 % of the amount applied. Steady state is reached briefly between 4 and 12 hrs before a depletion of the concentration of niacinamide in the donor chamber begins, an average flux was determined to be 1.135 ± 0.05823 µg/cm^2/hr.

$$Y = \frac{a}{1 + \left(x / x_{0+} \right)^b} \qquad 3)$$

3.4.1.3 Finite Study: From Formulation used In Vivo

As the formulation can have a large impact on the permeation characteristics of a compound, a finite permeation study was conducted on the complete product used for *in vivo* efficacy investigations, shown in Chapter Six.

The permeation cells were not occluded and the donor cream was described earlier in method Section 3.3.2.1. A plot of the cumulative percentage of the amount of applied niacinamide penetrating the skin versus time is shown below in Figure 6. The results consist of an average of 22 permeation experiments with three different skin donors.

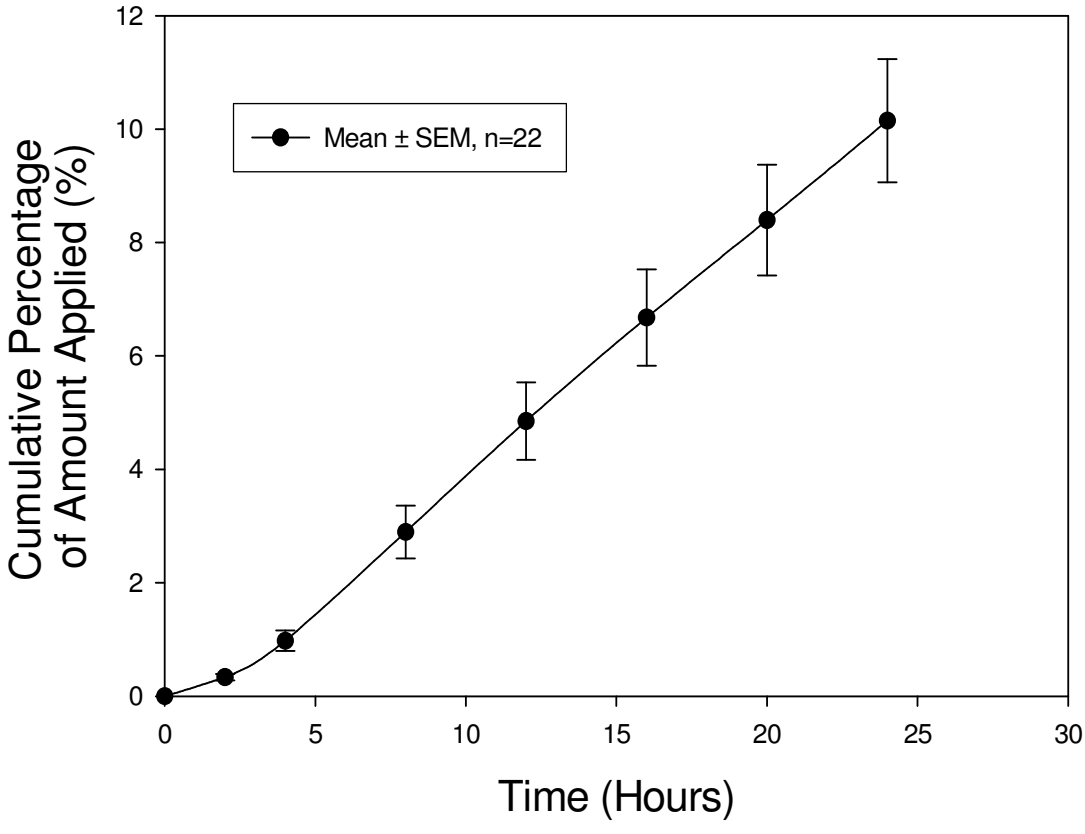

Figure 6. Permeation results for niacinamide out of cream used in the *in vivo* study over 24 hours, 3 donors age range = 37 - 54 years.

Although the *in vivo* formulation contains the same concentration of niacinamide (4.36 ± 0.53 %) as that used for the Simple Cream, permeation results shown in Section 3.4.1.2, the permeation characteristics have been altered significantly. Over the 24 hr permeation study an average maximum percentage of the applied amount was found to be 10.147 ± 1.0845 % and it appears as though dose depletion has not occurred. After 24 hrs less than a third of the amount of niacinamide penetrated compared to niacinamide from the Simple Cream formulation. The *in vivo* formulation is inhibiting the permeation of niacinamide compared to the Simple Cream formulation.

3.4.2 Genistein

For all permeation studies conducted on genistein a receptor solution of 0.5 % Volpo N20, 0.1 % sodium azide in water was employed, for reasons described in Chapter Two. All studies were conducted over 24 hrs and samples of the receptor solution were collected at 4 hr intervals, a sample was also collected at 2 hrs into the study to give a better view of the lag phase of the profile.

3.4.2.1 Infinite Study

As with niacinamide, initial permeation studies were conducted on a saturated solution of genistein in 100 % propylene glycol. The donor solutions were made up as per the Donor Solution Method Section 3.3.2.1. Over these permeation experiments all cells were occluded. The initial study of genistein was conducted under conditions favourable to permeation. The results of 16 permeation studies were averaged which

was performed on dermatomed skin with three different donors. A plot of the cumulative amount of genistein penetrating the skin versus time is shown in Figure 7.

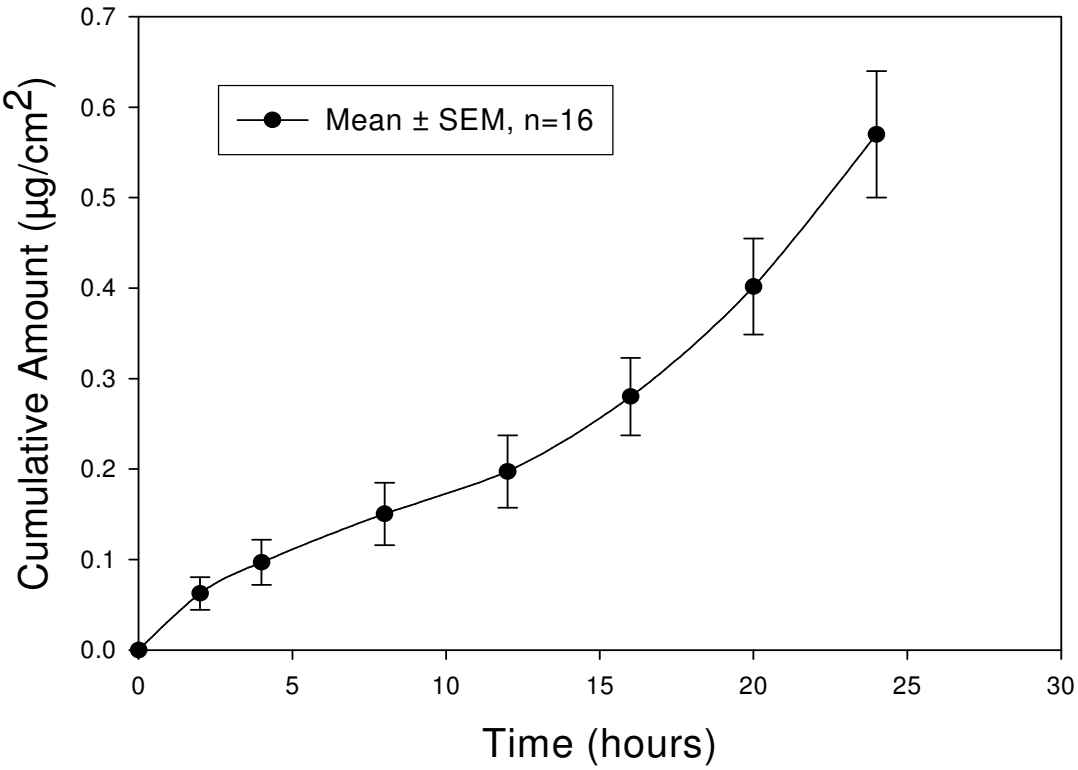

Figure 7. Mean genistein permeation over 24 hours, 3 donors age range = 47 - 57 years.

Exposure to a saturated solution of genistein in 100 % propylene glycol resulted in a cumulative permeation of 0.570 ± 0.070 µg/cm^2 (mean ± SEM), after 24 hrs. It appears as though genistein permeation has reach steady state after 16 hrs, the average flux under these conditions was determined to be 0.03625 ± 0.00544 µg/cm^2/hr. The recorded flux in this experiment was found to be one tenth of that reported in literature from 100 % propylene glycol [16]. However, in this experiment a saturated solution of soybean extract was used as the donor, a Franz Type diffusion cells was used for the experiment and the receptor solution consisted of 0.9 % NaCl Solution with Transultol®. Transcutol® is a penetration enhancer and it is possible that it may have diffused into the skin, which has the potential to cause penetration enhancement. The

cumulative permeation after 24 hrs and the flux of genistein from 100 % propylene glycol was found to be much lower than niacinamide under the same conditions. In assessing the relative potential for efficacy of an anti-wrinkle preparation it is necessary to consider both the penetration rate and the potency of the active ingredient. The profile of genistein permeation, shown in Figure 7, has an uncharacteristic jump in the amount of genistein permeating at time 2 hrs before entering the lag phase. This is likely to be caused by the amount of genistein penetrating at 2 hrs, which is close to the limit of detection of the HPLC assay.

3.4.2.2 Finite Study

After a basic understanding of the permeation of genistein was examined, a permeation study was then conducted on a 0.5 % genistein from the Simple Cream (a concentration commonly employed for studying genistein with regards to anti-aging effects) [17-19]. Permeation cells were not occluded. A plot of the cumulative percentage of the applied amount of genistein penetrating the skin versus time is shown in Figure 8. The results are an average of 21 permeation studies on three skin donors.

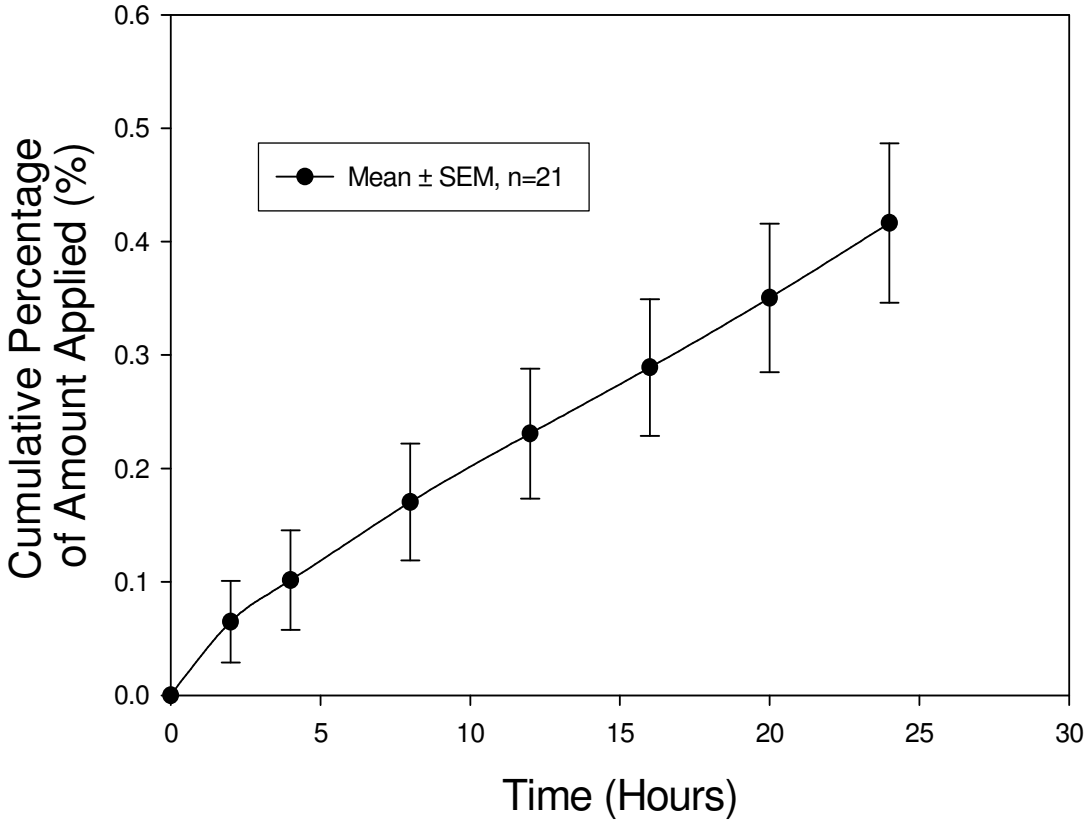

Figure 8. Permeation results for 0.5 % genistein out of a simple cream over 24 hours, 3 donors age range = 39 - 54 years.

After 24 hrs an average maximum of 0.4163 ± 0.07029 % of the applied dose was observed. The characteristic dose depletion plateau of a finite dose permeation study was not observed under these conditions. This was caused by the low amount of genistein permeating over the study period, as the proportion of genistein moving through the skin was less than the amount applied. A steady state was taken between 4 to 16 hrs and resulted in an average flux of 1.2960 ± 0.02807 %/cm^2/hr.

3.4.3 Palmitoyl tripeptide-5

For all permeation studies conducted on palmitoyl tripeptide-5 a receptor solution of 0.1 % sodium azide in water was employed for reasons described in Chapter

Two. All studies were conducted over 24 hrs and samples of the receptor solution were collected at 4 hr intervals. All studies conducted on the tri-peptide were finite dose studies. Cells were not occluded for all studies conducted on palmitoyl tripeptide-5.

3.4.3.1 Finite Study: Simple Solution

From the structure of palmitoyl tripeptide-5, it is predicted to have a low permeation rate through skin. Therefore, initial permeation studies were conducted using much larger concentrations than that recommended for cosmetic application to ensure the maximum chance of detecting permeating palmitoyl tripeptide-5.

Permeation studies were conducted on solution of 140, 280 and 560 μg/ml palmitoyl tripeptide-5 in water. Results shown in Figure 9 are an average of 24 permeation studies performed on dermatomed skin using three different donors. The data is plotted below as a percentage of the applied amount of palmitoyl tripeptide-5 penetrating the skin after 24 hrs.

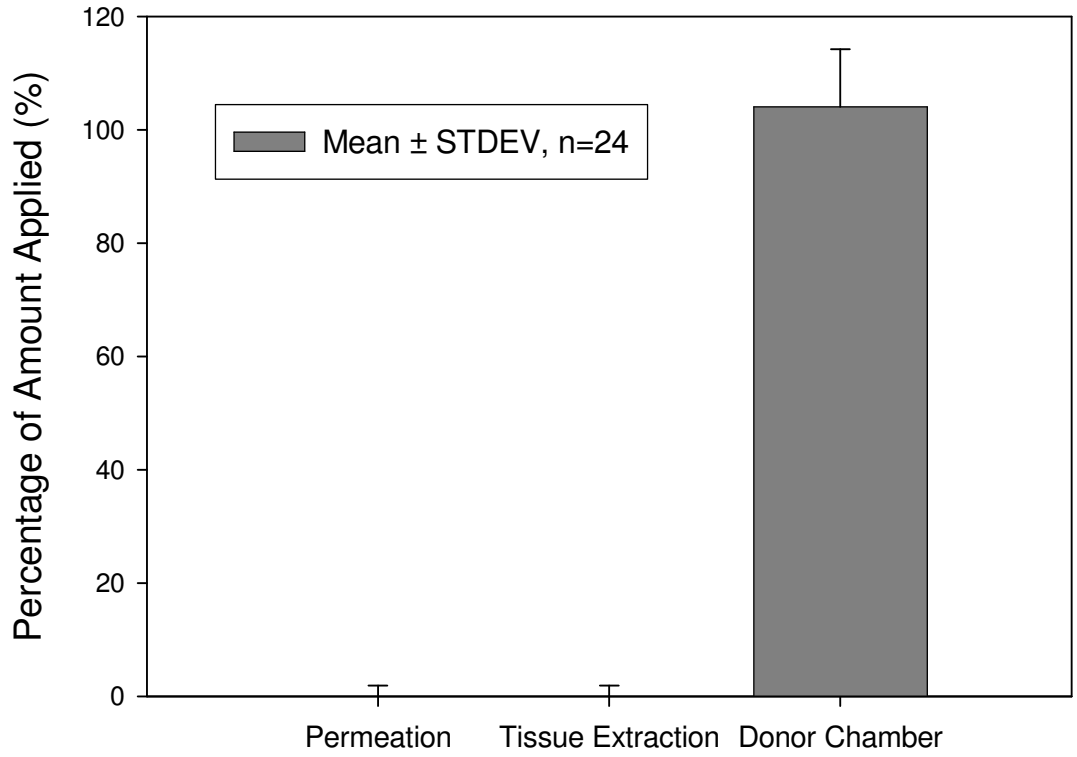

Figure 9. Mean palmitoyl tripeptide-5 permeation cumulative results after 24 hours, 3 donors age range = 39 - 54 years.

The concentration of palmitoyl tripeptide-5 in the collection vials during the 24 hr experiments was below the detection limit of the assay, indicating 0 % permeation after 24 hrs. The error was calculated from the limit of quantification, 6.581 µg/ml, as a percentage of the amount applied. This was the case for 140 µg/ml n = 8 (skin donors n = 1), 280 µg/ml n = 8 (skin donors n = 1) and 560 µg/ml n = 56 (skin donors n = 3). The concentration of palmitoyl tripeptide-5 extracted from skin tissue, performed as per methods Section 3.3.2.4, after 24 hrs of exposure to the solution was below the limit of assay detection, indicating 0 % tissue extraction. The average percentage recovery of palmitoyl tripeptide-5 in the donor chamber was determined to be 104.0734 ± 10.17271 % (mean ± STDEV). Permeation studies indicate that palmitoyl tripeptide-5 neither permeates nor penetrates the skin and therefore is unlikely to be able to activate production of collagen in the dermis.

3.5 Conclusion

The results presented in this chapter clearly demonstrate that niacinamide permeates the skin well and therefore it has the potential to reach its proposed site of action in significant concentrations. This indicates that niacinamide has the potential to cause antioxidant effects, stimulate collagen synthesis, epidermal cell growth and epidermal lipid production, once applied to the skin surface in a cosmetic product. This could lead to a number of anti-aging effects including: a reduction in the appearance, size and number of skin wrinkles. It was decided niacinamide would be a good compound to further investigate its anti-wrinkle efficacy *in vivo* based on its positive penetration results. Penetration was therefore conducted on niacinamide from the commercially available cream to be used in the small clinical study in Chapter Six. Although the cream did reduce the penetration of niacinamide by a third, there was still a high degree of penetration. Inhibition was likely caused by a less favourable release of niacinamide from the *in vivo* cream base compared the Simple Cream or 100 % propylene glycol. The variation between the two creams is likely to be related to the differences in the emulsion structure of the final creams. Both creams contain different emulsifiers and emulsion stabilisers which control the microstructure of the cream. The microstructure of the cream can severely impact the ability of an ingredient to be released from the formulation. Niacinamide, however, still has a high penetration potential from the *in vivo* cream base and therefore it has the capability of elicit an anti-wrinkle effect *in vivo*.

Genistein penetrates the stratum corneum slowly, under the conditions used in this study. Whether this will result in the desired anti-aging effect will depend heavily on the potency of genistein. It has been shown that topical treatment of the skin over 6

months with 0.01 % estradiol and 0.3 % estriol resulted in a reduction in wrinkle depth and an increase in moisture, elasticity and firmness of the skin [20] and an application of 0.625 mg/g of estradiol over 6 months resulted in a significant reduction in fine wrinkles compared to a placebo cream [21]. However, the estrogenic potency of genistein is ~1/10,000 and 1/50,000 of that of estriol and estradiol respectively [22].

Palmitoyl tripeptide-5 under permeation investigation showed an inability to penetrate or permeation the stratum corneum, therefore, it is unlikely to reach its site of action, within the dermis and is highly unlikely to be able to activate the production of collagen. This is confirmed in the analysis of the penetration of palmitoyl tripeptide-5 from applied concentrations much higher than that used in commercially available anti-aging creams. The levels penetrating the skin were much lower than those that have been shown to affect collagen synthesis. Manufacturers technical data has shown palmitoyl tripeptide-5 to affect collagen synthesis at concentrations in the range of 25 micro molar, which is equivalent to 15.30 µg/ml (palmitoyl tripeptide-5 MW 611.90), above the limit of detection of the assay. The studies were conducted on the compounds in simple solutions and therefore penetration enhancement protocols could dramatically alter the permeation of these ingredients. Although palmitoyl tripeptide-5 showed poor penetration potential it was still to be used for further *in vivo* anti-wrinkle efficacy investigation. Rather than the reported mechanism of action, collagen regeneration, *in vivo* efficacy studies can be used to investigate the potential for palmitoyl tripeptide-5 to superficially reduce the appearance, size and number of facial wrinkles. Its efficacy results can also be compared to a product that is likely to be able to elicit its anti-wrinkle mechanism of action, Niacinamide. For permeation studies to be best utilised they must be conducted using a situation that closely resembles that which would be used by the consumer.

These experiments illustrate permeation studies can be used by cosmetic companies as a tool for screening potential anti-wrinkling ingredients prior to conducting costly clinical trials. By coupling permeation study results with information on the concentration required for anti-wrinkle activity i.e. cell culture and toxicity results, they can demonstrate the potential for a product to get to where it's needed to perform its anti-wrinkle mechanism and also indicate potential safety concerns.

3.6 References

1. Benoit, I., et al., (2004) Oligopeptides to Smooth from Hibiscus Esculentus Seeds Expression Lines. *SOFW Journal,* **130**: 52-59.
2. Maia Campos, P., et al., (2008) *In Vitro* Antioxidant Activity and *In Vivo* Efficacy of Topical Formulations Containing Vitamin C and its Derivatives Studied by Non Invasive Methods. *Skin Res and Technol*, **14**: 376-380
3. Katayama, K, et al., (1993) A Pentapeptide from Type I Procollagen Promotes Extracelluar Matrix Production. *J Biol Chem*, **268**: 9941-9944
4. Barry, B.W., (1991) Lipid-Protein-Partitioning Theory of Skin Penetration Enhancement. *J Control Release*, **15**: 231-248
5. Elias, P.M., (1983) Epidermal Lipids, Barrier Function, and Desquamation. *J Invest Derm,* **80**: 44-49
6. Mitragotri, S., et al., (1995) A Mechanistic Study of Ultrasonically-Enhanced Transdermal Drug Delivery. *J Pharm Sci*, **84**: 697-706.
7. Weaver, J.C., et al., (1999) Theory of Electrical Creation of Aqueous Pathways across Skin Transport Barriers. *Adv Drug Del Reviews*, **35**: 21-39.
8. Walters, K.A. et al., (1993) Pharmaceutical Skin Penetration Enhancement. Drugs and the Pharmaceutical Sciences, Editor: Swarbrick, J., New York: Marcel Dekker, Inc.
9. Finnin, B.C. et al., (1999) Transdermal Penetration Enhancers: Applications, Limitations, and Potential. *J Pharm Sci*, **88**: 955-958.
10. Smith, E.W. et al., (1995) Percutaneous Penetration Enhancers. Florida: CRC Press, Inc.

11. Harrison, S.M., et al., (1984) Effects of Freezing on Human Skin Permeability. *J Pharm Pharmacol,* **36**: 261-262

12. Skelly, J.P., et al., (1987) AAPS/FDA Report of the Workshop on Principles and Practices of *In Vitro* Percutaneous Studies: Relevance to Bioavailability and Bioequivalence. *Pharm Res,* **4**: 265-267

13. Bronaugh, R.L. et al., (1985) Methods for In Vitro Percutaneous Studies: IV: The Flow-Through Diffusion Cell. *J Pharm Sci,* **74**: 64-67

14. Ostrenga, J., et al., (1971) Significance of Vehicle Composition. I. Relationship between Topical Vehicle Composition, Skin Penetrability, and Clinical Efficacy. *J Pharm Sci,* **60**: 1175-1179.

15. Gehring, W., (2004) Nicotinic Acid/ Niacinamide and the Skin. *J Cosmet Derm*, **3**: 88-93

16. Minghetti, P., et al., (2006) Evaluation of *Ex Vivo* Human Skin Permeation of Genistein and Daidzein. *Drug Del*, **13**: 411-415

17. Lin, J.Y., et al., (2008) Topical Isoflavones Provide Effective Photoprotection to Skin. *Photoderm Photoimm Photomed,* **24**: 61-66

18. Schmid, D., et al., (2003) Penetration and Metabolism of Isoflavones in Human Skin. *Cosmet Toiletries Mag,* **118**: 71-74

19. Wei, H., et al., (2003) Isoflavone Genistein: Photoprotection and Clinical Implications in Dermatology. *J Nutri*, **133**: 3811-3819S

20. Schmidt, J.B., et al., (1994) Treatment of Skin Ageing Symptoms in Perimenapausal Females with Estrogen Compounds. A Pilot Study. *Maturitas*, **20**: 25-30

21. Creudi, P., et al., (1994) Effect of a Conjugated Oestrogen (Premarin) Cream on Ageing Facial Skin. A Comparative Study with a Placebo Cream. *Maturitas,* **19**: 211-223

22. Shemesh, M., et al., (1972) Affinity of Rabbit Uterine Oestradiol Receptor for Phytoestragens and its use in a Competitive Protein Binding Radioassay for Plasma Coumestrol, *J Reprod Ferti,* **29**: 1-9

Chapter Four

Multiphoton Flourescence Lifetime Imaging a Potential Method for Evaluating Transdermal Diffusion

4.1 Introduction

As discussed in Chapter Three, transdermal delivery has become increasingly important within the cosmetic industry due to the emergence of so called "cosmeceutical" products [1]. To elicit their effect on the skin, the active ingredient must penetrate through the skin to reach its site of action. Recently multiphoton fluorescence microscopy has been used to visualise the penetration of drug molecules through the skin.

In order to gain a basic understanding of multiphoton fluorescence microscopy, confocal laser microscopy must first be explained. Confocal laser microscopy involves the investigation of a fluorescent specimen. A blue light is shone onto the sample, in this case skin [2]. When the light meets the fluorescent molecule, the light is absorbed causing it to jump into the excited state. As the molecule drops back to the ground state, fluorescent light is omitted. The fluorescent light is channelled through a pin-hole aperture, which only allows light from the focal point to reach the detector, excluding the out-of-focus background fluorescence of other molecules, shown below in Figure 1 (a). The theory of multiphoton fluorescent microscopy involves the use of a longer wavelength, a red light source, which has less energy than the blue light used in confocal laser microscopy. In this case when one photon of the light hits the fluorescent molecule it is excited into a pseudo-excited state. This molecule then requires a second photon of light at less energy to push it into the excited state [3]. Once it returns to ground state, fluorescence light is generated, which can then be absorbed by the detector. The laser focal point is the only location along the optical path where the photons are crowded enough to generate significant occurrence of two-photon excitation. A pin-hole aperture is, therefore, not required because it is unlikely any

background fluorescence will be created. The multiphoton system allows a greater proportion of the excitation light to reach the focal plane because the conditions for absorption are only met within the area of light concentration (at the focal plane), as demonstrated by the focal path below in Figure 1 (b). This means all the excitation energy reaches the focal plane, keeping the signal constant throughout the depth of the specimen and eliminating out-of-focus absorption. This method allows deeper analysis into the specimen [4] and reduces photo bleaching and photo damage, which is now strongly localized in the focal plane [5]. The red light suffers less scattering in the tissue than the blue light employed in confocal microscopy. Finally, in confocal microscopy out-of-focus fluorescence can be scattered into the pin-hole and then be detected. This will create a background fog and will reduce the image contrast, which is not seen in two-photon microscopy. Excitation photons can be scattered in two-photon microscopy, but the probability of two photons being scattered simultaneously to the same specimen location is extremely remote. However, the generated fluorescence, even if scattered, has an increased likelihood of being detected because there is no pin-hole present to block it from the detector [6-7]. One particular advantage of the technique for use in permeation studies is the ability of the longer wavelength photons to penetrate deeper into the dense tissue compared to the higher energy photons required for single photon excitation [3-4]. Furthermore, this technique has the potential for future development as a non invasive *in vivo* method for tracking permeation through human skin.

A schematic diagram illustrating the difference in confocal and multiphoton fluorescent microscopy is shown in Figure 1 (a) - (b).

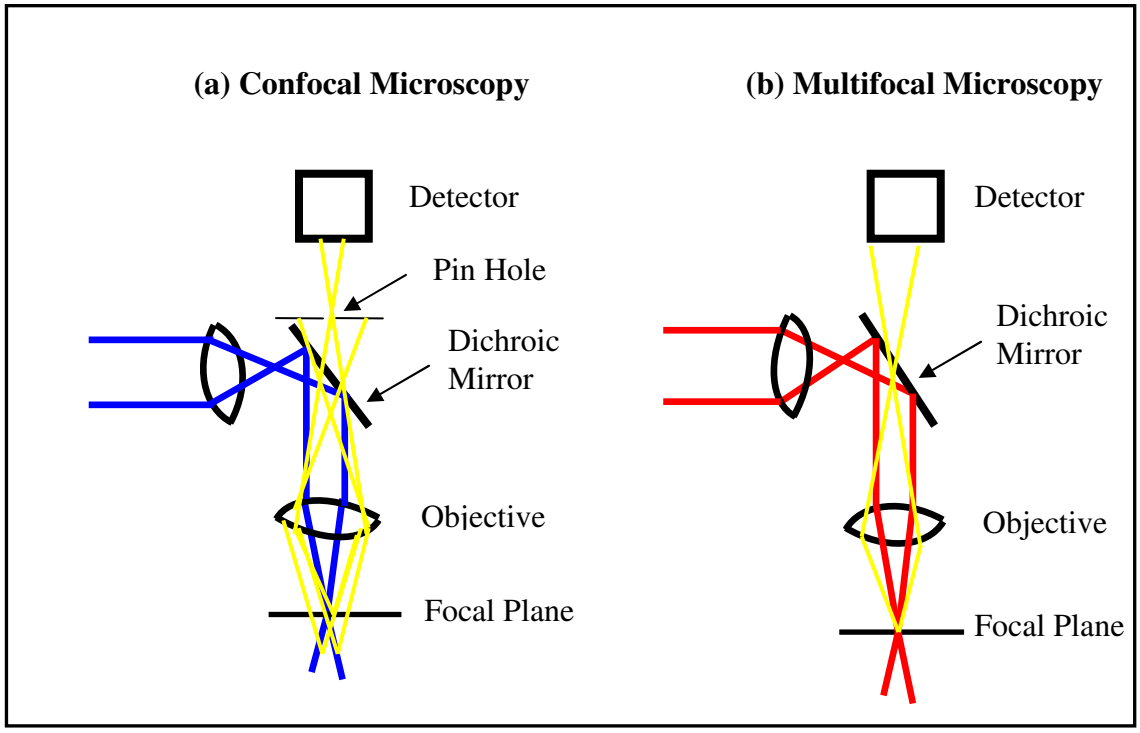

Figure 1. Schematic illustrations depicting (a) confocal fluorescent microscopy and (b) multiphoton fluorescent microscopy. The laser beam pathway is shown in either red or blue and the light path is shown in yellow.

The use of multiphoton fluorescence microscopy to study skin samples has traditionally targeted histological imaging of structures within the specimens [8-9]. More recently numerous studies have been conducted using multiphoton fluorescence microscopy to assess the transdermal permeation of drugs through the skin. However, as is the case with most studies to date, there is a major limiting feature; the studies only track the distribution of fluorescent probes after application to the skin. Because the compounds under investigation are generally non-fluorescent, multiphoton fluorescence imaging can not be used. A number of different methods have been utilised previously to overcome this problem. For example, fluorescent probes can be directly attached to the test compound [10], this treatment is generally undesirable, however, because it will

significantly alter the characteristics of the compound under investigation and thus may considerably affect the extent of compound penetration within the skin. Alternatively and most commonly, a model fluorescent compound can be used to simulate the expected permeation characteristics of the compound [11-14]. It is important that the model compound closely matches the characteristics of the compound it is modelling, in: size, charge, lipophilicity, membrane permeability and diffusion coefficient. This is a very difficult task as a change in one of these properties can significantly alter the way the model compound interacts with the skin.

Based on these major shortcomings, a technique which allows for the direct tracking of the test compound alone is currently being investigated. Of the many techniques available fluorescence lifetime imaging (FLIM) shows the most potential for solving this issue. The most common fluorescence technique involves the use of steady state fluorescence, examining the difference in specific fluorophores emission spectra i.e. fluorescence intensity. FLIM, however, produces an image based on the differences in the exponential decay rate of a fluorescent molecule. When a fluorescent molecule is excited by a photon or multiple photons it must then drop back to the ground state, releasing a photon (or multiple photons), this is done through the sum of a number of different decay pathways. Fluorescence lifetime is defined as the average amount of time a molecule spends in the excited state upon absorption of a photon of light [15]. Unlike steady state fluorescence the lifetime is usually reasonably independent of its concentration over modest ranges, and is to some extent immune to attenuation by re-absorption and / or light scattering [16-17].

Through the use of FLIM, researchers have gained insight into specific processes occurring within cells and tissues [9, 15, 18], which can not be performed with other florescence methods. FLIM has also been used for a range of studies these

include: monitoring and quantifying miscellaneous parameters [5, 19-20] i.e. pH which requires a number of fluorescent probes that can not be distinguished between using common methods [19], for diagnostics in oncology [21-23] and for histopathology [24].

In collecting fluorescence lifetime images a process known as single photon counting (SPC) is employed. Rather than the traditional method of capturing an image based on the fluorescence spectrum of the specimen, this method detects and counts individual photons of light. In simple terms an object that gives off a bright light is emitting lots of bundles of light energy, photons as a unit of time. While a dim light is emitting fewer photons in the same period of time. To measure the intensity of light from the object a count of the number of photons being emitted in a given unit of time can be performed, resulting in an exponential decay of the intensity over time. Intensity at each time point is a count of the number of photons being emitted from the sample at that time, after it was bombarded with a burst of light. Since the time scale of photon counting is in the order of a few hundred nanoseconds, the data can not be collected at this rate. Instead, a method known as time-correlated single-photon counting (TCSPC) is used. Under this method, for a given fluorescence event, one photon is detected and the time elapsed from the arrival of the excitation pulse at the sample, until that photon was detected, is recorded. This process is performed repeatedly millions of times and the information is collated. The resulting curve represents the intensity versus time decay curve [25-26]. As the laser scans the image, the data will generate a 3D matrix where the *x* and *y* axis is the position in the sample and the third dimension represents the arrival times of the photons associated with each pixel in the image [5].

Human skin has what is known as an intrinsic autofluorescence [27]. It originates from naturally occurring endogenous fluorescent biomolecules such as flavins, pyridine nucleotides metal free porphyrins and components of lipofuszin,

collagen, elastin and keratin [8, 28-29]. While imaging studies based on the fluorescence lifetime of the intrinsic autofluorescence from skin have been reported [28], few (if any) have been specifically tailored for transdermal delivery. The use of tracking the change in FLIM of the autofluorescence of the skin has mainly been studied as a tool for monitoring changes caused by a disease state.

It was proposed that by utilising an acquisition method previously published [19, 25] and by monitoring subtle changes in the fluorescence lifetime of the natural fluorophores in the skin, a method could be developed for determining the transdermal diffusion pathways and rates of a variety of applied formulations. It was thought this could be achieved by gaining a baseline of the fluorescence lifetime of the intrinsic fluorophores present in the intracellular region between corneocytes and those within the corneocytes. Subtle changes to the skins autofluorescence caused by a non-fluorescent compound / solvent formulation could then be detected.

4.2 Aim

To follow the penetration into the skin of applied compounds by observing the change in the FLIM images of endogenous skin components caused by the modification of the environment by the penetrating substance(s).

4.3 Materials and Methods

4.3.1 Sample Preparation and Handling Protocol

All skin samples used in this research were obtained from female patients who had undergone abdominal plastic surgery.

4.3.1.1 Full Thickness (Dermatomed)

Skin samples were sectioned to approximately 100 µm thicknesses. Samples were prepared as per the method described in Chapter Three, Section 3.3.1 (Skin Preparation and Handling) the Padgett Dermatome was, however, set to 100 µm.

4.3.1.2 Epidermal Membrane (Heat Separation)

Subcutaneous fat was removed from the skin samples using the blunt dissection method. The full-thickness skin was stored at -20 °C prior to use. Water was heated to 60 °C and stirred with a magnetic stirrer. The skin was placed in the heated water for 45 secs, allowing the epidermis to loosen from the dermis and aid separation. After which time the epidermal membrane was gently removed from the dermis with tweezers. The epidermal membrane was floated on water with the stratum corneum oriented up, then placed on an aluminium foil sheet to dry and stored at -20 °C. Prior to imaging, the frozen epidermis was left at ambient laboratory conditions (~22 - 24 °C and 40 - 60 % relative humidity) to thaw for ~1 hr. Excess moisture was removed by delicately blotting the skin. The sample was then placed on a 0.17 mm cover glass slide 24 x 60

mm from ProSciTech (Queensland, Australia) (stratum corneum oriented up) for multiphoton microscopy examination.

4.3.1.3 Stratum Corneum (Trypsin Separation)

Prior to stratum corneum separation, thawed epidermal membrane was cut into a series of sections, ~1 cm^2 in area. The epidermal membrane was floated on a trypsin solution (0.001 % trypsin from Sigma-Aldrich (Missouri, USA), 0.1 % sodium hydrogen carbonate from Merck Pty Ltd (Victoria, Australia) in water) and left overnight, stratum corneum oriented up. The epidermis was digested by the trypsin solution and sank, leaving the stratum corneum floating on the surface of the solution. The stratum corneum was placed on 0.17 mm cover glass slides and stored in a Pyrex Knob Top Desiccator containing silica desiccant beads both obtained from Sigma-Aldrich (Missouri, USA). In preparation for imaging, the stratum corneum was re-hydrated. The desiccant beads were replaced with a Petri dish containing a saturated solution of sodium sulphate anhydrous from Ajax Chemicals Pty Ltd (New South Wales, Australia) in water and the desiccator was subsequently sealed and left for a minimum period of 2 hrs. Examination by multiphoton microscopy immediately followed the re-hydration process.

4.3.2 Instrumentation

A detailed schematic diagram of the multiphoton FLIM system used for these imaging studies of human skin is shown in Figure 2 [30].

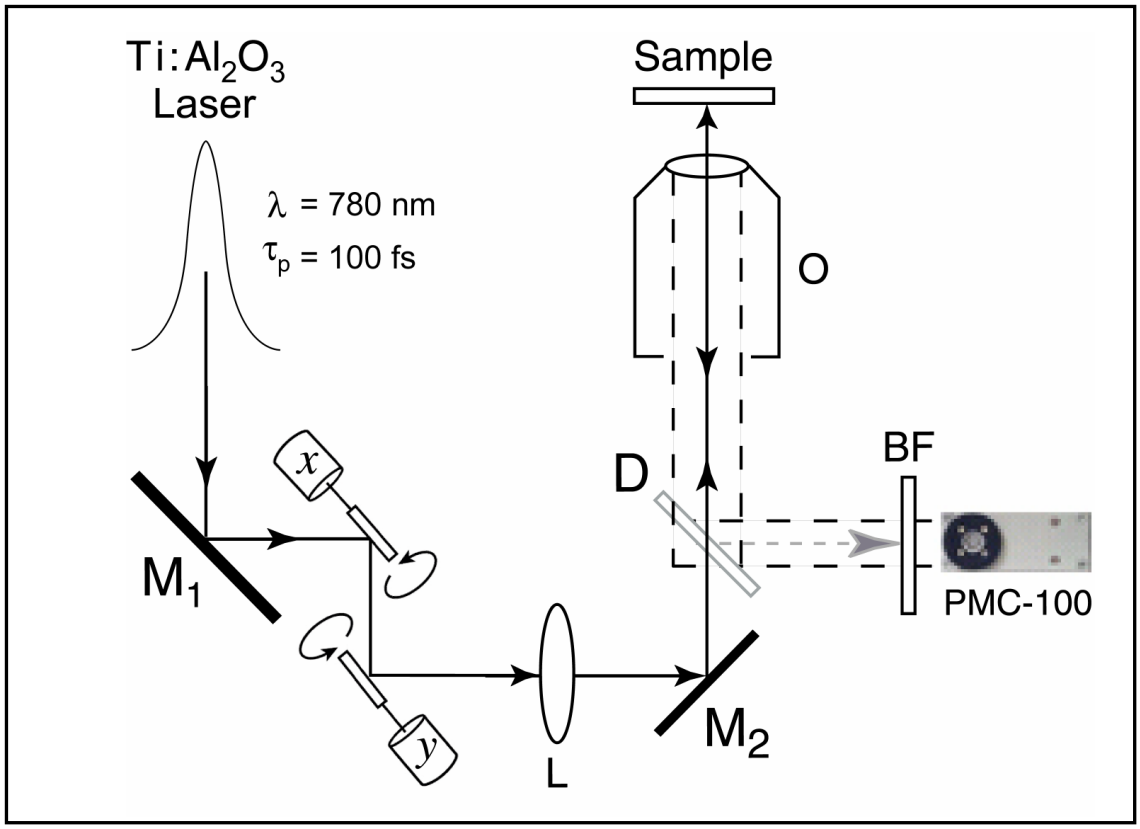

Figure 2. Schematic diagram of the multiphoton florescence lifetime microscope where; O: microscope objective, M_1.M_2: Mirrors, D: Dichromatic mirror, BF: IR-Blocker, PMC-100 photon-counting detector

The system details are described in depth elsewhere [30]. Briefly, it consists of a Ti:Sapphire mode-locked Mira 900F laser cavity from Coherent (California, USA), pumped by a 10 W solid-state Verdi V10 laser from Coherent (California, USA), an inverted IX-71 microscope from Olympus (Pennsylvania, USA), an Olympus, FV-300 confocal scanning unit (Pennsylvania, USA), a fast photon-counting PMC-1-100 detector from Becker & Hickl (Nahmitzer Damm, Berlin), dichromatic Cold Mirror from TFI Inc. (Massachusetts, USA), BF (BG39) blocking filter from TFI Inc. (Massachusetts, USA) and SPC-830 from Becker & Hickl (Berlin, Germany), a compact PCI card-based electronic system for recording fast light signals by TCSPC.

Synchronised fluorescence lifetime data collection on a pixel-by-pixel basis was achieved using the *x* and *y* laser scanning signals generated by the scanning unit.

4.3.3 Imaging Method

It is the significant endogenous fluorescence of keratin under two-photon excitation that enables imaging of the stratum corneum of human skin [10, 31]. The power was adjusted to a moderate 10 – 15 mW at the sample (~0.13 – 0.2 nJ per pulse) for imaging in order to obtain a sufficient photon count rate (~0.5 MHz). The skin samples were prepared as per Section 4.3.1 (Sample Preparation and Handling Protocol) and placed on the microscope cover glass with the stratum corneum oriented up, i.e. the stratum corneum is not in contact with the glass. This method was chosen since the aim of the experiment was to observe the absorption of ingredients topically applied to the skin, thus focusing through the bottom of the sample towards the stratum corneum, as illustrated in Figure 3. The orientation allowed the illumination beam to be focused at a certain depth, *z*, into the skin and to sequentially image before and after application of the formulation. Therefore, a formulation could be applied to the surface of the skin sample without disturbing the microscope focal point and images could then be captured at multiple time points after the application. Since an inverted microscope was used for all experiments, by orientating the specimen with the stratum corneum down, i.e. in contact with the cover glass, this would create a major practical issue for permeation studies. The skin would be required to be lifted off the cover glass after initially imaging the specimen to enable the formulation solution to be applied. To re-image, the sample would then have to be re-mounted on the cover glass, which would make the likelihood of recovering the original region of interest potentially non existent.

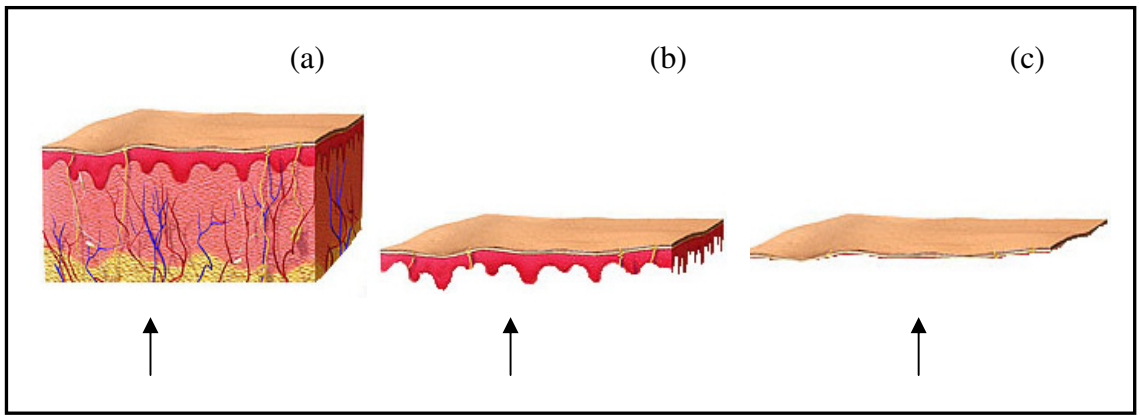

Figure 3. Schematic diagram of skin samples (a) full thickness, (b) epidermal membrane and (c) stratum corneum. The arrows below the samples indicate the microscope axial focal path of all three skin thicknesses referred to as, z, below [32].

Images were obtained on the multiphoton microscope for three skin thickness: full thickness, epidermal membrane and stratum corneum, illustrated in Figure 3. At each point the microscope was focused on a sample and a stack of images were collected, 10 images per stack separated by 1 μm in depth along the, *z*, axis. Three image stacks per sample were collected with a minimum spacing of ~5 mm. Photon counting per image section was 90 secs, with an average pixel dwell time of ~6.5 μs, which allowed for a sufficient photon count in order for the data to precisely fit to the curve. On average, it took ~20 mins to collect one image stack. Therefore, the total number of images acquired for each skin type: full thickness, epidermal membrane and stratum corneum, was a function of the number of stacks imaged per sample (three), the number of images in the stack (ten), and the number of skin samples for each skin type (five), giving a total of 150 images per skin type.

4.3.4 Data Processing and Validation

Analysis of the fluorescence lifetimes obtained from all skin types was performed using SPCImage software package from Becker & Hickl (Berlin, Germany), which is capable of modelling up to a three-exponential decay distribution. At each pixel the recorded fluorescence decay is fitted to a nonlinear model function. The fitted data is then displayed as a pseudo-coloured image with colour representing lifetime. A detailed description of the algorithmic functionality of the program has been given by the manufacturer [33].

For any given imaging session, stacks with the highest signal to noise ratio were selected. Each image was processed as per Figure 4, where six representative pixels along the junction, J (area where three or four corneocytes meet), were fitted to a two-exponential decay model with a binning factor, n = 1. Each pixel analysed is actually the sum of nine adjacent pixels, which is then fitted to an exponential decay. By using an average of the fluorescence lifetime decay of multiple pixels it has been shown to significantly increase the accuracy of the results [33]. Also within each corneocytes four representative pixels were analysed in the same manner, see Figure 4.

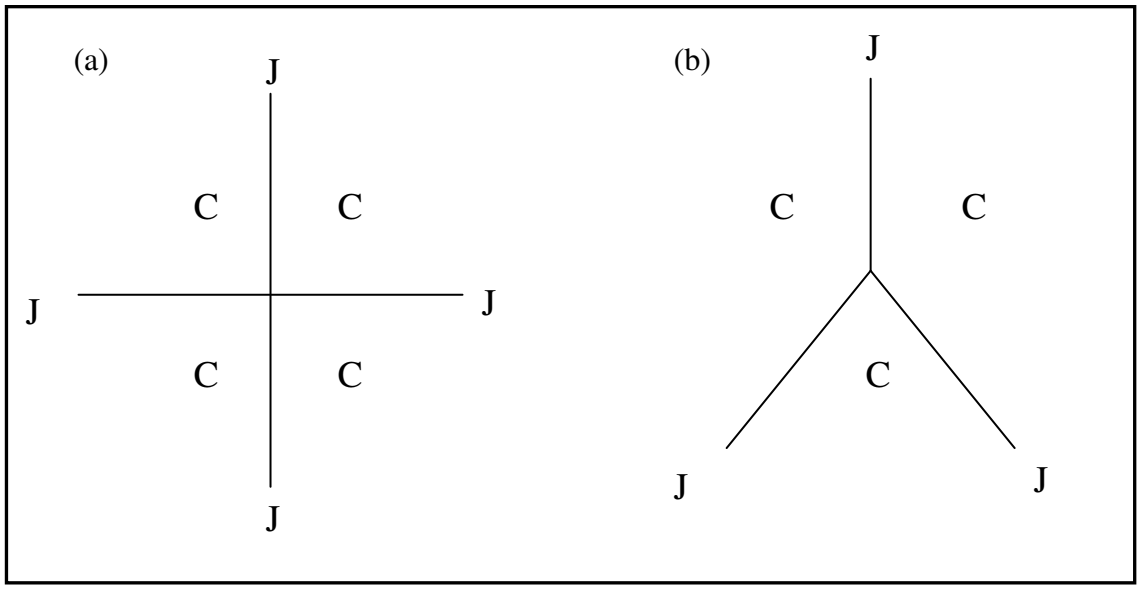

Figure 4. Schematic diagrams of the assessment criteria for each skin type. It was found corneocyte junctions either involved the coming together of (a) 4 corneocytes or (b) 3 corneocytes. Sites labelled (C) represents area within the corneocytes. In each area labelled (C) 4 pixels were analysed. Each line labelled (J) represents the junctions. Along each junction labelled (J) 6 pixels were analysed.

From each stack three images were analysed at depths approximately 2, 7 and 10 µm from the stratum corneum surface. FLIM data was analysed for three skin samples per skin type (full thickness, epidermal membrane and stratum corneum). For each representative pixel, the short (τ_1) and the long (τ_2) fluorescence lifetime components, the two components contributing to the double exponential decay, along with their relative contributions to the exponential sum, a_1 and a_2 respectively, were collected. A representative exponential decay curve obtained for each pixel is shown in Figure 5. The final presented results include the average values ± the standard deviation of each of these parameters, with a total of 160 pixels for junction sites and 88 pixels for corneocytes per sample thickness type.

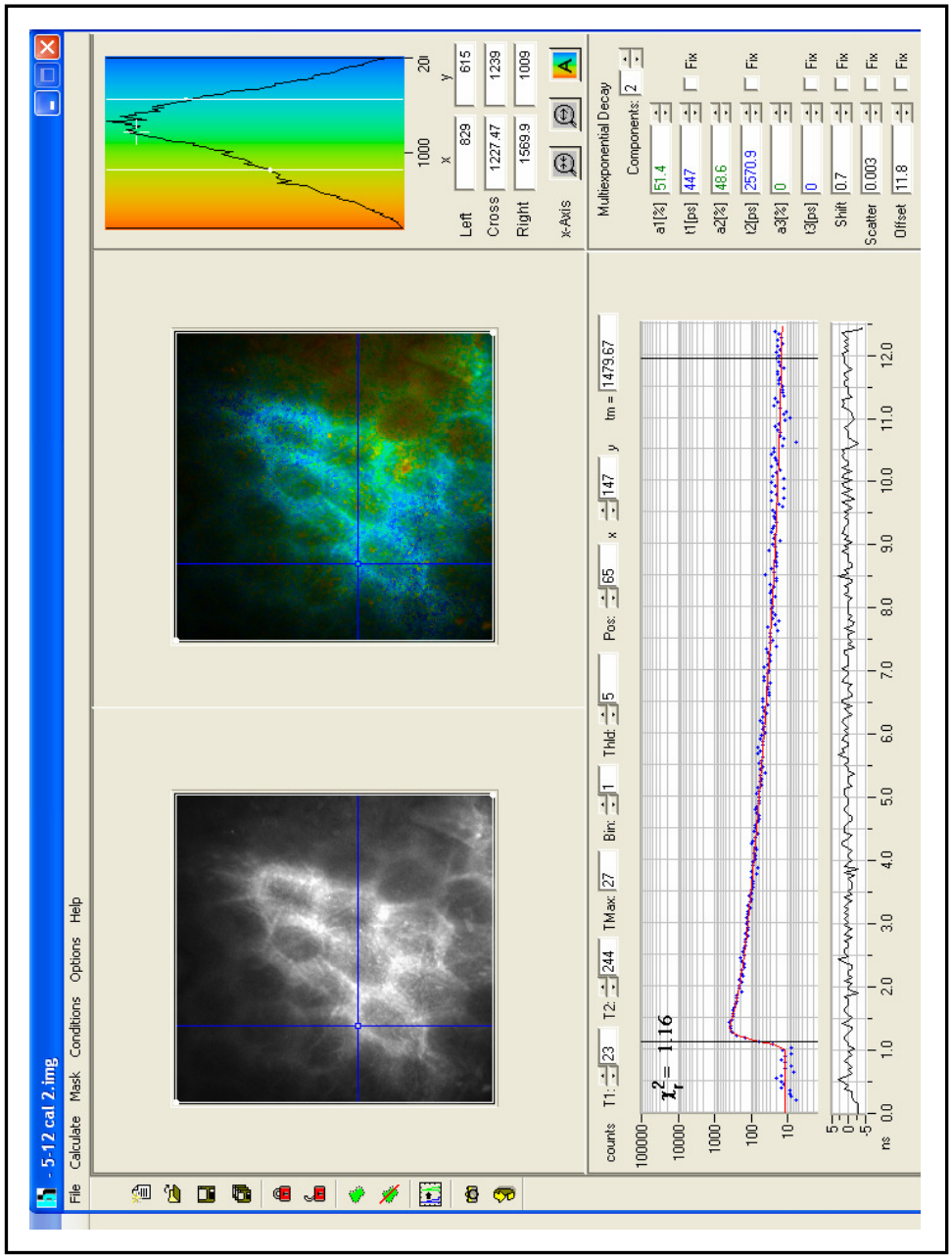

Figure 5. Representative exponential decay curve for a pixel, the short (τ_1) and the long (τ_2) fluorescence lifetime components indicated as. Their relative contributions are also determined from this graph as their exponential sum, a_1 and a_2, set up depicts that shown in Figure 7 (b).

The quality of the fit at any given pixel (and therefore the pseudo-coloured image) will depend greatly on the instrument response function (IRF) of the system, the instrumental accuracy. Therefore, if the imaging system is to have the ability to temporally resolve subtle changes in the fluorescence lifetime of fluorophores present in the skin, it is important to maintain an optimised IRF. The IRF of the optical system was determined at the beginning and at the conclusion of each imaging session held during the day to ensure the accuracy of the fluorescence lifetime results. This was performed by scattering a small portion of the illumination light to the photon counting detector. The measured the full width at half maximum (FWHM) of the IRF, used to describe the width of an object with non-sharp edges, was consistently determined on all days, before and after each imaging session, to be 240 ± 10 picosecond (ps). This measurement represents the response of the overall imaging system at the time. It takes into account the transient time spread of the detector, optical dispersion at the mirrors and through the lenses, and light scattering at the diaphragms.

Furthermore, a second instrument standard was used to ensure the integrity of the data collected over these experiments. The fluorescence lifetime of 20 μm diameter fluorescent latex beads from Polysciences Inc. (Pennsylvania, USA) was measured before and after the imaging session. Five to ten individual microspheres were imaged under the collection parameters of the imaging protocol, with the one exception an illumination power of ~1.0 mW at the sample (1.5 mW at the back of the objective). This level of power was used to yield a photon count rate comparable to that used in the imaging protocol (~0.5 MHz). On all days and all imaging sessions, the measured lifetime of the standard microspheres was 2.2 ± 0.1 ns, which is consistent with that reported in the literature [34-35].

4.3.5 Statistical Analysis

Statistical significance was determined using a One-Way Analysis of Variance (ANOVA). Post-Hoc Turkey tests were conducted to compare the differences between each group. A probability of $p < 0.05$ was considered statistically significant. All results shown in this chapter are presented as a mean $\pm$ STDEV unless otherwise stated.

4.4 Results and Discussion

4.4.1 Multiphoton FLIM of Human Skin

All fluorescence lifetime images, for each skin thickness type, have the dimensions 256 x 256 pixels. The yellow bar at the bottom right corner of the first image in each set represents a scale bar. For all images obtained for full thickness and stratum corneum skin types this scale bar corresponds to 25 µm, for Figures 6 and 9, however, for all images obtained for epidermal membrane skin type the scale bar corresponds to 10 µm, for Figures 7 and 8.

Representative fluorescence lifetime images of full thickness human skin sections prepared as per methods Section 4.3.1.1, with an imaging depth of approximately z = 2, z = 20 and z = 70 μm respectively are shown in Figures 6 (a) – (c). Therefore, image 6 (c) is closer to the stratum corneum than image 6 (a). All the images were obtained under identical experimental conditions, at 780 nm excitation and were pseudocolour mapped over the same range (0.2 – 1.0 ns). The mapping represents the weighted average of the short, τ_1, and the long, τ_2, exponential decays i.e. shown below in Equation 1.

$$\tau_m = \frac{\left(a_1\tau_1 + a_2\tau_2\right)}{\left(a_1 + a_2\right)} \qquad 1)$$

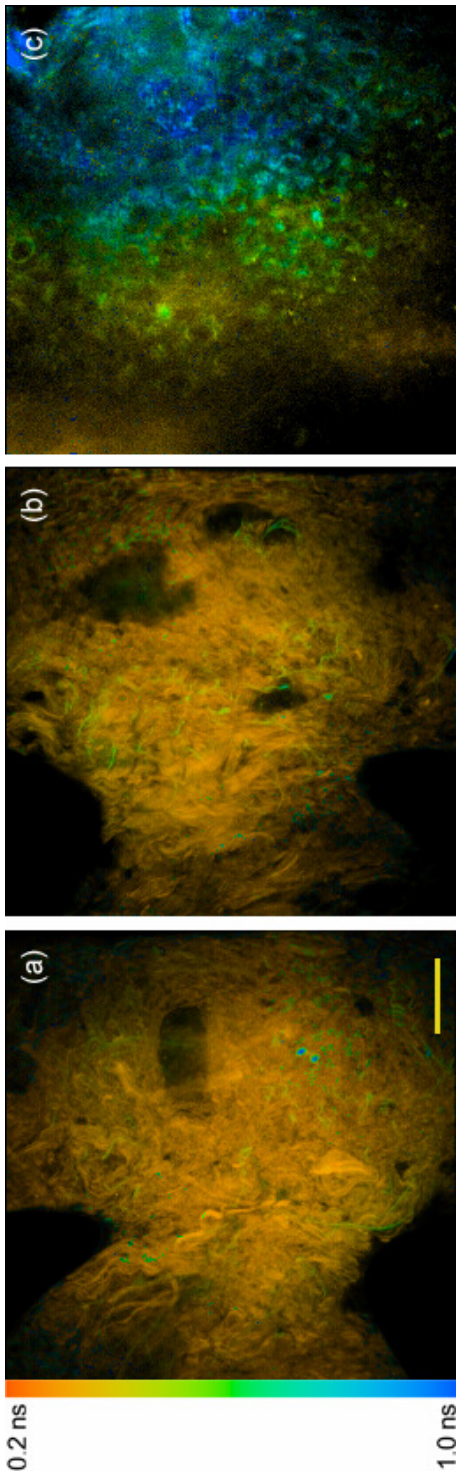

Figure 6. Representative fluorescence lifetime images of full thickness dermatomed sections of skin at an imaging depth of (a) z = 2 µm, (b) z = 20 µm and (c) z = 70 µm relative to the dermal layer. The yellow scale bar at the bottom right corner of (a) represents a distance of 25 µm and is valid for the three images.

In Figure 6 (a) collagen structures are clearly evident, represented by the orange coloured areas. This is understandable as collagen is present in the lower layers of the dermis. Collagen is known to produce a strong second harmonic signal [36-39], due to its highly crystalline triple helix structure, which means it is non centrosymmetric (lacks an inversion centre) and thus gives the molecule the ability to form second harmonic generation (SHG). For a simple understanding, when shining a pulsed deep red laser light through a quartz crystal ultraviolet light is produced, this light is the second harmonic of the original light [40]. The measured value of τ_m reflects the SHG signal, which is in the order of the instrument response (~0.26 ns). This is clearly demonstrated at a depth of z = 2 μm into the full thickness sample where the dominant signal is that of second harmonic signal generated by the collagen located in the area captured in the image.

As for Figure 6 (b) at a depth of ~20 μm into the sample from the base, the second harmonic is still the dominant signal. However, as the focus is moved deeper into the full thickness sample, Figure 6 (c), ~70 μm, the value of τ_m increases significantly to ~0.9 ns, indicating a reduction in the second harmonic contribution. We can visually see this change in Figure 6 (c) with the emergence of the epithelial cells seen in blue. The aim of this work was focused on developing a method for tracking the permeation of topically applied compounds through the stratum corneum. Since the autofluorescence of the corneocytes junctions is distorted, mainly caused by the second harmonic generated (SHG) signal from the collagen fibres and fluorescence caused by the epithelial cells, full thickness sample preparation is not a viable choice.

The use of full thickness skin samples does, however, have the potential for future development in studying the efficacy of anti-aging products. Collagen regeneration is one of the three main mechanisms by which cosmeceutical products

claim to treat skin aging. Therefore, by applying products claiming collagen regeneration capabilities to the surface of full thickness skin samples and visualising effects caused to the collagen fibres using multiphoton fluorescence lifetime imaging there is a potential to develop a method to evaluate product efficacy. As seen in Figure 6 (a) and 6 (b) this highly sensitive method provides well detailed images of the skins collagen ribbons. A change in the mass of the collagen ribbons could be used to identify an effect a test formulation may have on collagen regeneration over time. The method also has the potential for *in vivo* evaluation of collagen regeneration. Patients undergoing facial plastic surgery could apply a test product 4 - 8 weeks prior to surgery, containing an anti-wrinkle ingredient (claiming collagen regeneration properties) to a site being removed, while another site would be left untreated. The collagen content of the two excised sites, treated and untreated could be used to asses the collagen regeneration efficacy of the test ingredient using the multiphoton FLIM apparatus.

Figures 7 (a) – (c) shows representative fluorescence lifetime images obtained from epidermal membrane sections pseudo-colour mapped to the weighted average of the short and long components, τ_m. Figures 8 (a) – (c) show the same epidermal membrane images, however, they are pseudo-colour mapped to the long exponential component, τ_2, excluding the short component.

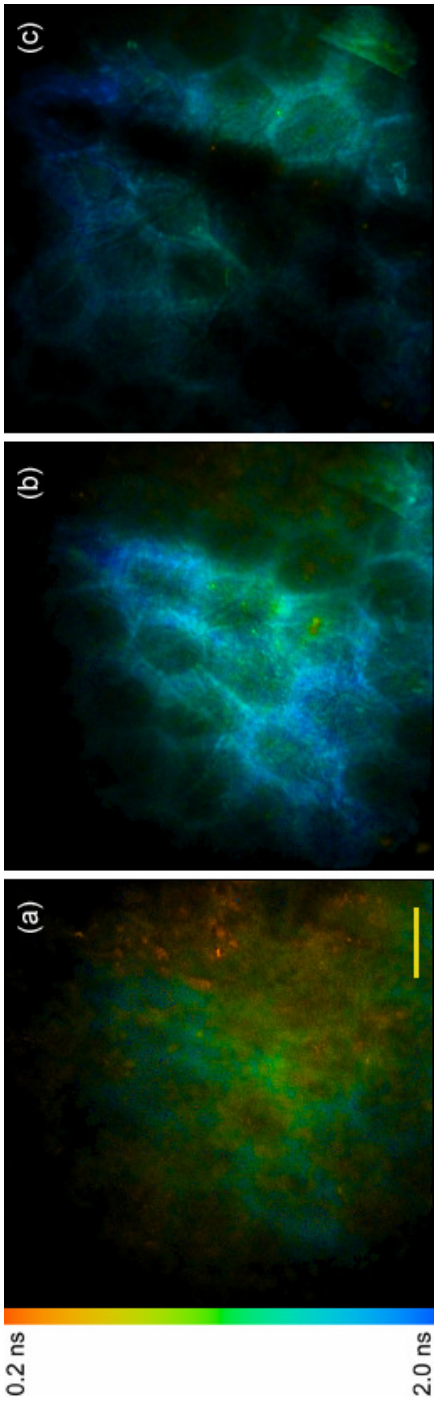

Figure 7. Representative fluorescence lifetime images of human skin obtained from epidermal membrane skin sections. The yellow scale bar at the bottom corner of (a) represents a distance of 10 µm for the three images.

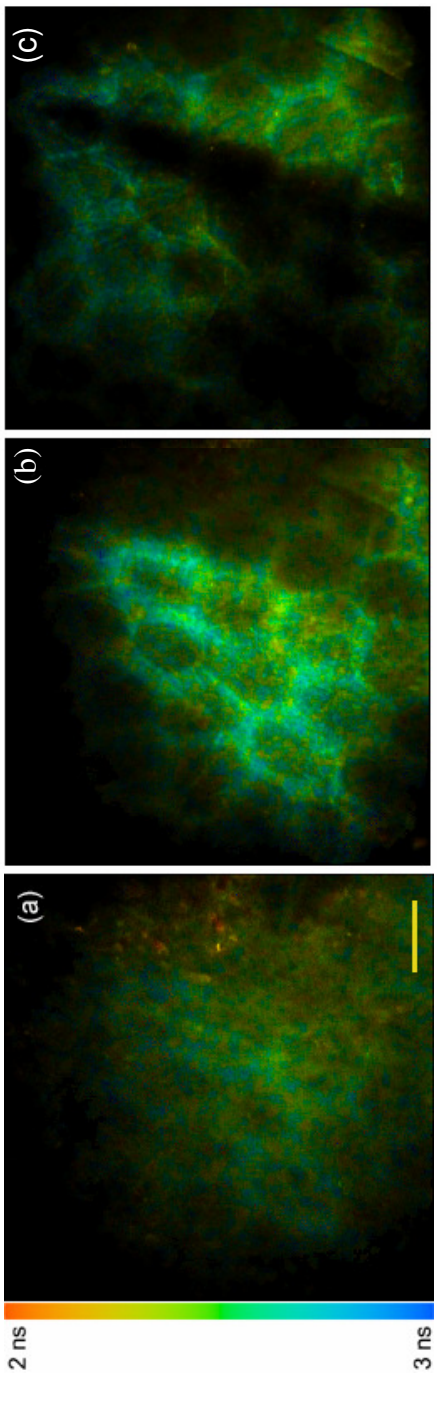

Figure 8. The same representative fluorescence lifetime images of human skin obtained from epidermal membrane skin sections given in Fig. 7, pseudocoloured to only the long, τ_2 exponential component. As with Fig. 7 the yellow scale bar represents a distance of 10 µm.

Figure 7 (a) again reveals a SHG signal from collagen structures, seen in the orange colour to the right of the image. The contribution of the SHG signal is significantly reduced compared with Figures 6 (a) and (b); this is because the epidermal skin preparation technique, where the dermis is removed through a heat separation process, removes most of this tissue type. It is important to note in Figure 7 (b) and (c) at a depth of approximately z = 45 μm and z = 51 μm into the skin sample (which corresponds to a depth of ~12 and ~6 μm relative to the skin surface, respectively) the corneocytes of the stratum corneum are clearly visible, which was not seen in Figure 6. On closer inspection of Figure 7, we see there is a clear change in the measured weighted average fluorescence lifetime from the intracorneocyte junctions in Figure 7 (a) compared to those of Figures 7 (b) and (c). In Figure 7 (a) the intracorneocytes junctions are depicted in a green colour indicating a τ_m is of ~1.0 ns. Where as, in Figures 7 (b) and (c) the intracorneocyte junctions are depicted in a blue colour, indicating a τ_m value of ~1.7 ns. The significant shortening in the value of τ_m is caused by the SHG process dominating in the exponential sum via the a_1 coefficient. By excluding the short exponential component when creating the pseudo-colour map, shown in Figure 8, we can justify this statement. Figures 8 (a) – (c) clearly show there is virtually no change in the fluorescence lifetime of the emitting species present in the intracorneocyte junctions when the short, τ_1 component is omitted from the pseudo-colour map. While the existence of a short-lived emitting species present in the junction cannot be discounted, compared to the magnitude of the scattering signal, it is reasonable not to consider it.

Figures 9 (a) – (c) depicts the representative fluorescence lifetime images reconstructed purely from the autofluorescence present in the stratum corneum at an imaging depth of approximately z = 2, z = 6 and z = 14 μm respectively, relative to the base of the sample.

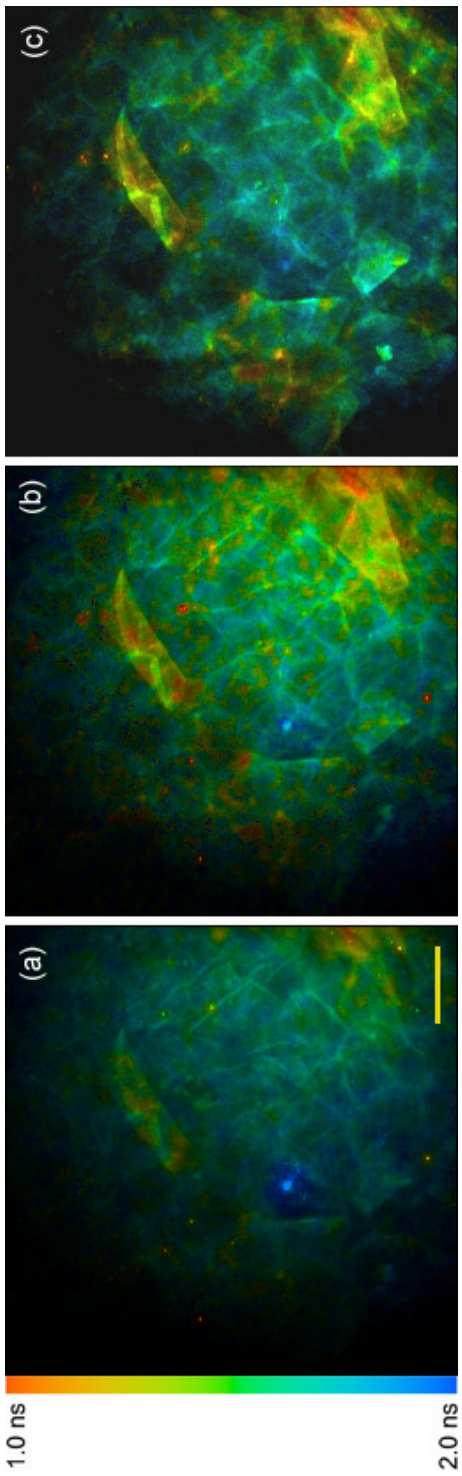

Figure 9. Representative fluorescence lifetime images of human skin obtained from stratum corneum skin sections. The yellow scale bar at the bottom right corner of (a) represents a distance of 25 µm for the three images.

The sample thickness has been reduced to ~10 – 20 μm. Almost all of the connective tissue structures (i.e. collagen) have been entirely removed. The reduction in sample thickness has caused the short lifetime component, $\tau_{1,}$ to be reduced to ~0.7 ns and a reduction in the relative contribution of, a_1, to ~50 %. Therefore, we would assume the values given from the stratum corneum skin sections are a better representation of the true fluorescence lifetimes of the emitting species in the intracorneocyte junctions because the measured decay function does not include emission or scatter caused by other species / structures (i.e. collagen, elastin or epithelial cells).

Figure 10 is a summary of the combined average fluorescence lifetimes and standard deviations for the imaging sessions for each skin type. Each skin type represents the short and long lived emitting species, along with the ratio of these two forms (a_1:a_2) for an average of 160 pixels per skin sample.

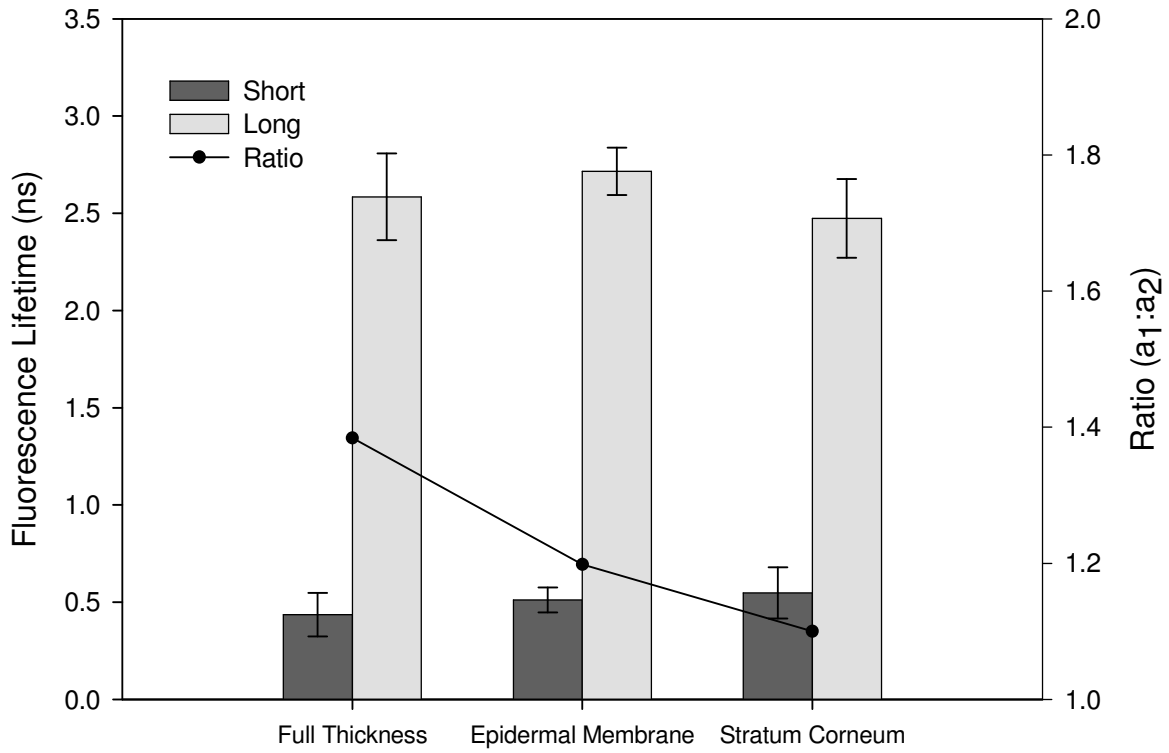

Figure 10: The average fluorescence lifetimes ± STDEV at the intracorneocyte junction as a function of sample type.

From the graph it is evident there is a large reduction in the ratio (a_1:a_2) by a factor of ~2 for stratum corneum compared to both the full thickness and epidermal membrane skin types. Confirming what is depicted above in the fluorescence lifetime images in Figures 6, 7 and 9. As most of the connective tissue, i.e. collagen and elastin, has been removed in the process of heat treatment to separate the stratum corneum from the epidermis, the second harmonic generation signals and light scattering has been reduced, if not eliminated.

For all skin types a range of ~0.4 – 0.6 for the short and 2.5 – 3.2 ns long fluorescence lifetime components was found at the intracorneocyte junctions. The values agree well with that reported in literature [28, 41], which from only one example

has been calculated as short component of 0.3 ns, long component of 1.7 ns and a ratio (a_1:a_2) of 1.56. There is excellent consistency in the fluorescence lifetimes over the five independent imaging sessions, which is evident with a standard deviation of 0.2 ns. Statistical significance, p = 0.000, was found between the average fluorescence lifetimes of the species responsible for the short (τ_1) component present in the intracorneocyte junctions for the different sample thicknesses. The average fluorescence lifetime of the short (τ_1) component for full thickness skin is significantly less than that of the stratum corneum, increasing from 0.44 to 0.55 ± 0.1 ns respectively, while it remains relatively unchanged when compared with the epidermal membrane sections. The long (τ_2) component remains approximately constant across all sample types, the measured average being 2.6, 2.72 and 2.47 ± 0.2 ns, for full-thickness, epidermal membrane and stratum corneum sections respectively.

As stated previously, excised, frozen and thawed human skin was used in this study. The properties of these skin specimens treated here may differ from skin *in vivo* and freshly excised biopsies, as for example, the pH depth profile changes rapidly after excision [42]. However, previous investigations have shown that no change in the penetration characteristics occurs during a storage time of 6 months [42-43].

4.5 Conclusion

This study has comprehensively characterised the autofluorescence of junctions between corneocytes for a number of skin thicknesses. This work has demonstrated the potential of fluorescence lifetime imaging by TCSPC as a method for monitoring the transdermal diffusion pathway and diffusion rate of topically applied compounds in

human skin. Indicating a change in the fluorescence lifetime within intracorneocyte junctions can be used to determine the presence of a topically applied non fluorescent compound, such as genistein, niacinamide or palmitoyl tripeptide-5. The method relies on observing subtle changes in the fluorescence lifetime of the intrinsic fluorophores present in the intracellular region between corneocytes of the stratum corneum using high-resolution spatially and temporally resolved images. This new technique does not rely on fluorescent model compounds or fluorescent reporting probes. The method could, therefore, directly visualise the path of an applied formulation, including non-fluorescent compounds, such as most cosmeceutical or pharmaceutical ingredients. As this method is able to observe what is happening within the skin throughout the penetration period we will be able to gain a better understanding of the process and may also gain a more accurate method of monitoring diffusion rates through the skin.

On completion of the characterisation of the autofluorescence of the junctions between the corneocytes within the stratum corneum, an issue arose with regards to the availability of the multiphoton FLIM apparatus. Without the equipment further development of this innovative *in vitro* method for evaluating the permeation of the anti-aging test compounds was not possible.

This work has, however, identified a number of challenges associated with the use of this method as a means of investigating compound penetration. Such as, the microscope focal orientation must be from below the skin sample, whereas previous studies generally, if not always, imaged through the stratum corneum, which is a much simpler process. This was done so a formulation could then be applied to the surface of the skin sample and after a specific time another image could be taken capturing the permeation process over time. By imaging from below the skin sample it was discovered the only skin preparation technique that would reduce interference of SHG

and light scattering was to use stratum corneum skin samples prepared by trypsin separation.

The method may be useful for studying the efficacy of anti-aging ingredients at stimulating collagen regeneration after topical application through full thickness skin or after *in vivo* application. This highly sensitive method was able to provide well detailed images of collagen ribbons shown in Figures 6 (a) and (b). A change in the mass of the collagen ribbons could be used to identify collagen regeneration effects caused by a test formulation as a function of time. All of the compounds under investigation in this research have the potential to increase collagen content within the skin. Again, due to the unavailability of the equipment, further development of these innovative *in vitro* and *in vivo* methods for evaluating collagen regeneration was not possible.

The weighted average fluorescence lifetimes in the three types of skin sections: full thickness, epidermal membrane and stratum corneum, have been consistently measured to be 1.4, 1.2 and 1.1 ± 0.03 ns (SEM) respectively. The method may be useful for future studies where the diffusion rate and pathways of a variety of applied formulations could be investigated by monitoring such parameters at multiple axial depths as a function of time.

4.6 References

1. Choi, C.M. et al., (2008) Cosmecetuicals. *Semin Cutan Med Surg,* **25**: 163-168.
2. Sun, Y., et al., (2004) Multiphoton Polarization and Generalized Polarization Microscopy Reveal Oleic-Acid-Induced Structural Changes in Intercellular Lipid Layers of the Skin. *Optics Letters*, **29**: 2013-2015.
3. Denk, W., et al., (1990) Two-Photon Laser Scanning Fluorescence Microscopy. *Science*, **248**: 73-76
4. Centonze, V.E. et al., (1998) Multiphoton Excitation Provides Optical Sections from Deeper within Scattering Specimens than Confocal Imaging. *Biophys J,* **75**: 2015-2024.
5. Lakowicz, J.R. (1999) Principles of Fluorescence Spectroscopy, 2nd ed. Kluwer Academic / Plenum Publishers, New York.
6. Mulholland, W.J., et al., (2004) Characterization of Powdered Epidermal Vaccine Delivery with Multiphoton Microscopy. *Phys Med Bio*, **49**: 5043-5058.
7. Williams, R.M., et al., (2001) Multiphoton Microscopy in Biological Research. *Current Opinion Chem Bio*, **5**: 603-608
8. Masters, B.R., et al., (1998) Optical Biopsy of *In Vivo* Human Skin: Multiphoton Excitation Microscopy, *Laser Med Sci,* **13**: 196-203
9. Cubeddu, et al., (1999) Fluorescence Lifetime Imaging: An Application to the Detection of Skin Tumors. *IEEE J Sel Top Quantum Electron*, **5**: 923-929.
10. Stracke, F., et al., (2006) Multiphoton Microscopy for the Investigation of Dermal Penetration of Nanoparticle-Borne Drugs. *J Invest Derm,* **126**: 2224-2233.

11. Yu, B., et al., (2003) Visualization of Oleic Acid-Induced Transdermal Diffusion Pathways using Two-Photon Fluorescence Microscopy. *J Invest Derm*, **120**: 448-455.

12. Yu, B., et al., (2003) Evaluation of Fluorescent Probe Surface Intensities as an Indicator of Transdermal Permeant Distributions using Wide-Area Two-Photon Fluorescence Microscopy. *J Pharm Sci,* **92**: 2354-2365.

13. Lo, W., et al., (2004) Monitoring Chemically Enhanced Transdermal Delivery Pathways of Luminescent Quantum Dots by Multiphoton Microscopy. *APBP 2004: The Second Asian and Pacific Rim Symposium on Biophotonics*, 74-75.

14. Luengo J., et al, (2006) Influence of Nano-Encapsulation on Human Skin Transport of Flufenamic Acid. *Skin Pharmacol Physiol*, **19**: 190–197.

15. Bastiaens, P.I. et al., (1999) Fluorescence Lifetime Imaging Microscopy: Spatial Resolution of Biochemical Processes in the Cell. *Trends Cell Biol.* **9**: 48-52.

16. Wang, X.F., et al., (1992) Fluorescence Lifetime Imaging Microscopy (FLIM): Instrumentation and Applications. *Crit Rev Anal Chem,* **23**: 365-369.

17. Becker, W., et al., (2004) Fluorescence Lifetime Imaging by Time-Correlated Single-Photon Counting. *Micro Res Technol*, **63**: 58-66.

18. Dickinson, M.E., et al., (2003) Multiphoton Excitation Spectra in Biological Samples. *J Biomed Opt*, **8**: 329-338.

19. Hanson, K.M., et al., (2002) Two-Photon Fluorescence Lifetime Imaging of the Skin Stratum Corneum pH Gradient. *Biophys J,* **83**: 1682-1690.

20. Periasamy, A., (2001) Methods in Cellular Imaging. Oxford University Press, New York.

21. Wagnieres, G.A., et al., (1998) *In Vivo* Fluorescence Spectroscopy and Imaging for Oncological Applications. *Photochem Photobiol,* **68**: 603-632.

22. Dowling, K., et al., (1998) Fluorescence Lifetime Imaging with Picosecond Resolution for Biomedical Applications. *Opt Lett*, **23**: 810-812.

23. Bird, D.K., et al., (2005) Metabolic Mapping of MCF10A Human Breast Cells via Multiphoton Fluorescence Lifetime Imaging of the Coenzyme NADH. *Cancer Res,* **65**: 8766-8773.

24. Eliceiri, K.W., et al., (2003) Analysis of Histology Specimens using Lifetime Multiphoton Microscopy. *J Biomed Opt*, **8**: 376-380.

25. Becker, W., (2005) Advanced Time Correlated Single Photon Counting Techniques. Springer, Germany

26. Canberra Nuclear, Connecticut, USA. An Introduction to Time Correlated Single Photon Counting for Fluorescence Decay Time Studies. Available at: http://www.canberra.com/. Accessed: Feb 2010

27. Masters B.R., (1996) Three-Dimensional Confocal Microscopy of Human Skin *In Vivo*: Autofluorescence of Normal Skin. *Bioimages,* **4**: 13-19.

28. König, K., et al., (2002) Optical Tomography of Human Skin with Subcellular Resolution and Picosecond Time Resolution using Intense Near Infrared Femtosecond Laser Pulses. *Proc SPIE,* **4620**: 191-201.

29. Masters, B.R., et al., (1997) Multiphoton Excitation Fluorescence Microscopy and Spectroscopy of *In Vivo* Human Skin. *Biophysical J*, **72**: 2405-2412.

30. Bird, D.K., et al., (2008) Navigating Transdermal Diffusion with Multiphoton Fluorescence Lifetime Imaging. *J Micro,* **230**: 61-69.

31. Pena, A.M., et al., (2005) Spectroscopic Analysis of Keratin Endogenous Signal for Skin Multiphoton Microscopy. *Opt Exp,* **13**: 6268-6274.

32. iVillage Inc. a company of NBC Universal, California, USA, Burns. Available at: http://yourtotalhealth.ivillage.com/burns.html. Accessed: Sep 2009

33. Bergmann, A., (2006) Data Analysis Software for Fluorescence Lifetime Imaging Microscopy. Becker & Hickl GmbH, Berlin.

34. Schönle, A., et al., (2000) Four-Dimensional Multiphoton Microscopy with Time-Correlated Single-Photon Counting. *Appl Opt,* **39**: 6306-6311.

35. Bird, D.K., et al., (2004) Simultaneous Two-Photon Spectral and Lifetime Fluorescence Microscopy. *Appl Opt,* **43**: 5173-5182.

36. Roth, S. et al., (1981) Optical Second-Harmonic Scattering in Rat-Tail Tendon. *Biopolymers,* **20**: 1271-1290.

37. Campagnola, et al., (2001) Second-Harmonic Imaging Microscopy of Living Cells. *J Biomed Opt,* **6**: 277-286.

38. Zoumi, A., et al., (2002) Imaging Cells and Extracellular Matrix *In Vivo* by using Second-Harmonic Generation and Two-Photon Excited Fluorescence. *Proc Nat Acad Sci USA,* **99**: 11014-11019.

39. Cox, G., et al., (2003) 3-Dimensional Imaging of Collagen using Second Harmonic Generation. *J Struct Biol,* **141**: 53-62.

40. Franken, P.A., et al., (1961) Generation of Optical Harmonics. *Phys Rev Lett,* **7**: 118-119.

41. Masters, B.R. et al., (1999) Multi-photon Excitation Microscopy and Confocal Microscopy Imaging of *In Vivo* Human Skin: A Comparison. *Microsc Microanal,* **5**: 282-289.

42. Wagner, H., et al., (2004) Effects of various vehicles on the penetration of flufenamic acid into human skin. *Europ J Pharm Biopharm*, **58**: 121-129.

43. Bronaugh, R.L., et al., (1986) Methods for *In Vitro* Percutaneous-Absorption Studies VII: Use of Excised Human Skin. *J Pharm Sci,* **75**: 1094-1097.

Chapter Five

Development of a Suitable *In Vivo* Clinical Study Method for Assessing Anti-Wrinkle Efficacy

5.1 Introduction

In vivo human clinical studies are used to assess the efficacy of a product at producing a claimed response, in this case an anti-wrinkle response. The best way to demonstrate how well an ingredient or product is at reducing wrinkles, is by examining its effects on participants in a strictly controlled manor. Chapter One Table 2 illustrates that there are many *in vivo* techniques employed for assessing the efficacy of anti-wrinkle products, and these techniques vary vastly with regards to method and cost. Almost all efficacy experiments utilize subjective Visual Image Analysis, where by changes caused by the product are determined by visually assessing digital photographs taken over a study period. This method is important because the main outcome a consumer is concerned with is that a visual improvement in the appearance of their wrinkles has been accomplished. The method is relatively inexpensive and simple to perform. There are, however, a number of issues relating to Visual Image Analysis, these include: the method is subjective, the assessor can be biased, and also if strict conditions are not maintained for each photograph, the assessment may be affected.

Based on these issues an objective method of assessing change in the size and number of wrinkles is a way of eliminating bias. Various methods have been utilised for this purpose, refer to Chapter One Table 2. On selecting an objective *in vivo* wrinkle analysis method the following considerations must be taken into account:

i. Cost of the equipment
ii. Equipment availability
iii. Accuracy
iv. Reproducibility
v. Sensitivity

In vivo clinical studies were conducted on two out of the three common anti-wrinkle ingredients under investigation in this research, niacinamide and palmitoyl tripeptide-5, referred to as the anti-wrinkle formulas, "AWP-N" and "AWP-P" respectively.

5.1.1 Direct Skin Surface Analysis

Direct wrinkle assessment, where a subject's skin surface is analysed for imperfections (namely wrinkles), has recently been implemented to assess the variation in cosmetic and surgical methods that focus on ameliorating wrinkles. These methods require an objective measurement of wrinkle size in order to assess effectiveness and monitor wrinkle depth changes over time. Methods that have been previously employed include: a "Haptic Finger" device, which utilises a polyvinylidene fluoride piezopolymer film as a receptor to create a signal that illuminates the roughness and hardness of skin surfaces [1]; 3D Morphometric Analysis, which is an innovative optical system where wrinkle depth information is determined on the basis of triangulation from the images obtained (many scans are conducted and an overall 3D image is created of the skin surface [2]); an Optical 3D device, where a parallel stripe pattern is projected onto the skin surface of a subject and a recording chip detects small differences in elevations on the skin (this is then converted into a 3D image of the skin surface to give information on mean roughness and mean depth of roughness on the test site [3-4]) and; a Photonumerical Scale method, where digital photographs are taken and a visual comparison is made between day 0 and subsequent study days [5-7].

With the exception of the Photonumerical Scale method and Direct Skin Surface Analysis, the direct wrinkle assessment methods have generally only recently been

developed. These methods eliminate the error associated with accurately replicating a section of skin surface as an impression. These highly technical methods are advanced, rapid and very sensitive. However, they require expensive and highly specific equipment that are not readily available. Also, these more technical methods have not been fully assessed for accuracy and reproducibility.

5.1.2 Indirect Skin Surface Analysis

Indirect skin surface analysis methods involve taking an impression of the skin, which can then be analysed at a later date. The impression involves taking a physical replica of the skin using a silicone impression material that can then be assessed in a number of ways. The impressions can be analysed using numerical image analysis of photographs involving a standardised optical system that captures an image of the silicone negative imprint and wrinkle depth, which is assessed as a correlation with the average surface area of shadow generated by the wrinkle in the image [8]. 3D Morphometric Analysis and Optical 3D Analysis can also be used for the assessment of silicone moulds, as discussed above for direct skin surface analysis. Alternatively, a silicone impression can be analysed under a microscope (i.e. Silicone Skin Replication). There are two distinct methods for measuring wrinkle depth using a microscope, either analysing the surface or analysing the cross section of a mould. For surface analysis, the silicone impression mould is placed facing upward on the microscopic stage. Initially, the ridge representing an individual wrinkle can be brought into focus. At that point, the reading on the focus knob is noted. After this initial reading the region at the base of the ridge, which corresponds to the normal skin surface, is brought into focus. The difference in the reading on the fine focus, relative to the initial reading and the final

reading, is equivalent to the depth of the wrinkle [9]. For cross section analysis, the silicone moulds are sectioned prior to microscopic analysis. The segments are placed on the microscopic stage with the cut face facing upwards, creating a cross sectional view of the skin surface. The wrinkle creates a peak in the mould and the peak height can be measured. To date, Silicone Skin Replication is the easiest, least expensive and most accurate method for investigating skin surface morphology and microanatomy [10].

The Photonumerical Scale and Silicone Skin Replication methods involve relatively inexpensive, easily accessible equipment, can be conveniently applied, are non-invasive for the participant and give a reproducible and sensitive measurement of topographic changes in the surface of the skin. Together, these methods give both an evaluation of the change in wrinkle appearance over time (an attribute of great significance to the final consumer) and provide an objective assessment of variation in the size and number of wrinkles.

5.2 Aim

To develop quantitative *in vivo* methods for investigating changes in the appearance and size of mild to moderate facial wrinkles caused by anti-wrinkle products.

5.3 Material and Methods

5.3.1 Study Population

Using Equation 1 shown below, it was determined that a sample size of 20 people would be suitable to detect a 20 % difference in wrinkle measurement [11].

$$n \geq \frac{15.68\sigma^2}{\Delta^2} \qquad 1)$$

Where Δ is the difference to be detected (0.2) and σ^2 is the error of variance or (coefficient of variance) ^{2}. The error of variance that was used to calculate the sample size required for this study was taken from a previous study conducted by Hatzis [10], where it was found that the measured mean wrinkle depth had a coefficient of variance of 0.209.

The volunteers were recruited into either an 8 week study for palmitoyl tripeptide-5 or a 12 week study for niacinamide, following approval from *The Standing Committee on Ethics in Research Involving Humans*, Monash University, Victoria, Australia. Each volunteer was provided with an Explanatory Statement, shown in Appendix A Section 5.7.1, which briefly outlined the nature of the study and the potential risks involved. All volunteers were required to sign an Informed Consent form, shown in Appendix B Section 5.7.2, and a Photographic Release Authorisation form, shown in Appendix C Section 5.7.3, prior to commencing the study. The volunteers were aged between 30 and 55 years and exhibited mild to moderate dynamic

facial wrinkles bilaterally distributed across their face. The volunteers had no history of dermatological disease (such as dermatitis or acne), were not using topical medications on their face that could not be discontinued throughout the course of the study (such as topical antibiotics or corticosteriods), and they had no known allergy to any of the components of either of the "AWPs", silicone or silicone containing products. When an area of skin was tested with these products, the volunteers did not show any signs of sensitivity.

5.3.2 Study Protocol

5.3.2.1 Washout Period

In order to ensure that the areas of skin examined during the study were treated in the same manner and to minimise inter-subject variability, a one-week washout period was undertaken prior to treatment with the "AWP". During this washout period, all of the participants refrained from applying any topical formulations to their face, with the exception of a cleanser, moisturiser and if required sunscreen. The participants were advised to standardise their daily facial cleanser and moisturiser regime by applying their regular products twice daily, which they continued to apply throughout the course of the study. A standardised moisturiser and cleanser were not included into the study protocol. The study aimed at assessing changes made to the participants wrinkles caused by the "AWPs" alone. Altering the cleanser and / or moisturiser may have severely altered the hydration state of the participants' skin, adding another variable to the study.

5.3.2.2 Study Design

In each study the participants were randomly assigned to one of two contralateral split-face study groups: a comparison of the "AWP" versus untreated skin (Group one, n = 10) and a comparison of the "AWP" versus an identical formulation, excluding the compound under investigation (either niacinamide or palmitoyl tripeptide-5), the control formulation (Group two, n = 10). Participants in each group received the treatment(s) in containers labelled with: the batch number of the formulation, the participant's study identification number, the formulation manufacture date and directions as to which half of the face to apply the formulation to (either the right or left hand side), which was randomly determined before the study.

On the first day of the treatment period (day 0), a baseline evaluation of each participant was conducted. This was used as a comparison of the degree of change in wrinkle size and appearance over the study. Standardised digital photographs of the participant's face and two silicone moulds of the left and right side of their face were taken. A photograph was taken of the silicone application sites to assist in subsequent applications. A measurement was also recorded as the application distance from a specific location i.e. the distance from the centre of the participant's nose.

Each morning and night throughout the 8 or 12 week study periods (starting from day 0), the participants washed their faces with their cleanser and then applied the formulation(s) to either the left or right half of the forehead and the orbital regions, as indicated on the label of the jar. The participant then applied their moisturiser over their face. In order to prevent cross-contamination of the application site(s) and to ensure the formulation(s) was applied in a standardised manner, the participants were instructed to rub the formulation(s) onto the test area(s) with alternate or freshly washed hands. In

order to reduce loss of the formulation(s) from the skin surface by rubbing off onto the bed linen, the formulation(s) were applied at least 15 mins before retiring.

Subjects returned any unused formulations to the investigator on the final day of the study, either day 56 or 84. They were provided with more of the formulation(s) if required. Containers were weighed before distribution and after collection, which acted as a method for evaluating subject compliance. The investigator also questioned participants at every evaluation point i.e. days 14, 28, 42, 56, 70 and 84 as to whether any application points were omitted, which was recorded in a diary. These monitoring procedures ensured that the subjects were complying with the study protocol.

5.3.3 Efficacy Assessment

Efficacy evaluations were performed on the same day of each week, at approximately the same time of day, because skin mobility and wrinkling can vary during the day due to emotional or somatic factors [10, 12]. These evaluations were started from day 14 of the study. Subjective efficacy evaluations were based on visual comparisons made between the standardised digital photographs taken of each participant at baseline (day 0) and each of the evaluation days. Visual changes in the size and depth of the dynamic wrinkles on the forehead and around the orbital area were assessed based on a Photonumerical Scale method detailed in Section 5.3.3.1.4 and recorded on an Efficacy Evaluation form, shown in Appendix D Section 5.7.4. The efficacy evaluation process consisted of two parts: i) an evaluation performed by the investigator and ii) an evaluation performed by an independent assessor. Both assessors were blinded to which side of the face had been allocated treatment with the “AWP”.

The average score obtained from the evaluations was used to assess the efficacy of each treatment.

Objective efficacy evaluations were performed using a technique referred to as Silicone Skin Replication. This technique involved two silicone impressions taken of both the left and right side of the participant's face. The impressions were taken after photography. The moulds were dissected and examined for wrinkle depth and the number of visible wrinkles in the selected analysis area. The analysis area was selected from the moulds, the area was marked on the mould and the previous evaluation day was used as a template to ensure the exact area was assessed every time.

In order to assess the potential of the topical "AWP" to elicit an adverse local skin reaction, the participants were asked to document whether they experienced any adverse skin reactions (e.g. a feeling of warmth on the skin, burning, stinging, redness, dryness, flakiness, itching, a rash and / or any other skin reaction) in Safety Evaluation forms, shown in Appendix E Section 5.7.5, which were completed fortnightly [6, 13]. In the instance where a participant experienced a local skin reaction, they graded its intensity according to a 4-point scale, where 1 = mild, 2 = moderate, 3 = severe, and 4 = very severe. Cases of irritation were treated, if required, and participants could remove themselves from the study if necessary. Irritation was also assessed on completion of the study where by digital images were analysed for changes in the intensity of the red colour on each side of the participant's face.

5.3.3.1 Digital Photography

5.3.3.1.1 Subject Preparation

The participants were asked to remove any jewellery prior to photography. A black headband was used to pull any hair off the participant's face. The same headband was used for all photographs throughout both studies. The participants were supplied a facial cleansing wipe, 15 x 20 cm Pure & Soft by Sej (Zhejiang, China), to remove make-up and / or grime at each evaluation point. Sebaceous lipids from the skin surface and damp skin can reflect light and may thus cause large areas of brightness over the skin (and hence obscure wrinkle detail in the photographs). Therefore, after using a cleansing wipe each participant blotted his / her forehead and orbital regions of their face with a facial tissue, Kleenex by Kimberly-Clark Australia Pty Ltd. (New South Wales, Australia), immediately before photography. A black cape was used to cover the participants clothing to eliminate variation that might be caused by light reflecting differently from different clothing.

5.3.3.1.2 Photography Optimisation

Digital photography was conducted using a Canon EOS 400D Digital Camera with a Canon Zoom Lens EF 75-300 mm, 1:4-5.6 III from Canon Inc. (Tokyo, Japan). In order to produce images of the participants' fine lines and wrinkles for examination, the photography technique and apparatus was optimised to enhance wrinkle detail. The following tests were conducted and the images analysed to determine the best photographic apparatus set-up to produce the highest wrinkle definition:

1. Camera location: the camera distance from the participant.

2. Camera settings: zoom, focal distance, area to be photographed (full face or only the upper portion) and with or without a flash.

3. Lighting: the choice between two halogen work lights or one fluorescent ring light. The effect of changing the position of the light on the participant's wrinkles appearance was examined by altering the lights' distance behind the camera.

4. Photographs were taken under standardised conditions throughout the study and were indirectly optimised for each participant to create the greatest wrinkle definition. The contrast, colour balance and sharpness were adjusted to the same extent for each participant's images.

5.3.3.1.3 Photography Apparatus

Three photographs were taken of each participant's face during a photography session: a frontal view, a left and a right oblique view. Photographs of each participant captured an area of just below their nose to the top of their forehead. For assessment all three photographs were compared to the baseline photographs.

The same photographer, using identical equipment and lighting conditions, photographed all of the participants in the same location. In order to ensure that the participant's face was in a standardised position for each photograph, the participant was seated in a swivel chair, the chair height was optimised for each participant for comfort and the height was recorded. The seat height was adjusted prior to each sitting. For each photograph the participant was seated behind a table 71 cm in height, directly in front of a custom-designed head-positioning apparatus. The head-positioning apparatus consisted of: a metal tripod support, with a rubber chin rest 38 cm in height

from the table surface, see Figure 1. The participant's head was re-positioned to the same orientation throughout the study by printing the frontal, left and right oblique views of the participant's baseline photographs, these images were used as a reference to adjust the participant's head to match the baseline positioning. An image of a card, which consisted of a red, blue and yellow colour scheme, was captured before each photograph session in order to ensure that brightness, contrast, colour intensity and colour balance were standardised.

The digital photographs were taken at the same focal distance, using the same camera, which was held in a standardised position by means of an aluminium camera tripod 124 cm in height, placed 193 cm from the front of the chin support to the centre of the camera tripod. The same camera settings were used for each photograph. The in-built flash was switched off to prevent large areas of brightness on the participants' faces (obscuring the appearance of the test wrinkles).

The lighting apparatus used during photography was one V-Tec® Fluorescent Ring Light 40W from Vanbar Imaging (Victoria, Australia), adjusted to 144 cm from the ground to the top of the light with an adjustable light stand. The lighting apparatus was positioned 174 cm from the front of the chin support. The light allowed for a detailed image of participants fine lines and wrinkles to be captured.

For the adjustment and standardisation of all images, three set-up positions were marked on the table. The three legs of the head positioning apparatus were placed on all three marks at position 1 for frontal image, position 2 for the right oblique and position 3 for the left oblique images, 200 cm to the left and right of the centre for each oblique image. Participant positioning was also maintained by markers placed on the floor and in the eye line of the participant. A cross was placed on each wall, measured for the same angle on each side. The participant lined themselves up, placing their feet either

side of the floor line and lining up the centre of their head with the line on the wall. When the participant was in the correct position they closed their eyes gently. Minor adjustments to the positioning were made based on baseline images and the photograph was then captured.

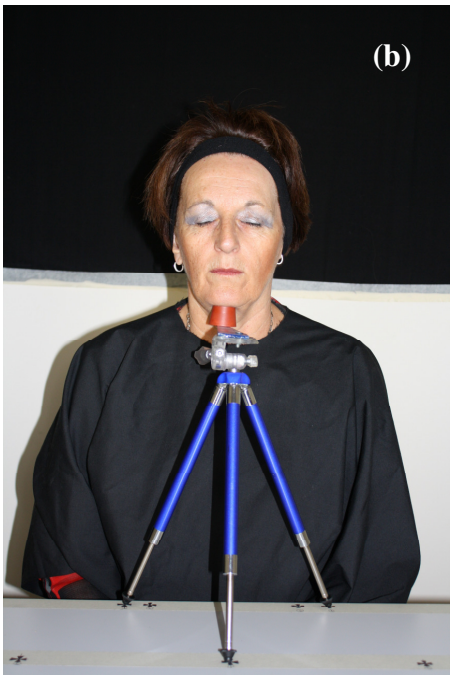

Figure 1. Photography apparatus (a) Entire apparatus set up (b) frontal photographic setup (c) left oblique photographic view set up (d) close up of head-positioning apparatus and (e) light and camera setup

5.3.3.1.4 Photographic Assessment

The assessment of change in the wrinkle appearance caused by an anti-wrinkle product was conducted by capturing and analysing digital photographs for change over time. The Photonumerical Scale method was employed [5-7]. The method involves digital photographs being taken under standardised conditions. A visual comparison was made between the photographs taken of each participant at baseline (day 0) and photographs taken on each day of analysis i.e. every second week over the study period. The photographs were graded on a 7-point scale, where: -3 = much worse, -2 = moderately worse, -1 = slightly worse, 0 = no change, +1 = slightly improved, +2 = moderately improved and +3 = much improved. Photographic assessment was performed by the investigator and an independent assessor. All images were coded to

eliminate bias, so that both of the assessors were blinded as to which was the anti-wrinkle treated, the control treated or the untreated sides of the face.

5.3.3.1.5 Redness Assessment

Safety Evaluation forms, as shown in Appendix E Section 5.7.5, were completed by each participant fortnightly as to whether or not they experienced any local side effects throughout the study period. Since redness in the skin is the most common sign of skin irritation, a quantitative method was also employed to assess whether any local skin reactions were caused by the product or more specifically the anti-wrinkle ingredient under investigation. The method involved the analysis of the digital photographs used for photographic assessment of anti-wrinkle efficacy. Adobe Photoshop CS3 Extended version 10.0, by Adobe (California, USA) was used to investigate a change in redness of either the left or right side of each participants face compared to day 0. The histogram function was used, which examines the image for the distribution of pixels at a specific intensity. The Red channel was selected and a 500 pixel diameter area on each side of the participant's face for the frontal image and on each orbital image was examined. The mean intensity value was recorded for each image. The results were presented as an average mean of both the orbital and frontal image results. Skin sensitivity is regarded as an increase in the mean Red intensity from baseline.

5.3.3.2 Silicone Skin Replication Technique

5.3.3.2.1 Moulding Technique

In order to make an assessment of change in wrinkle depth and skin roughness over a period of time, the skin can be measured directly or alternatively an impression of the participants' skin can be acquired. For an impression the mould must be taken accurately as analysis is made from the mould. The method used for this study was loosely based on a method developed by Hatzis (2004) [10], which involved an impression being taken with a silicone material. The impression was covered with a second silicone material, which was then dissected perpendicular to the wrinkle to produce 5 mm sections that were studied under a stereomicroscope. For the studies reported in this thesis, the silicon medium selected was a hydrophilic polyether dental impression material of soft consistency known as Impregum^{TM} Soft from 3M ESPE, (New South Wales, Australia). In the work conducted by Hatzis [10], a second layer of impression medium was applied to the original mould for analysis. Based on tests conducted in my honours thesis titled, "Efficacy Evaluation of an Anti-wrinkle Formulation", a second layer was not used in these studies.

The computer software, Adobe Photoshop CS3 Extended version 10.0, by Adobe (California, USA), was employed for determining wrinkle size and skin roughness from the impression moulds, this method required calibration. Calibration was achieved by capturing images of a microscopic slide marked with circles of varying diameters of 0.07, 0.6, 0.15 and 1.5 mm, known as etched graticules. The etched graticules were captured under the standardised conditions used for microscopic analysis performed on the participants' silicon mould slice samples.

The microscope settings, light intensity on the sample, zoom and focus, were optimised for the greatest definition of the moulds. Constraints were made on the area analysed in order to determine variation in the number of wrinkles over the study period.

5.3.3.2.2 Application Technique

An application technique was developed for applying the impression material onto the participants' faces. Previous studies utilised adhesive rings to delineate sampling sites [13]. However, these were time consuming to position, created difficulty in dissecting the sample and presented some discomfort to the test subjects. A technique was adapted where by a ruler was used to reference two application zones. It was determined that the technique was highly reproducible in taking a sample of the same site. Therefore, only one impression was taken of each side of the participant's face at each evaluation point of the study, unless a problem occurred when the mould was taken.

The silicone impression material consisted of two parts: a base and a catalyst. The tubes are designed with different opening diameters such that the correct proportions were obtained by expressing the same length of material from each tube. Strands of base and catalyst of ~3 cm in length were placed on a glass mixing pad. The pastes were mixed together, with a plastic spatula, by picking up the paste and spreading it against the mixing board for ~45 secs. Mixing was continued until a uniform colour was obtained. Half of the mixed paste was placed on the spatula. The participant closed their eyes gently, allowing wrinkles and facial expression to retain comparability to baseline. Wrinkle-wrinkle distance reflects the degree of relaxation

[10], shown in Figure 2, which becomes more consistent when the participants eyes are closed.

Figure 2. (a) A profile of the 'wrinkle area' in relaxed state and (b) a profile of the 'wrinkle area' in maximum wrinkled state.

An area on each side of the participant's face was selected to analyse wrinkles, either in the orbital or forehead region depending on their wrinkle distribution. For application on the forehead, a 35 mm wide ruler was centred on the participant's nose and the silicone was applied with a spatula to the left side and right side of the ruler in a rectangular area of ~2 cm in width, ~3 cm in height and ~0.3 cm in depth, shown in Figure 3. For orbitale analysis the silicone was applied with a spatula to a rectangular area on the left and right side of the participant's crows feet eye area ~2 cm width, ~3 cm height and ~0.3 cm depth, see Figure 3. A digital photograph was captured at day 0 of the silicone application areas and any specific information about the application site was noted for standardisation in subsequent evaluation points. The silicone was then left to set for ~5 mins or until the silicone was not tacky to touch. The moulds were then removed from each side, the top of the mould marked for determining the orientation for analysis and placed into a labelled plastic pocket. The plastic pockets were labelled with the subjects: study number, the side of the face the mould was taken from and the day of the study (i.e. 0, 14, 28, 42, 56, 70 or 84).

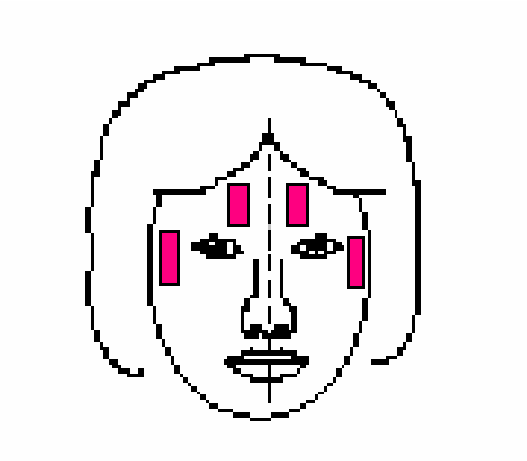

Figure 3. A diagram representing the application of silicone to a participant's forehead where the rectangles ( ) indicate the silicone impression material.

5.3.3.2.3 Sectioning the Mould

An analysis area was marked on the moulds, based on the previous evaluation day as a template to ensure the exact area was assessed every time. The analysis area consisted of three segments ~5 mm in width and ~23 mm in length. The three segments were sliced from the analysis area using a Snape Off Knife Utility Blade 18 x 107.95 mm from Stanley Works, (Connecticut, New Britain). A tool to section the segments into smaller slices was required and not stated in previous works. In my honours work, a Victorinox Pairing knife was used, however, due to the large uneven thickness of the slices, which impeded definition between wrinkles, an alternative method was sourced. A piece of equipment that is regularly employed for sectioning tissue prior to microscopic analysis was selected, known as a cryostat [14-16].

The segments were placed in a Tissue-Tek® Cryomould® 25 x 20 x 5 mm from Sakura Finetek (California, USA) and embedded in cryostat medium, Tissue-Tek® OCT Compound from Sakura Finetek (California, USA). The cryomoulds including mould segments were then placed in the freezer at below -10 °C to freeze the OCT embedding

compound. Once frozen 60 µm thick slices of each segment were made using the cryostat, Leica CM1850 with a low profile disposable blades 80 x 8 mm from Leica Microsystems Pty Ltd (New South Wales, Australia), shown below in Figure 4. Each slice was placed on a 25 x 75 x 1.0 mm Menzel-Glaser Superfrost® Plus microscope slide from Thermo Fisher Scientific Inc. (Massachusetts, USA) and a 24 x 64 mm Medoglass Coverslip No. 1 from Lomb Scientific (New South Wales, Australia), was placed over the slice, the OCT medium melted and held the cover slip in place.

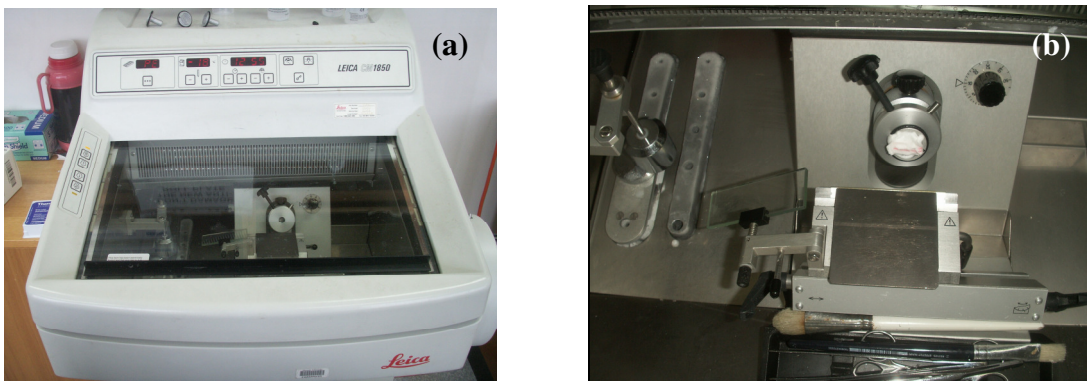

Figure 4. Cryostat (a) the entire apparatus set up and (b) an enhancement of the slicing plate and chuck holding a mould segment embedded in OCT.

5.3.3.2.4 Mould Imaging

Cross sectional slices were viewed and imaged under the Olympus BX60F5 microscope from Olympus Optical Co. Ltd. (Tokyo, Japan) coupled with a RT Slider Spot camera from Diagnostic Instrument Inc. (Michigan, USA), shown in Figure 5. For microscopic imaging a 4x /0.16 UIS2 /-/FN26.5 UPlanSApo objective from Olympus Optical Co. Ltd (Tokyo, Japan) was used, the camera brightness was set at 6, no filter was used, the camera was set on black and white and ND25 was set on light and LBD and ND6 on dark. The images were captured using the Spot Advanced Version 3.5 software from Diagnostic Instruments Inc. (Michigan, USA). The imaging processing

method used for every image consisted of: a grayscale, 8 bits per pixel, binning of 2 x 2, an exposure time of 1.1970 ms and a gain of 1. Images were taken with a 25 – 40 % overlap to allow for the images to be knitted together using Adobe Photoshop CS3 Extended version 10.0, by Adobe Systems Inc. (California, USA). Approximately 15 images were captured for each slice of silicone mould.

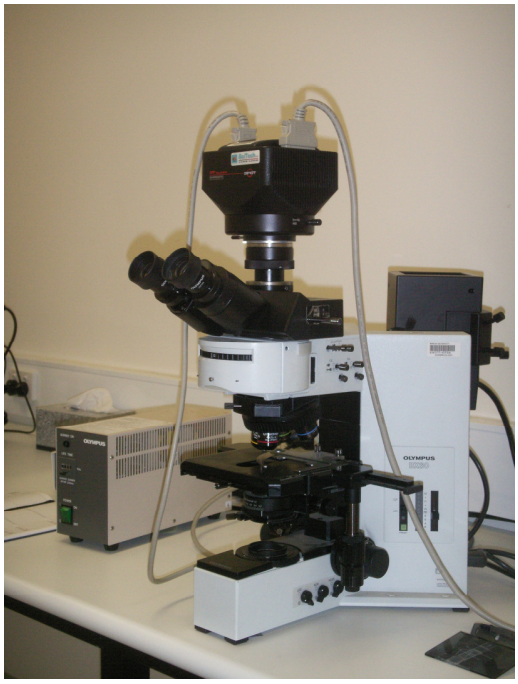

Figure 5. Microscopy unit used for silicone mould analysis

5.3.3.2.5 Sample Analysis

Images collected from the microscope were analysed using Adobe Photoshop CS3. Silicon Skin Moulding was performed at study time points 0, 28 and 56 for palmitoyl tripeptide-5 and 0, 56 and 84 for niacinamide. At each time point images were analysed for three slices from both the left and right moulds of each participant. The images for each slice, ~15, were merged using the automatic photomerge function. If difficulties arose with the automated process the images were manually repositioned and blended. The final image was then saved; an example of a merged slice from a

participant is shown in Figure 6. All the slice lengths from the merged images for a particular participant on a particular side of their face were measured. The minimum length was used as the analysis length. A mark was placed on each merged slice image for the particular side of a participant's face and the image was analysed up to the mark for wrinkle height and number. For accuracy, the merged image was zoomed to 200 % prior to analysis. The measurement scale was set based on the validation procedure described in Section 5.3.3.2.6.

Wrinkle depth was determined for each slice based on the height of the resultant peaks. A baseline was drawn for each peak, shown in Figure 6, and the peak height was measured from the baseline to the tip of the peak. Between 50 to 120 peaks were analysed per slice. The number of peaks measured in the standardised analysis area was determined, as was the surface roughness (the sum of the peak heights). Results were reported as a % Difference. A comparison can only be made for roughness and the total number of wrinkles for the same side of each participant's face compared to the baseline of the same side because there is a large variation in results between participants.

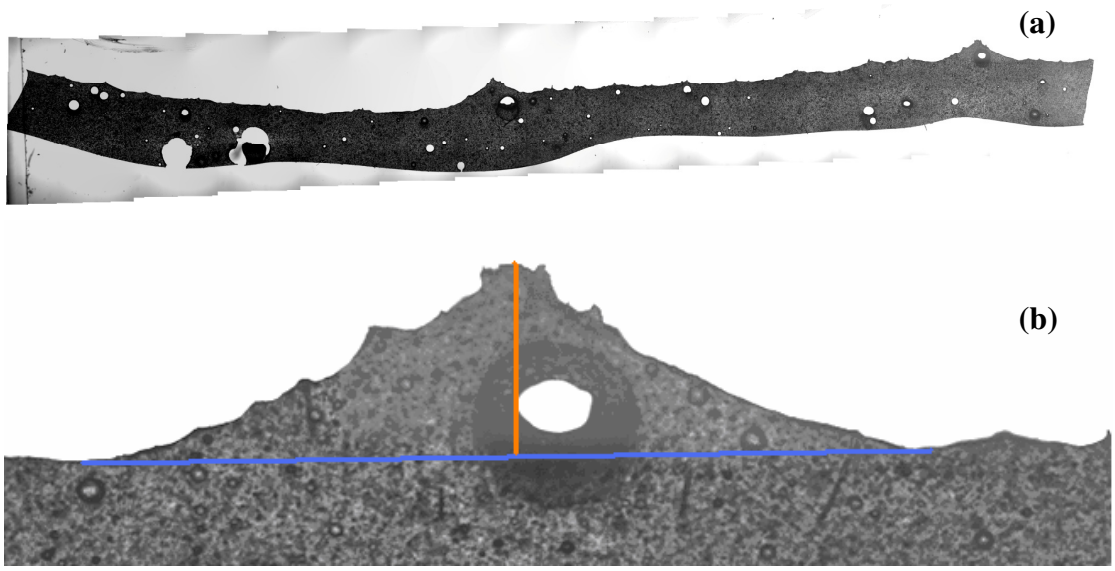

Figure 6: (a) Representation of a cross section of a silicone skin impression mould of a study participant and (b) measurement of a sample wrinkle from a mould slice, where the baseline is shown in blue and the peak height shown in orange

5.3.3.2.6 Method Validation

The linearity, precision and accuracy of both the Silicone Skin Replication technique and the analysis method of measuring wrinkle depths were assessed.

The impression technique for measuring wrinkle depth was evaluated using a gauge devised by S.G. Prittie precision Gauges (Victoria, Australia) to the specifications outlined by the investigator and is shown in Figure 7. The gauge consisted of four triangular depressions of depths: 10.0 µm, 19.5 µm, 50.0 µm and 100.5 µm ± 0.8 and widths 30 µm, 34 µm, 68 µm and 122 µm ± 2µm respectively. The silicone impression material was applied to the wrinkle depth gauge and left to set for ~5 mins or until no longer tacky to the touch. The mould was removed and embedded in OCT medium, frozen and sectioned as per the method described in Section 5.3.3.2.3. The slices were then analysed under the microscope using the method described in

Section 5.3.3.2.4 and analysed as per the method in Section 5.3.3.2.5. The wrinkle depth gauge was cleaned with ethanol, before each impression, to remove any residual impression material.

Figure 7. Winkle depth gauge developed by S.G. Prittie precision Gauges.

Precision and accuracy of the analysis technique used for measuring wrinkle depth by Adobe Photoshop CS3, was assessed with a microscope stage slide. The slide consisted of circular graticules with diameters 0.07, 0.15, 0.6 and 1.5 mm, shown below in Figure 8. Each graticule was imaged under the microscope and the images captured. The diameters of the graticules were then assessed as per the method described in Section 5.3.3.2.5.

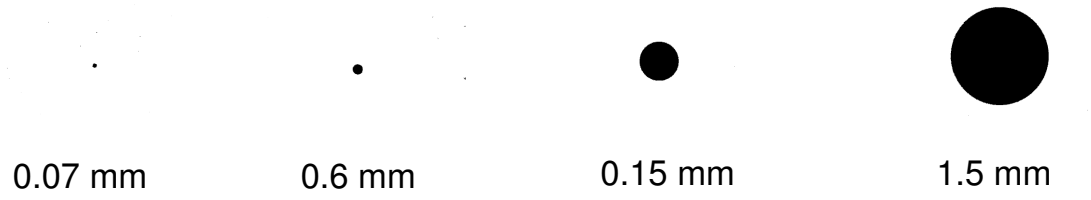

Figure 8. Image analysis standards of microscopy stage graticules captured at 4x zoom, enlarged to double the size

5.3.3.2.6.1 Intra- and Inter-day Validation

Intra-day precision and accuracy for the Silicone Skin Replication technique was determined by imaging three different slices from the same mould and measuring the number of pixels for each resultant depression from the depth gauge. For the analysis method using Photoshop CS3, intra-day precision and accuracy was determined by imaging each graticule under the microscope and capturing the images. Repeated measurements of the graticule diameters from the images was conducted n = 5.

Inter-day precision and accuracy for the impression technique was determined by analysing slices taken from different impression of the depth gauge on three different days. For the analysis method, inter-day precision and accuracy was determined by measuring the graticule diameters from the images on three different days.

Linearity of each method was assessed through calibration plots where by the measured number of pixels was compared to known measurements. A linear relationship between the length and number of pixels was confirmed by the correlation coefficient generated by the linear regression of the plot, using a least squares method.

Intra-day precision was evaluated by repeated analysis at different lengths on the same day. Precision is expressed as a co-efficient of variation (CV), shown in Equation 1.

$$CV = \frac{\text{Standard Deviation}}{\text{Mean}} \times 100 \qquad 1)$$

Inter-day precision was determined over three different days and plots were constructed for pixels versus length. The precision was determined as the CV of the

resultant slopes. For acceptability the mean value at each measurement and slope was not to exceed ± 15 % CV.

Accuracy is expressed as the deviation of the measured length from the actual length (bias), and was calculated using Equation 2.

$$\text{Accuracy} = \frac{\text{Calculated Measurement - Actual Measurement}}{\text{Calculated Measurement}} \times 100 \qquad 2)$$

The intra-day accuracy was evaluated by determining the calculated measurements i.e. gauge depression depth or graticule diameter at varying lengths and two plots of pixels versus length were constructed. A regression line of the plots was determined and the mean intercept and slope was used to calculate the measurement from the number of pixels measured, shown below in Equation 3.

$$\text{Calculated Measurement} = \frac{\text{No. of Pixels - Mean Intercept}}{\text{Mean Slope}} \qquad 3)$$

Inter-day accuracy was determined over three different days. The plots of number of pixels versus actual measurement were conducted on the three days. The regression lines were used to determine the mean slope and intercept, which was used to determine the calculated measurement from each measured number of pixels. Accuracy was to be within ± 15 % of the actual measurement to be acceptable.

5.3.3.3 Statistical Analysis in Skin

All statistical analysis was performed using SPSS version 16.0 scientific software (Illinois, USA). Differences between the efficacy scores over the study between "AWP" treated and either untreated or control treated skin were analysed by a Wilcoxon Signed Rank Test. A Wilcoxon Signed Rank Test was then performed at each time point of the study. This was done to assess if there was a significant difference between "AWP" treated and untreated or control treated skin over time. For each test day, "AWP" and untreated or control treated results were assessed using the One Sample T-Test, showing whether the results were statistically different to 0, therefore indicating an improvement over the baseline. A non linear statistical analysis method was utilized, since the results were measured on a 7-point scale. This in turn led to the results not fitting the criteria for parametric statistical analysis i.e. will not have a normal distribution of results.

Differences between the skin redness (irritation), skin roughness and the number of visible wrinkles given to the side of the face treated with the "AWP" and the side left untreated or treated with the control formulation was analyzed by a Paired-samples T-test. This was done for the results as a whole and at each time point. For each test day "AWP" and untreated or control treated results were also assessed using a One Sample T-Test, showing whether the results were statistically different to 0, therefore, indicating an improvement over the baseline.

In all cases statistical significance was defined as a p-value less than 0.05.

5.4 Results Silicone Skin Replication Method Validation

5.4.1 Impression Technique

The gauge developed consisted of four triangular depressions of depths: 10.0 µm, 19.5 µm, 50.0 µm and 100.5 µm ± 0.8 and widths 30 µm, 34 µm, 68 µm and 122 µm ± 2µm respectively. Figure 9 demonstrates the resultant images captured from the mould taken of the depth gauge, respective gauge depressions are shown.

Figure 9. Images of impression take from depth gauge (a) 10.0, (b) 19.5, (c) 50 and (d) 100.5 µm

Linearity of the impression technique was assessed by a calibration curve. A calibration curve for pixels versus the depth intervals 0.1, 0.195, 0.5 and 1.005 mm is shown in Figure 10.

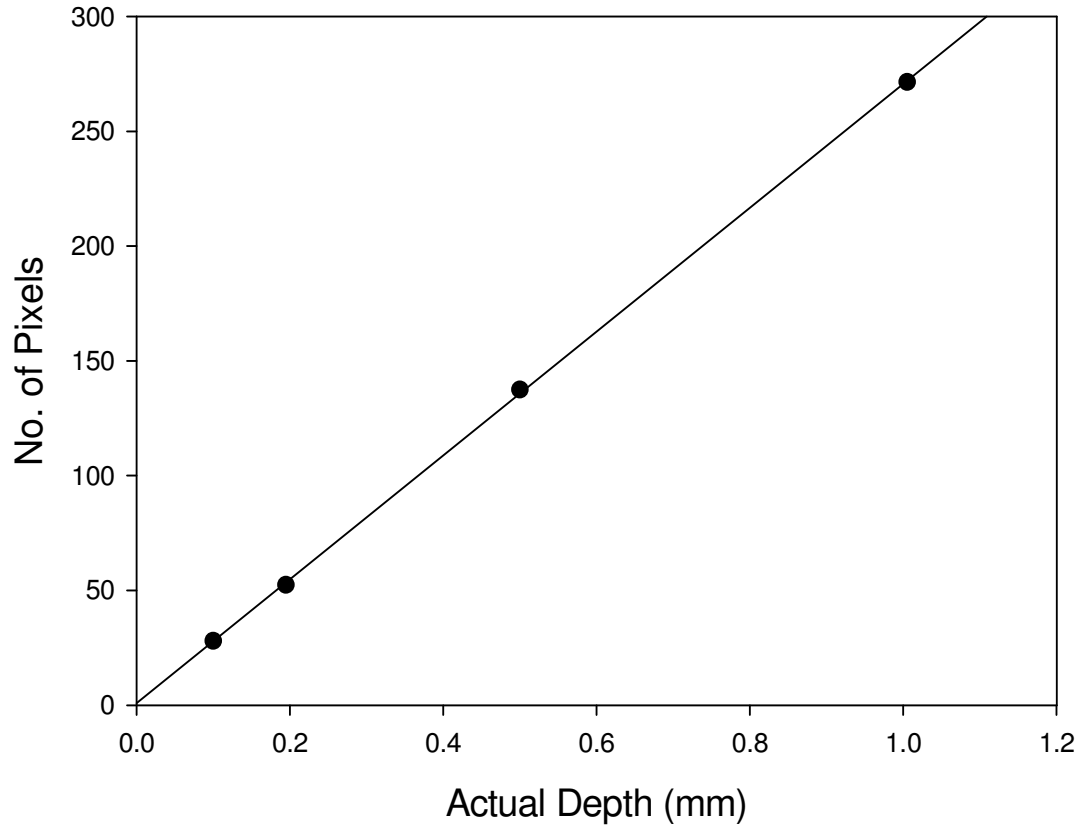

Figure 10. Calibration curve for the number of pixels versus the depth of the gauge depressions. Each point represents the mean and the error bars represent the standard deviation of the mean (STDEV), n = 3.

The linearity of the standard curve was excellent with an $r^2 = 0.9999$. Intra-day precision % CV was found to be 1.20, 5.55, 3.73 and 3.27 % at 0.1, 0.195, 0.5 and 1.005 mm respectively. Accuracy was determined to be - 0.58 ± 1.25, - 2.39 ± 5.95, 0.55 ± 3.71 and 1.97 ± 3.16 % ± STDEV, for 0.1, 0.195, 0.5 and 1.005 mm respectively.

The method was found have an inter-day precision of 2.43 % and accuracy of 0.26, - 2.37, 1.16 and - 0.24 % at 0.1, 0.195, 0.5 and 1.005 mm respectively.

5.4.2 Sample Analysis Technique

Accuracy and precision of the analysis technique used for measuring wrinkle depth by Adobe Photoshop CS3, was assessed through microscope stage graticules with diameters of: 0.07, 0.15, 0.6 and 1.5 mm, shown in Figure 8. The validation technique also allowed the determination of a conversion factor from pixels to the peak height, therefore allowing for wrinkle depth to be assessed.

A calibration curve of pixels versus graticule diameter (length) is shown below in Figure 11.

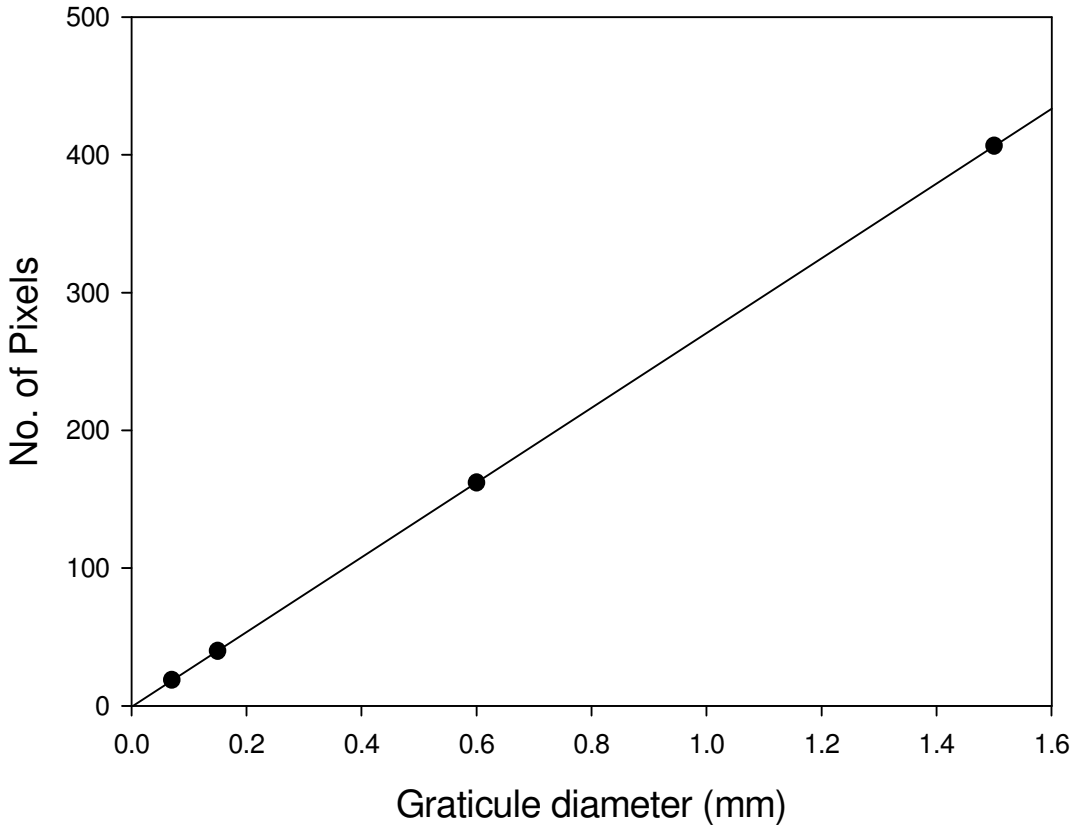

Figure 11. Plot of the number of pixels versus the graticule diameter

The linearity of the analysis method was excellent with an $r^2 = 1$. Intra-day precision % CV was found to be 0.50, 0.65, 0.09 and 0.00 % at 0.07, 0.15, 0.6 and 1.5

mm respectively. The method accuracy was determined to be – 1.32 ± 0.49, - 0.05 ± 0.65, 0.05 ± 0.09 and 0.02 ± 0.00 % ± STDEV for 0.07, 0.15, 0.6 and 1.5 mm respectively.

The method was found to have an inter-day precision of 0.29 % CV and accuracy of – 0.33, - 0.19, -0.09 and – 0.15 % for 0.07, 0.15, 0.6 and 1.5 mm respectively.

The conversion factor from the number of pixels equivalent to measurement was found to be, 1 pixel = 6.085 µm.

5.5 Conclusion

This study has comprehensively developed quantitative *in vivo* methods for investigating changes in the appearance, size and number of mild to moderate facial wrinkles caused by anti-wrinkle products. The *in vivo* protocol has been optimized and extensively characterized. The objective Silicon Skin Replication Technique has been validated and it was shown to have excellent intra- and inter-day precision and accuracy for both the impression method and the analysis method. This work has demonstrated the potential for using Photographic Assessment and Silicone Skin Replication techniques as methods for monitoring changes in the appearance, number and size of facial wrinkles over each study period. These methods will be used to investigate the efficacy of products containing the anti-wrinkle agents, niacinamide and palmitoyl tripeptide-5 at eliciting an anti-wrinkle effect on human subjects.

5.6 References

1. Tanaka, M., et al., (2003) The "Haptic Finger" A New Device for Monitoring Skin Conditions. *Skin Res Technol*, **9**: 131-136.
2. Akazaki, S., et al., (2002) Age-Related Changes in Skin Wrinkles Assessed by a Novel Three-Dimensional Morphometric Analysis. *Br J Derm*, **147**: 689-695.
3. Jacobi, U., et al., (2004) *In Vivo* Determination of Skin Surface Topography Using an Optical 3D Device. *Skin Res Technol*, **10**: 207-214.
4. Nardin, P., et al., (2002) Automation of a Series of Cutaneous Topography Measurements from Silicon Rubber Replicas. *Skin Res Technol*, **8**: 112-117.
5. Gupta, A.K. et al., (2003) Critical Review of the Manner in which the Efficacy of Therapies for Rosacea are Evaluated. *Int J Derm*, **42**: 909-916.
6. Beitner, H., (2003) Randomized, Placebo-Controlled, Double Blind Study on the Clinical Efficacy of a Cream Containing 5 % α-Lipoic Acid Related to Photoageing of Facial Skin. *Br J Derm*, **149**: 841-849.
7. Tsukahara, K., et al., (2000) Determination of Age-Related Changes in the Morphological Structure (Sagging) of the Human Cheek using a Photonumeric Scale and Three-Dimensioal Surface Parameters. *Int J Cosmet Sci*, **22**: 247-258.
8. Benoit, I., et al., (2004) Oligopeptides to Smooth from Hibiscus Esculentus Seeds Expression Lines. *SOFW Journal*, **130**: 52-59.
9. Grover, R., et al., (1998) A Quantitative Method for the Assessment of Facial Rejuvenation: A Prospective Study Investigating the Carbon Dioxide Laser. *Br J Plastic Surg*, **51**: 8-13.
10. Hatzis, J., (2004) The Wrinkle and its Measurement: A Skin Surface Profilometric Method. *Micron*, **35**: 201-219.

11. Bissett, D.L., et al., (2004) Topical Niacinamide Reduces Yellowing, Wrinkling, Red Blotchiness, and Hyperpigmented Spots in Aging Facial Skin. *Int J Cosmet Sci*, **26**: 231-238.

12. Tsukahara, K., et al., (2004) A Study of Diurnal Variation in Wrinkles on the Human Face. *Arch Derm Res*, **296**: 169-174.

13. Traikovich, S., (1999) Use of Topical Ascorbic Acid and it's Effects on Photodamaged Skin Topography. *Arch Otolaryngol-Head Neck Surg*, **125**: 1091-1098.

14. Seité, S., et al., (2005) Histological Evaluation of a Topically Applied Retinol-Vitamin C Combination. *Skin Pharmacol Physiol*, **18**: 81.

15. Barthel, L. et al., (1990) Improved Method for Obtaining 3-Microns Cryosections for Immunocytochemistry. *J Histochem Cytochem*, **38**: 1383-1388.

16. University of Bristol Veterinary Pathology School of Clinical Veterinary Science, Freezing Tissue for Cryostat Sectioning. Available At: http://www.bristol.ac.uk/vetpath/cpl/cpl.htm Internet Accessed: June 2008.

5.7 Appendix

5.7.1 A: Explanatory Statement

EXPLANATORY STATEMENT

Effects of common Anti-Aging Products on Human Skin

This information sheet is for you to keep

My name is Anita Schneider and I am studying for a Doctor of Philosophy (PhD) degree in the Department of Pharmaceutics at the Faculty of Pharmacy and Pharmaceutical Sciences, under the supervision of Prof. Barrie Finnin.

The aim of this project is to examine whether the anti-aging products actually visually reduce or eliminate the appearance and size of facial lines and wrinkles compared to untreated skin or skin treated with the product excluding the active ingredient. In essence we are determining whether the product can scientifically be proven to do what it claims.

We are planning to conduct a small study using human volunteers that will run for a period of 8 – 12 weeks in order to determine whether commercially available anti-aging product can in fact reduce the appearance and size of facial wrinkles. These results will be presented in my final thesis to go towards obtaining my PhD and will hopefully lead to publications in relevant journals. The project is receiving funding from Dr LeWinn's Private Formula.

Aging of the skin results in the formation of facial lines and wrinkles, which are a concern for many individuals. Thus, due to a great demand to "look younger for longer", the cosmetic industry has focused on developing novel formulations for reducing the signs of skin aging. One commonly claimed mechanism of action for such anti-aging preparations is collagen regeneration. While laboratory tests conducted by the cosmetic companies are cited in support of these claims there is little or no substantiation in the peer reviewed scientific literature. Many of the products in this category are expensive and widely used and it is important to determine whether the expenditure on these products can be justified. This study proposes to test products for their ability to improve skin appearance and reduce the size of surface wrinkles therefore we will be able to determine whether the ingredient claimed to exhibit anti-aging can be substantiated.

I am seeking volunteers aged between 30 and 70 years, who have mild to moderate visible facial line or wrinkles on their face preferably around the eye area or forehead and are able to donate some of their time to participate in this study. However, if you have a history of skin disease (such as eczema or psoriasis), are using any medicated products on your face that cannot be stopped during the course of this study, if the area to be tested is damaged and /or scarred, and /or you have had any reactions (including allergic reactions) to any of

the ingredients in the formulations to be tested and /or silicone you will be unable to participate in the research.

Those who agree to participate in the research will be required to apply an amount of two formulations sufficient to cover a ten cent piece to two test sites: a base cream will be applied to one area and the test formulation to the other or in some cases, only one side of your face. Each formulation will be applied twice a day over the study period (8 – 12 weeks). You will perform this procedure yourself, in your own home, and it should only take a couple of minutes to complete. You will be required to complete a safety evaluation form at the end of the study or if any adverse reactions occur. Since the ingredients of the products to be tested are currently available to the public there should not be any major side effects. However, you will be given the opportunity to test the silicone mould and the test products on your skin to ensure you are not sensitive or allergic to any of its components. Although it is unlikely that the product will cause an adverse skin reaction you will be given a supply of anti-histamine tablets and a tube of hydrocortisone cream, which should be used if you experience an adverse skin reaction. Every second week, on the same day of each week, you will be required to travel to either 381 Royal Parade Parkville or 17 Rees Rd Sunbury, where I will take 3 digital photographs of your face (front and both profile views). After the photograph is taken, I will then take silicone moulds of two test sites either on the forehead or around the eye area. This procedure involved the application of a dental impression medium to the test area in a paste form it is then left for a few minute until it sets. The mould is then removed, labelled and analysed at a later date. It is completely painless and does not cause any discomfort.

The entire process will only take about 15 minutes to complete and will be carried out at a time that is convenient for you. If you participate in this study travel costs up to per visit $20 will be reimbursed, also a cosmetic package containing products from Dr LeWinn’s Private Formula will be supplied at the end of the study.

In order to ensure that your identity remains anonymous, you will only be identified by a coded number, which will be used on any written document generated during the study, such as the safety and efficacy evaluation forms. Only I and Barrie Finnin will know the names of the participants and their ages and only the combined results of all participants will be published. In order to ensure that the information gathered from this study remains confidential, the only people who will have access to the original information will be myself and Barrie Finnin. Completed safety and efficacy evaluation forms will be stored in a locked filing cabinet in a locked office at Monash University and hard copies of the digital photographs will only be stored on my computer’s hard drive (access is password-protected). The information gathered from this study will be stored for at least five years as stated by Monash University regulations. After this time, the information will be discarded in a confidential manner.

In order to demonstrate the efficacy of this formulation, with your prior permission a photograph of your face may be used in my thesis and other publications and presentations. You will be given the opportunity to sign a form that states whether or not you will allow the publishing of photographs of your face.

Participation in this research is entirely voluntary and, if you agree to participate in this study, you may withdraw at any stage during the study without further consequence. Although it is not essential, it

would be helpful if you could tell us whether you want to withdraw from the study due to an adverse reaction to the formulation or due to other reasons (which do not need to be specified). The entire study period will be approximately 8 – 12 weeks.

If you have any questions or concerns about the study or wish to obtain results of the project, please feel free to contact either myself or Barrie Finnin (Ph: (03) 9903 9520 Fax: 9903 9583 Email: Barrie.Finnin@vcp.monash.edu.au)

Should you have any complaint concerning the manner in which this research (Project Number: CF08/0308 – 2008000115) is conducted, please do not hesitate to contact the Monash University Standing Committee on Ethics in Research Involving Humans at the following address:

The Secretary
The Standing Committee on Ethics in Research Involving Humans (SCERH)
Building 3d
Research Grants & Ethics Branch
Monash University VIC 3800
Tel: +61 3 9905 2052 Fax: +61 3 9905 1420 Email: scerh@adm.monash.edu.au

Regards

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5.7.2 B: Informed Consent Form

CONSENT FORM

Effects of common Anti-Aging Products on Human Skin

I agree to take part in the above Monash University research project. I have had the project explained to me, and I have read the Explanatory statement, which I will keep for my records. I understand that agreeing to take part means that I am willing to:

- Apply the "Anti-Wrinkle Formulation" to my face twice a day for a period of 12 weeks
- Meet with the student researcher prior to study commencement and every second week up to 12 weeks
- Have a silicone mould taken of both test sites on my face every second week of the study
- Have photographs of my face taken by the student researcher of the study (please refer to the **Photographic Release and Authorisation Form**
- Supply the student researcher with my personal information including: name, age and contact information, although I understand this will be kept confidential and only combined results will be published

Please tick all appropriate boxes:

❑ I understand that I will be given a choice as to whether any photographs that are taken of my face will be published

❑ I understand that my participation is voluntary, that I can choose not to participate in part or all of the project, and that I can withdraw at any stage of the project without being penalised or disadvantaged in any way

Please tick the appropriate box:

❑ The information I provide cannot be used by other researchers without asking me first

❑ The information I provide cannot be used except for this project

Name:……...(please print)

Signature:……. Date…….

5.7.3 C: Photographic Release Form

PHOTOGRAPHIC RELEASE and AUTHORISATION FORM

Effects of common Anti-Aging Products on Human Skin

I agree to the taking of photographs of my face during the above Monash University research project. I understand that such photographs may need to be published in print, visual and / or electronic media including, but not limited to, a thesis paper and oral presentation, possible medical journals and scientific presentations, in order to inform the medical and / or pharmaceutical profession and / or the general public about the project I have participated in.

I understand that in agreeing to have the photographs of my face published means that although I will not be identified by name in any publication, the photographs will portray features that may make my identity recognisable. I also understand that the researchers will attempt to conceal some parts of the photograph in order to make my face less recognisable (i.e. they will insert a "black box" over the eye area of the face) and that I will have the right to approve the actual images used.

I understand that while I agree to have the photographs of my face published, I have the right to revoke this authorisation in writing at any time, and if I do so it will have no effect on any actions taken prior to my revocation. I understand I may refuse this authorisation and such refusal will be without any consequence.

Please tick the appropriate box:

- ☐ I agree to the taking of photographs of my face during the above Monash University research project and I also agree to the publication of these photographs in any print, visual and / or electrical media subject to my approval of the actual images. I grant this consent as a voluntary contribution in the interest of this research project

- ☐ Although I agree to the taking of photographs of my face during the above Monash University research project, I DO NOT agree to the publication of these photographs in any print, visual and / or electronic media

Name:……...(please print)

Signature:……. Date…….

5.7.4 D: Efficacy Evaluation Form

STUDY RECORDS: EFFICACY EVALUATION FORM

Effect of common Anti-Aging Products on Human Skin

INVESTIGATOR \ INDEPENDENT ASSESSOR-EVALUATION FORM

(To be completed by the chief / co-investigator and the research student comparing day 0 to final day of the study)

STUDY DETAILS

Participant Code No: _____

Formulation applied to left hand side of face:_____(*This is a coded number*)

Formulation applied to right hand side of face:_____(*This is a coded number*)

EFFICACY EVALUATION

a) *LEFT HAND SIDE OF FACE*

Compared with the baseline photograph, how would you rate the appearance of the wrinkles on the left hand side of the participants face?

Please circle the most appropriate number:

3	Much improved
2	Moderately improved
1	Slightly improved
0	No change
-1	Slightly worse
-2	Moderately worse
-3	Much worse

b) *RIGHT HAND SIDE OF FACE*

Compared with the baseline photograph, how would you rate the appearance of the wrinkles on the right hand side of the participants face?

Please circle the most appropriate number:

3	Much improved
2	Moderately improved
1	Slightly improved
0	No change
-1	Slightly worse
-2	Moderately worse
-3	Much worse

5.7.5 E: Safety Evaluation Form

STUDY RECORDS: SAFETY EVALUATION FORM

Effects of common Anti-Aging Products on Human Skin
(To be completed by the participant if any side effects present or on completion of the study)

STUDY DETAILS

Participant Code No: _____

Formulation applied to left hand side of face: _____

Formulation applied to right hand side of face: _____

SAFETY EVALUATION

Has the formulation caused any of the following side effects? Please tick the appropriate box if any below.

If you answered "yes" to any of the side effects below, please grade the severity of the side effect by circling the appropriate number:

*

1 = mild

2 = moderate

3 = severe

4 = very severe

	YES	*NO*	*SEVERITY**
Feeling warmth on the skin	☐	☐	1 2 3 4
Burning	☐	☐	1 2 3 4
Stinging	☐	☐	1 2 3 4
Redness	☐	☐	1 2 3 4
Dryness	☐	☐	1 2 3 4
Flakiness	☐	☐	1 2 3 4
Itching	☐	☐	1 2 3 4
Rash	☐	☐	1 2 3 4
Other (please state)_____	☐	☐	1 2 3 4

Chapter Six

In Vivo Evaluation of Commercially Available Anti-Aging Products

6.1 Introduction

Of the three common anti-wrinkle compounds investigated in the *in vitro* diffusion studies, niacinamide and palmitoyl tripeptide-5 were chosen for *in vivo* evaluation. Niacinamide demonstrated the highest rate of percutaneous permeation (results shown in Chapter Three). On the other hand palmitoyl tripeptide-5, under the conditions tested, showed an inability to penetrate or permeation the stratum corneum (results shown Chapter Three). Therefore, it is not likely to reach its site of action where it claims to cause collagen regeneration, in the dermis. By examining the ability of both ingredients to elicit an anti-wrinkle effect on human skin the following can be assessed:

i. Whether an anti-wrinkle effect is seen from the commercially available products, over the study periods.
ii. Whether the ingredients under investigation are responsible for the anti-wrinkle effect, or the effect are caused by the vehicles.
iii. Whether the ingredients are likely to be causing the anti-wrinkle effect by the proposed mechanisms of action i.e. collagen regeneration or antioxidant behaviour, or if the ingredients are simply causing a surface effect i.e. moisturisation.

6.2 Aim

To determine whether a cream containing niacinamide (niacinamide anti-wrinkle product, "AWP-N") and a cream containing palmitoyl tripeptide-5 (palmitoyl tripeptide-5 anti-wrinkle product, "AWP-P") reduce the appearance, size and number of mild to

moderate facial wrinkles compared to either skin left untreated or skin treated with a control (the "AWP" excluding either niacinamide or palmitoyl tripeptide-5).

6.3 Methods

6.3.1 Formulations

6.3.1.1 Niacinamide

The "AWP-N" analysed for anti-wrinkle efficacy consisted of 5 % niacinamide in a cream formulation. Table 1 below, lists all the ingredients present in the "AWP-N" and their reported function(s).

Table 1: "AWP-N" Cream

Chemical	Reported Function
Water	Solvent, Water Component and Moisturiser [1]
Glycerin	Solvent and Humectant [1-3]
Almond Oil	Emollient and Oil Component [2]
Crambe Oil	Emollient and Oil Component [2]
Ethyl Macadamiate	Emollient and Oil Component [2]
Octyl Salicylate	Sunscreen Agent [2, 4]
Xanthan Gum	Thickening Agent [2-3]
EDTA	Chelating Agent [2-3]
Cetearyl Alcohol	Co-Emulsifier and Emollient [1, 3]
Hydrogenated Olive Oil	Skin Conditioning and Thickening Agent [3]
Olive Oil	Emollient, Antioxidant and Moisturising Agent [2-3]
Olive Oil Unsaponifiable	Emollient and Skin Conditioning Agent [3]
Cetearyl Olivate	Hair Conditioning Agent [3]

Sorbitan Olivate	Emulsifier [3]
Dimethicone	Slipping and Humectant, Emollient and Wound Healing [2-3]
Bisabolol	Anti-Irritant and Skin Conditioning Agent [2-3]
Lecithin	Emollient and Humectant [2]
Borage Oil	Emollient, Antioxidant and Cell Regulator [5-6]
Caprylic / Capric Triglyceride	Emollient [1, 3]
Retinyl Palmitate	Active: Antioxidant and Skin Cell Regulator [2, 7-10]
Tocopheryl Acetate	Active: Antioxidant and Skin Conditioning Agent [2-3, 10-12]
Ascorbyl Palmitate	Active: Antioxidant [13] and stimulates collagen synthesist [14]
Alcohol	Humectant [2]
Niacinamide	Active: Antioxidant and NAD Precursor [15-17]
N-Acetyl Glucosamine	Active: Reduce Hyperpigmentation [18]
Mica	Pearling / Opacifying Agent [2-3]
Titanium Dioxide	Thickening, Whitening, Lubricating and Sunscreen Agent [2-3]
Tin Dioxide	Opacifying Agent [19]
Pea Extract	Active: Skin Tension, Moisturising and Firming [20]
Pineapple Extract	Fragrance and Exfoliant [2]
Carrot Oil	Emollient [2]

6.3.1.2 Palmitoyl Tripeptide-5

The "AWP-P" under investigation for this *in vivo* clinical study, contained 1 % of palmitoyl tripeptide-5 solution in a cream formulation. The ingredients of the "AWP-P" are shown below in Table 2, and are presented in descending order of concentration.

Table 2: "AWP-P" Cream

Chemical	Reported Function
Water	Solvent, Water Component and Moisturiser [1]
Ceteareth-6 Olivate	Emulsifier [3]
Glycerin	Humectant [1]
Dicaprylyl Carbonate	Emollient [1]
Sorbitol	Humectant, Thickening and Slipping Agent [2]
Glyceryl Stearate	Co-Emulsifier [1]
PEG-100 Stearate	Thickening Agent [2]
Dimethicone	Solvent [1, 3]
Cetyl Alcohol	Co-Emulsifier [1]
Stearic Acid	Emollient [2]
Sodium Ascorbyl Phosphate	Active: Antioxidant [21-22] and stimulates collagen synthesist [14]
PPG-12 / SMDI Copolymer	Skin Conditioning Agent, Emulsifier, Solvent and Surfactant [2]
Punica Granatum Extract	Active: Antioxidant when taken orally [23-24]
Crithmum Maritimum Extract	Active: Antioxidant [25]
Mushroom Extract	Active: Antioxidant [26]
Palmitoyl Tripeptide-3	Active: Collagen and GAG Synthesis [2]
C12-15 Alkyl Benzoate	Emollient and Thickening Agent [2]
Tribehenin	Skin Conditioning Agent [2]
Ceramide 2	Active: Restore Skin's Barrier Function [27-29]
PEG-10 Rapeseed Sterol	Emulsifier [3]
Palmitoyl Oligopeptide	Active: Stimulate Collagen Production and Encourage Production of Intercellular Matrix Substances [2]
Caprylic / Capric Triglyceride	Emollient and Thickening Agent [2]
Linoleic Acid	Emollient and Thickening Agent [2]
Glycine Soja (Soybean) Sterols	Active: Mimics Estrogen [2]
Phospholipids	Emollient and Humectant [2]
Olea Europaea (Olive) Leaf Extract	Skin Conditioning and Perfuming Agent [3]
Brassica Campestris / Aleurites Fordi Oil Copolymer	Emollient, Antioxidant, Film Forming and Skin Conditioning Agent [3, 30]
Stearyl Alcohol	Co-Emulsifier [1]

Zinc Gluconate	Antiviral / Acne Treatment [2]
Magnesium Aspartate	Skin Conditioning Agent [3]
Copper Gluconate	Active: Antioxidant, Collagen and Elastin Synthesiser [31-35]
Xanthan Gum	Thickening Agent [2-3]
Phenoxyethanol	Preservative [2-3]
Parabens (Methyl, Butyl, Ethyl, Propyl, Iso-Butyl)	Preservatives [1-3]
Magnesium Aluminium Silicate	Thickening Agent [2-3]
Tocopheryl Acetate	Active: Antioxidant [36-38]
Gamma Aminobutyric Acid	Active: Muscle Relaxant (claim) [2]
Disodium EDTA	Chelating and Thickening Agent [2-3]
Fragrances	

6.3.2 Study Protocol

The small clinical studies were conducted and anti-wrinkle efficacy was assessed, as per the protocol and the methods explicitly outlined in Chapter Five. Briefly, participants aged between 30 and 55 years were recruited into the human efficacy studies to assess whether the "AWP" either containing niacinamide ("AWP-N") or palmitoyl tripeptide-5 ("AWP-P") markedly reduce or eliminate the visible appearance, size and number of wrinkles when compared to either untreated skin or skin treated with the identical formulation without the active ingredient.

The effects of the formulations were assessed by two means. Firstly, a subjective assessment of the change in the visible appearance of the dynamic facial wrinkles was performed through a Photonumerical Scale method. The Photonumerical Scale method provided an efficacy evaluation based on visual comparisons made between the standardised digital photographs taken of each participant at baseline (day 0) and on days 14, 28, 42, 56 and (70 and 84, where applicable). The evaluation consisted of two

parts: an evaluation performed by the investigator and an evaluation performed by a blinded independent assessor. Secondly, an objective assessment of the variation in wrinkle depth (skin roughness) and the number of wrinkles over the study duration was performed. The change in the depth and number of wrinkles was assessed through a method referred to as Silicone Skin Replication, which was developed and validated prior to commencing the study (results presented in Chapter Five). The technique involved moulds, using a polyether impression material, being taken of the left and right side of the participants' face, of either the forehead or around the eye (crow's feet) area. The selection of the analysis area was based on each participant's wrinkle distribution. The wrinkle depth was then determined from cross sections of the mould by examination under a microscope. The number of wrinkles was determined by the number of visible wrinkles in the analysed sample area. Objective assessments were performed at the beginning, middle and end of the study periods.

6.3.3 Study Population

6.3.3.1 Niacinamide Cream Split Face Clinical Study

The niacinamide split face clinical study consisted of 22 volunteers, of which one volunteer pulled out from the clinical study after one week due to an adverse reaction. The participant experienced swelling around the eye area and their skin felt tight on the sites of application. The participant, therefore, requested to remove themselves from the study. Pregnant women were also excluded from this clinical study due to the presence of retinyl palmitate in the "AWP-N". Retinyl palmitate has been

shown in animal studies to cause reproductive and developmental issues at low doses [39].

The niacinamide clinical study consisted of two application groups:

i. Group one: Contra-lateral side of face left untreated

ii. Group two: Contra-lateral side of face treated with a control formulation

Group one participants applied the "AWP-N" cream to either the left or right side of their face (to the forehead and crows feet areas), while the other side was left untreated (with the exception of use of a cleanser and moisturiser), which was randomly determined. Application was conducted twice daily over the study period of 84 days (12 weeks). Group one consisted of 11 female participants, of which after one week, one did not continue due to an adverse response caused by the "AWP-N".

Group two involved participants applying the "AWP-N" cream to either the left or right side of their face, which was randomly determined, while the alternate side of the face was treated with the control cream (the "AWP-N" excluding niacinamide). Application of both creams was conducted twice daily over the 84 day study period. Group two consisted of 11 participants: 2 male and 9 female.

At day 0, baseline images and silicone moulds were taken of the participants, the creams were supplied and the study protocol was explicitly explained to the participants. The participants returned to the evaluation site every second week of the clinical study, days 14, 28, 42, 56, 70 and 84. On each evaluation day digital images were taken of the frontal and both orbital views of the participants' upper face, along with silicone impression moulds of either their forehead or crow's feet wrinkles. Digital photographs were assessed for visual changes to the participants' dynamic facial wrinkles comparing each evaluation day to the baseline (day 0) photographs. Silicone

moulds were assessed for changes in the size and number of wrinkles at day 56 and 84 compared to day 0.

6.3.3.2 Palmitoyl tripeptide-5 Cream Split Face Clinical Study

The palmitoyl tripeptide-5 cream split face clinical study consisted of 20 volunteers with two application groups:

i. Group one: Contra-lateral side of face left untreated
ii. Group two: Contra-lateral side of face treated with a control formulation

Group one involved participants applying the "AWP-P" cream to either the left or right side of their face, which was randomly determined, while the alternate side of the face was left untreated (with the exception of use of a cleanser and moisturiser). Ten female participants completed the clinical study for group one.

Group two involved participants applying the "AWP-P" cream to either the left or right side of their face, which was randomly determined, while the alternate side of the face was treated with the control cream, the "AWP-P" excluding palmitoyl tripeptide-5. Group two consisted of 10 participants: 5 male and 5 female.

Participants from both groups travelled to the evaluation site, at day 0, where baseline images and silicone moulds were captured of their face, the creams were supplied and study protocol explicitly explained to them. The participants applied the creams to the allocated sides of their face twice daily over the 56 days (8 weeks) study period. Participants returned to the evaluation site every second week of the clinical study, days 14, 28, 42 and 56, where digital images were taken of the frontal and both orbital views of the participants' upper face, along with silicone impression moulds taken of either their forehead or crow's feet wrinkles. Digital photographs were assessed

for visual changes in the participants' dynamic facial wrinkles comparing each evaluation day to baseline (day 0) photographs. Silicone moulds were assessed for changes in the size and number of wrinkles at day 28 and 56 compared to day 0.

6.3.4 Statistical Analysis

Statistical Analysis of the visual change in wrinkles by Visual Image Analysis, the assessment of safety and change in both wrinkle size and number of wrinkles was assessed through the methods described in Chapter Five Section 5.3.3.3. All statistical analysis was performed using SPSS version 16.0 scientific software (Chicago, Illinois). A probability of $p < 0.05$ was considered statistically significant. All results shown in this chapter are presented as mean $\pm$ SEM (standard error of the mean) calculation shown below in Equation 1, unless otherwise stated.

$$\mathrm{SEM} = \frac{\mathrm{STDEV}}{\sqrt{(n-1)}} \qquad 1)$$

6.4 Results and Discussion

6.4.1 Niacinamide Cream Split Face Clinical Study

6.4.1.1 Safety Assessment

On each evaluation day, days 14, 28, 42, 56, 70 and 84, the participants were questioned as to whether they experienced any adverse skin reactions, if so they were required to complete a safety evaluation form (Chapter Five Section 5.7.5 Appendix E).

Over the first fortnight of the study, 6 participants out of 22 recorded adverse reactions. One participant recorded an adverse reaction so severe they withdrew themselves from the clinical study after one week. Reactions included: tingle / sting on application, slight swelling (only seen on week one) and one record of an increased sensitivity on exposure to light (feeling hot / burn at the application site in sunlight).

To quantitatively assess skin redness that may have been caused by the "AWP-N" or niacinamide itself, digital photographs were assessed for a change in skin redness over the study period, indicating skin sensitivity. Assessment was conducted as per the method described in Chapter Five Section 5.3.3.1.5 using Adobe Photoshop CS3. Skin redness was recorded as a percentage change in the mean intensity of the red histogram at day X compared to day 0. The results are presented below in Figures 1 and 2, for groups one and two respectively.

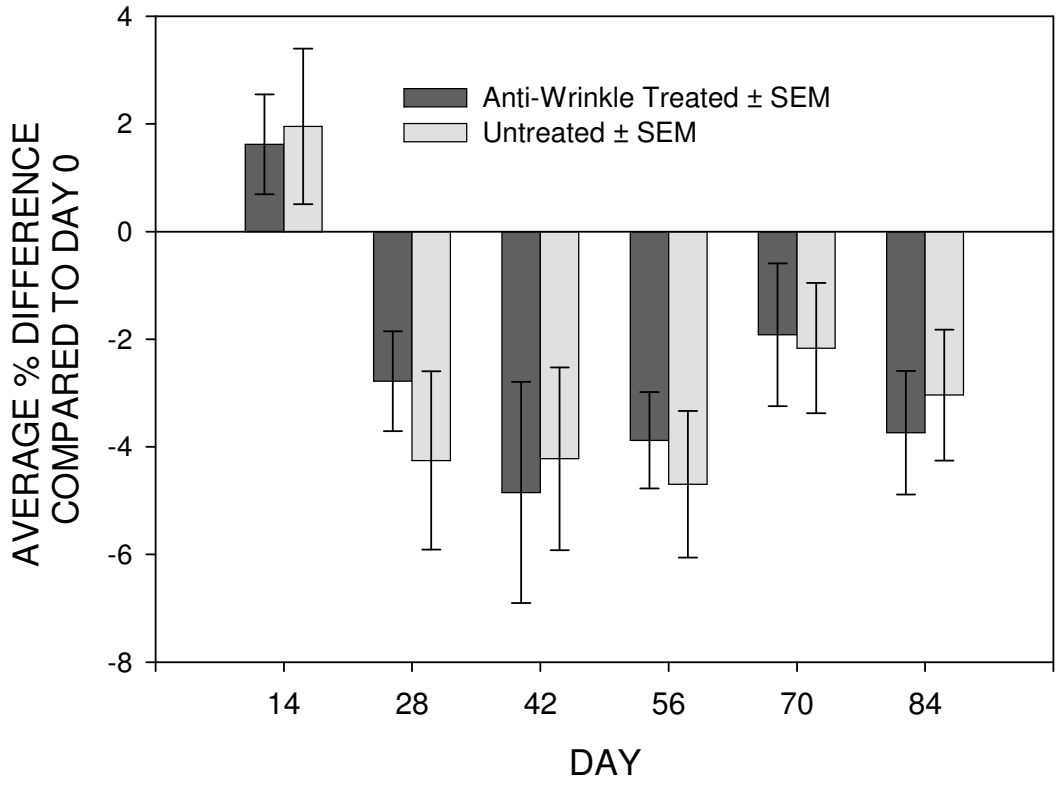

Figure 1. Skin redness results for "AWP-N" treated versus untreated skin as a function of average % difference in redness intensity at each evaluation day compared to day 0 over 84 day. The error bars represent the standard error of the mean (SEM), n = 10.

No difference was observed over the study between skin treated with the "AWP-N" and skin left untreated with regards to skin redness. However, redness was significantly reduced, $p < 0.05$, at days 42, 56, 70 and 84 compared to day 0, this was not the case for untreated skin, with the exception of day 56. Skin redness can vary in response to factors other than skin sensitivity. Such factors can include: temperature, emotional state and exercise. The thinness and extensiveness of the skin make it an effective radiator of heat when the body temperature rises above ambient temperature. In warm temperatures and after exercise, heat is transferred from the body to the external environment by vasodilation, the flow of warm blood through capillary loops near the skin surface, which lead to a reddening of the skin [40]. Cutaneous blood flow

is also affected by sympathetic nerve activity, which is controlled by the brain and can be affected by emotions. Emotions, such as nervousness, can cause vasodilatation and lead to an event referred to as blushing (skin reddening), this may have occurred with a number of participants on day 0 of the study [40].

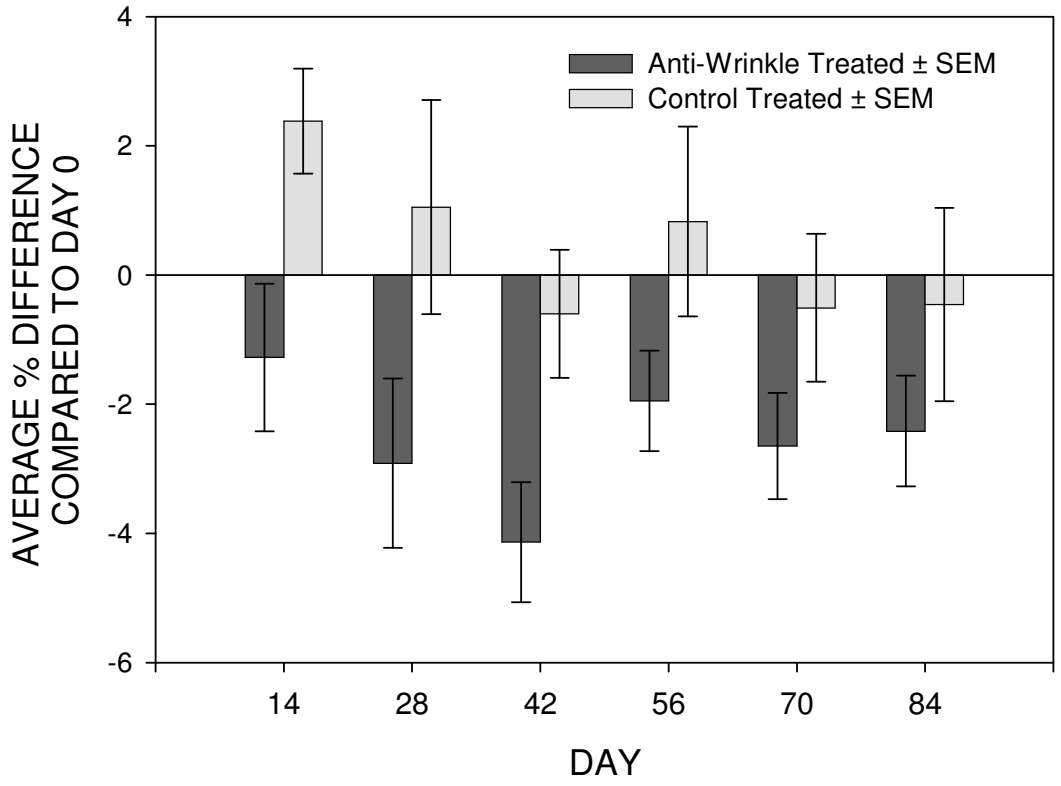

Figure 2. Skin redness results for "AWP-N" treated versus control treated % ± SEM, n = 11.

Skin treated with the "AWP-N" was found to significantly reduce skin redness over control treated skin, $p < 0.05$, at days 14, 42 and 56. "AWP-N" treated skin was also found to significantly reduce skin redness, $p < 0.05$, at days 42, 56, 70 and 84 compared to day 0, this was not the case with skin treated with the control. The results demonstrate the control cream has caused little change on skin redness, whereas the "AWP-N" has caused a reduction in skin redness over the study period.

Quantitative assessment of changes in skin redness showed neither the "AWP-N" nor niacinamide itself had any effect on increasing skin redness over the study period, indicating it was unlikely to be causing skin irritation. The safety assessment results for group two indicate that niacinamide may be responsible for reducing skin redness, which supports the use of niacinamide in products used to treat erythema. A variety of disorders such as rosacea can cause skin inflammation, which can lead to skin reddening [41-42], niacinamide, therefore, could be used to treat these disorders.

6.4.1.2 Participant Compliance

On each evaluation day participants were asked whether they had forgotten to apply the "AWP-N" and control cream (if applicable) over the previous fortnight, and the information was recorded in a compliance diary. At the completion of the study any unused creams were returned to the investigator and the containers were weighed.

The container weights did not indicate any compliance issues. It was discovered from the compliance diary that the participants' applied the formulation(s) 99.38 % of all application points. The largest percentage of non compliance was found to be over the first fortnight of the study, recorded at day 14. It can be assumed this lack of compliance over the first two weeks of the study was related to the fact that the participants had not formed a habitual behaviour of applying the product(s). Habitual behaviour is associated with the repetition of a function both frequently (at least twice a month) and extensively (at least 10 times) [43]. Therefore, habitual behaviour of applying the test product(s) twice daily was achieved over the first fortnight of the clinical study.

6.4.1.3 Participant Perception

On each evaluation day the participants were asked if they had noticed a change in the visible depth and size of their dynamic forehead and crow's feet wrinkles, comparing one side of their face to the other. A change was recorded on a 7-point scale, where: -3 = much worse, -2 = moderately worse, -1 = slightly worse, 0 = no change, 1 = slightly improved, 2 = moderately improved or 3 = much improved. Results, shown below in Figures 3 and 4, are represented as the mean of the perceived efficacy assessment for each group, for groups one and two respectively.

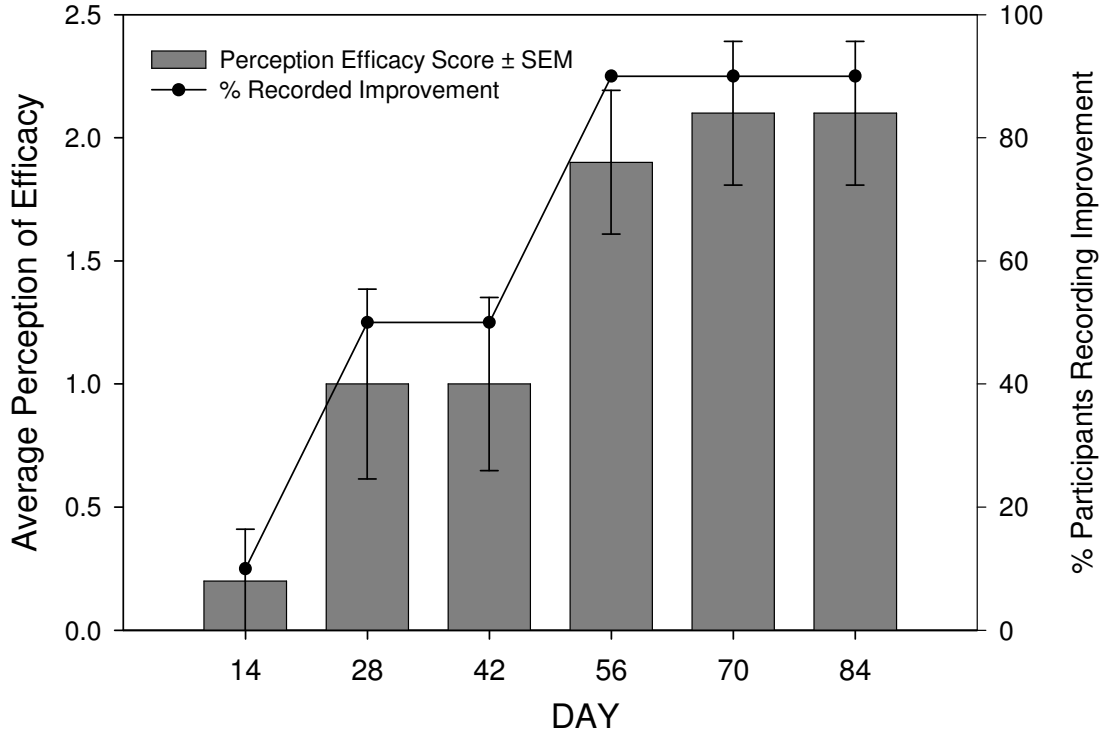

Figure 3. Participant perceived efficacy results for "AWP-N" treated versus untreated skin. Each bar represents the mean of the perceived improvement of the participants ± SEM, n = 10. The line represents the percentage of participants at each time point that recorded any perceived improvement.

From day 56 onwards, 90 % of the participants recorded an improvement in skin treated with the "AWP-N" over skin left untreated. At day 84 of the clinical study, of the 90 % of participants, 67 % recorded moderate improvement, while 33 % recorded much improvement over untreated skin.

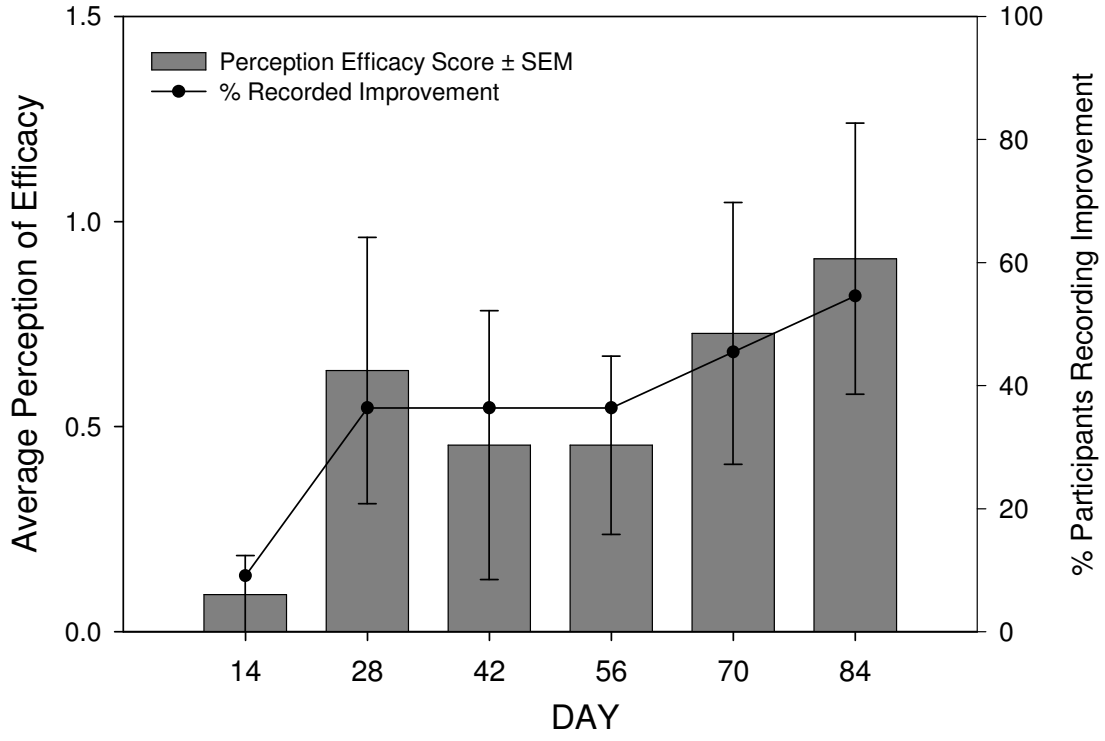

Figure 4. Participant perceived efficacy assessment results for "AWP-N" treated versus control treated skin n = 11.

At day 84 of the clinical study, 82 % of the participants recorded some improvement compared to day 0. While at day 84, 55 % of the participants recorded an improvement in skin treated with the "AWP-N" over control treated skin. Of the 55 % of participants at day 84, 33 % recorded moderate improvement, while 17 % recorded much improvement of "AWP-N" over control treated skin.

Participant perception of the efficacy of the "AWP-N" resulted, on average, in a moderate visible improvement compared to untreated skin after 84 days. However, compared to control treated skin, the "AWP-N" only resulted in a perception of slight

improvement on average. From a consumers perspective it seems as though the vehicle rather than niacinamide itself has caused the largest impact on altering the visible appearance of dynamic forehead and crow's feet wrinkles. There is, however, an added bias for participant perception of improvement. It is possible for a participant to see an effect because they have a desire to do so, caused by the power of suggestion. This phenomenon has been well documented in pharmaceutical clinical trials, demonstrated by the placebo effect [44-46].

6.4.1.4 Digital Photography

The assessment of change in wrinkle appearance caused by an anti-wrinkle formulation or anti-wrinkle agent was conducted by capturing and analysing digital photographs for change over time, as per method Chapter Five Section 5.3.3.1. The photographs were graded on a 7-point scale at each evaluation day compared to day 0, by the investigator and an independent assessor. The results are shown below in Figure 5 and 6, for groups one and two respectively.

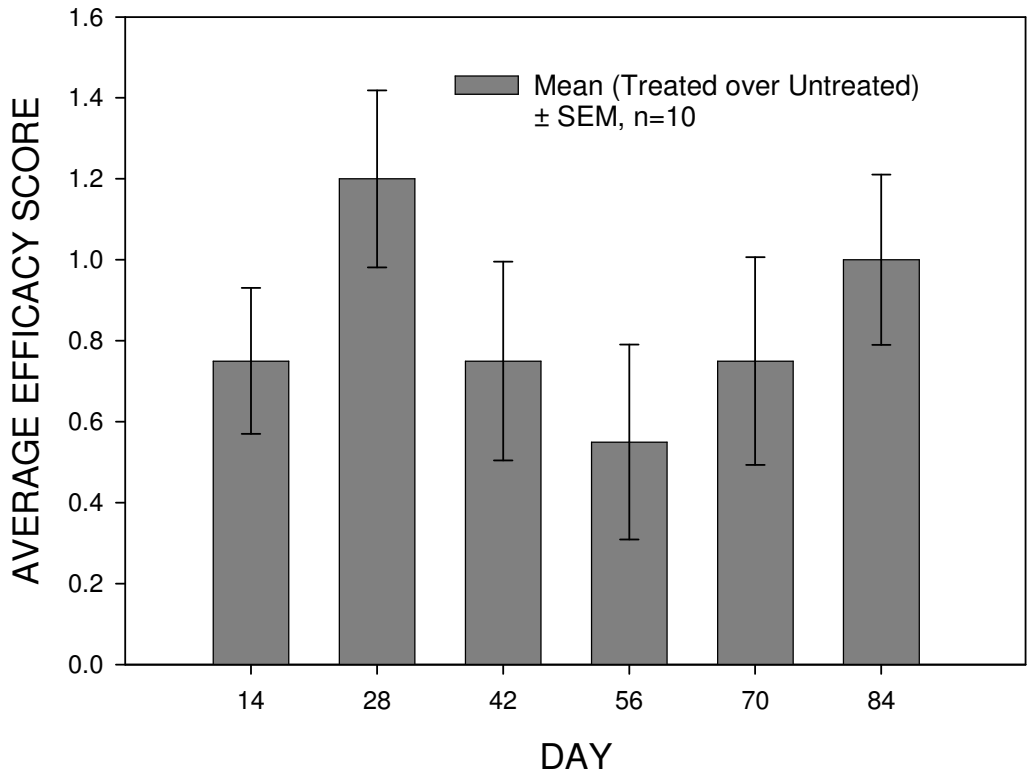

Figure 5. Photographic assessment results for "AWP-N" treated versus untreated. Each bar represents the mean efficacy score of the treated skin minus the untreated skin, therefore, results given are the improvement of the treatment over skin left untreated ± SEM, n = 10.

Over all, skin treated with the "AWP-N" was shown to have a significant improvement over skin left untreated, p=0.000. This was found to be true for all time points of the study, $p < 0.05$. Using a One Sample T-Test it was found on all test days skin treated with the "AWP-N" was significantly improved compared to day 0, $p < 0.05$, this was not the case for skin left untreated.

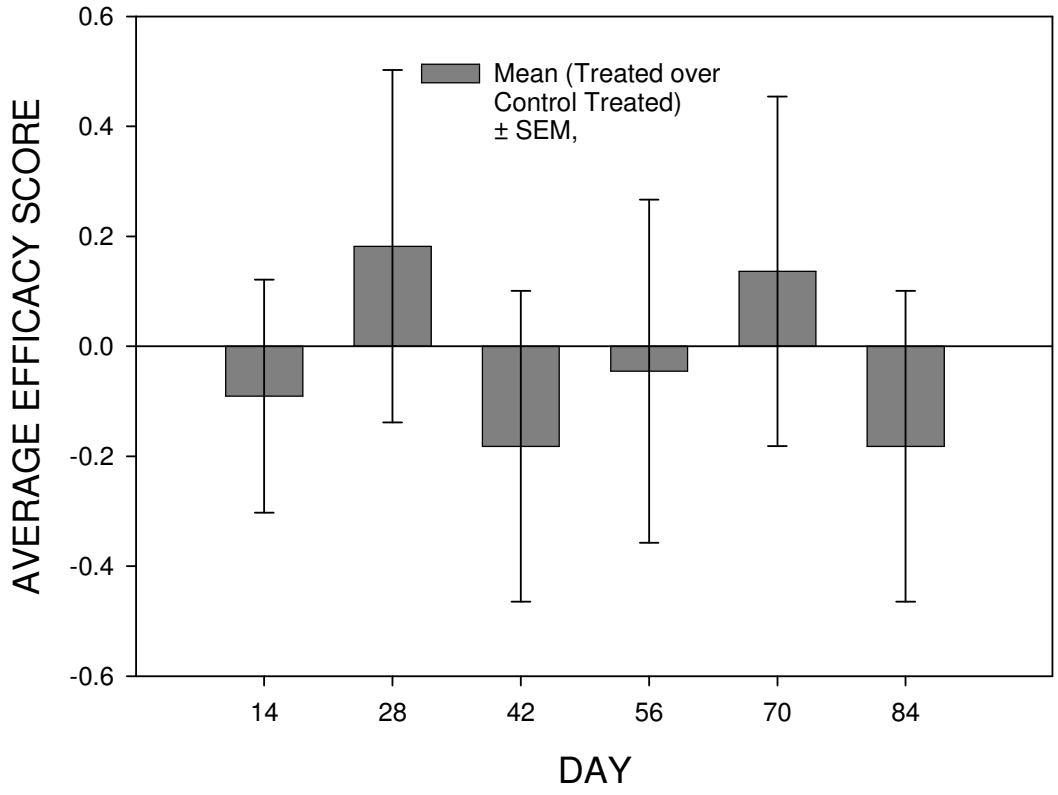

Figure 6. Photographic assessment results for "AWP-N" versus control treated skin. Each bar represents the mean efficacy score of the treated skin minus skin treated with the control, therefore, results given are the improvement of the treatment over control treatment ± SEM, n = 11.

No significance difference was found between "AWP-N" and control treated skin, $p > 0.05$.

Digital photographic assessment over the 84 day study period showed the commercially available "AWP-N" containing 5 % niacinamide caused a visible reduction in the appearance of facial wrinkles. The results, however, suggest that this is a function of the vehicle and not niacinamide specifically which is responsible for the anti-wrinkle behavior, demonstrated by Figure 6. Participant perception results, shown in Section 6.4.1.3, are consistent with the subjective Visual Image Analysis results.

6.4.1.5 Silicone Skin Replication Technique

6.4.1.5.1 Roughness

Wrinkle depth was determined as the peak height of a selected length of silicone mould slice for each participant. Between 50 and 120 peaks were analysed per slice. The roughness of each slice was expressed as a total of all the peak heights per slice. Three slices were analysed for each participant per side of the face, the left and the right, at each evaluation point; day 0, 56 and 84. The average roughness for each side, of each participant, at each evaluation day was compared as a percentage different to baseline (day 0). The three roughness values at each evaluation day were averaged and compared to day 0, as a percentage difference. The results, shown in Figures 7 and 8, are a function of the average percentage difference of all participants at each evaluation day, for group one and two respectively.

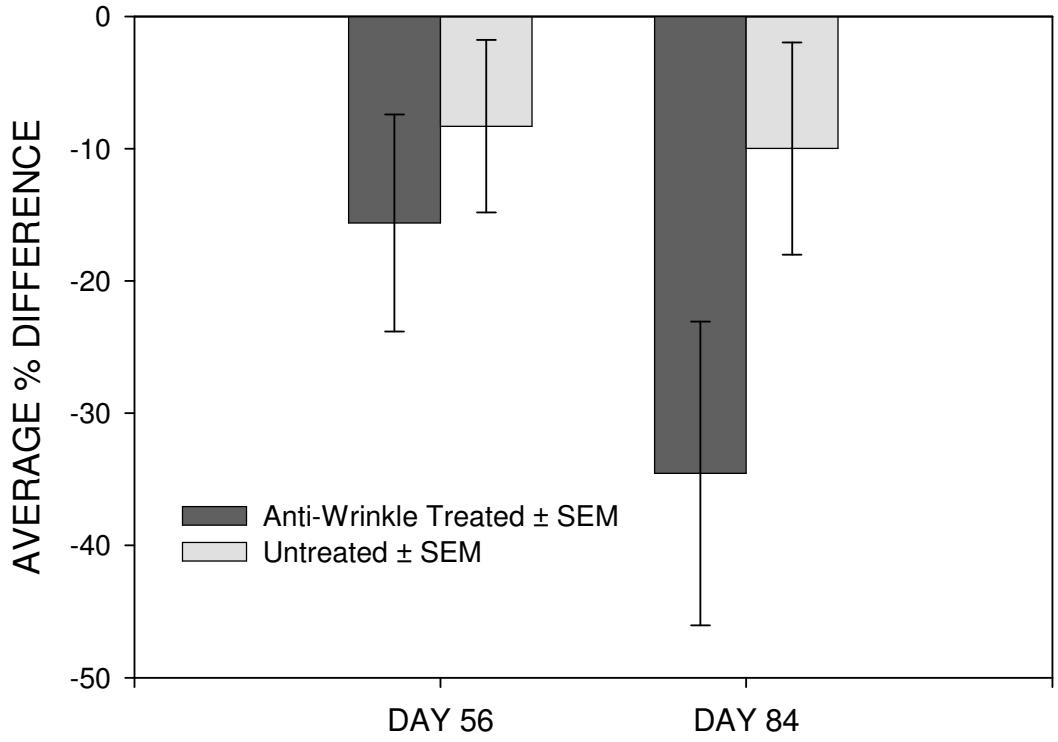

Figure 7. Skin roughness results for "AWP-N" treated versus untreated skin. Each point represents the mean percentage difference of each participant compared to their baseline ± SEM, n = 10.

Skin treated with the "AWP-N" at day 84 of the clinical study was shown to significantly reduce skin roughness compared to skin left untreated, $p = 0.02$. It was also demonstrated that skin roughness was significantly reduced at day 56 and day 84, $p = 0.038$ and 0.006 respectively compared to day 0, which was not the case for skin left untreated. The average reduction, from day 0, in skin roughness for skin treated with the "AWP-N" was found to be 15.62 ± 8.21 and 34.55 ± 11.49 % at day 56 and 84 respectively.

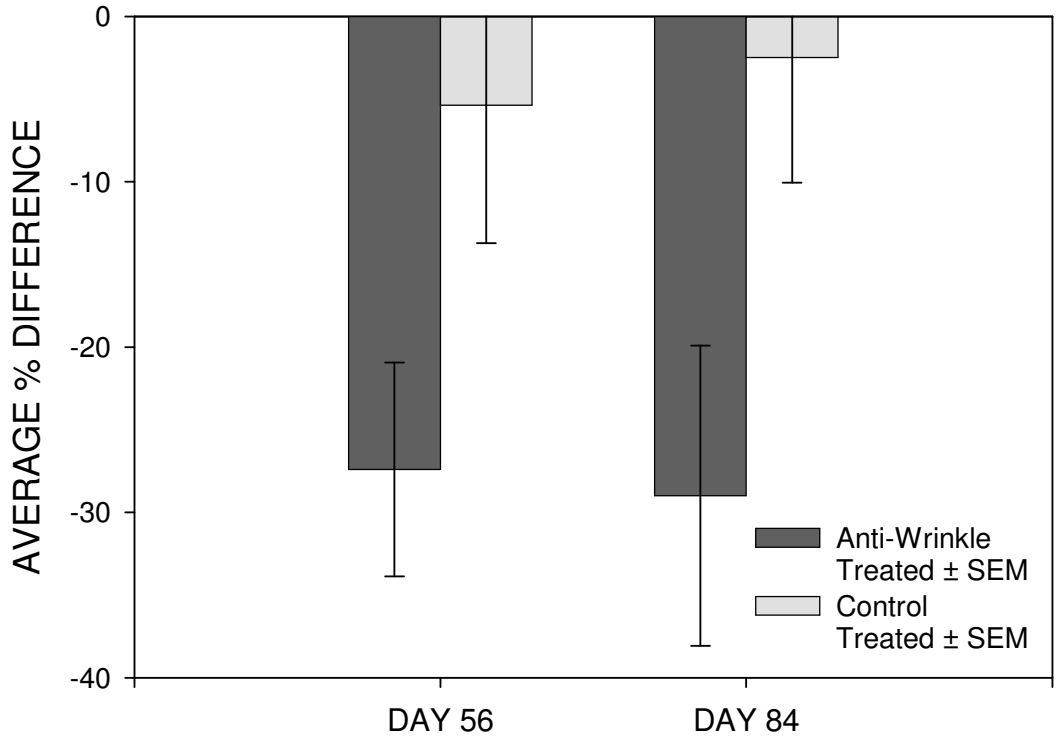

Figure 8. Skin roughness results for "AWP-N" treated compared to control treated skin. Each point represents the mean percentage difference of each participant compared to their baseline ± SEM, n = 10.

In group two, "AWP-N" treated skin showed a significant improvement over skin treated with the control at day 56 and day 84, p = 0.007 and 0.001 respectively. Skin treated with the "AWP-N", at both days 56 and 84, were found to be significantly reduced with respect to skin roughness compared to day 0, p = 0.0005 and 0.0025 respectively, using a One Sample T Test. Skin treated with the "AWP-N" was shown to reduce the average skin roughness, for days 56 and 84 by 27.40 ± 6.47 and 28.99 ± 9.09 % respectively, compared to baseline.

Skin treated with the "AWP-N" significantly reduced skin roughness and the size of the participants' wrinkles, over both untreated and control treated skin. The results indicate the "AWP-N" can reduce the size of facial wrinkles as early as 56 days.

Finally, although niacinamide itself was not shown to visibly reduce the appearance of wrinkle depth and size, it has been shown to cause a reduction in the size of wrinkles over the study period, compared to the vehicle.

6.4.1.5.2 Wrinkle Number

Wrinkle number was calculated from the total number of peaks analysed per slice for the same side of each participants face. The results, presented below in Figures 9 and 10 for groups one and two respectively, are expressed as an average of all participants in each group as a percentage difference of the average number of wrinkles analysed at each evaluation day compared to day 0.

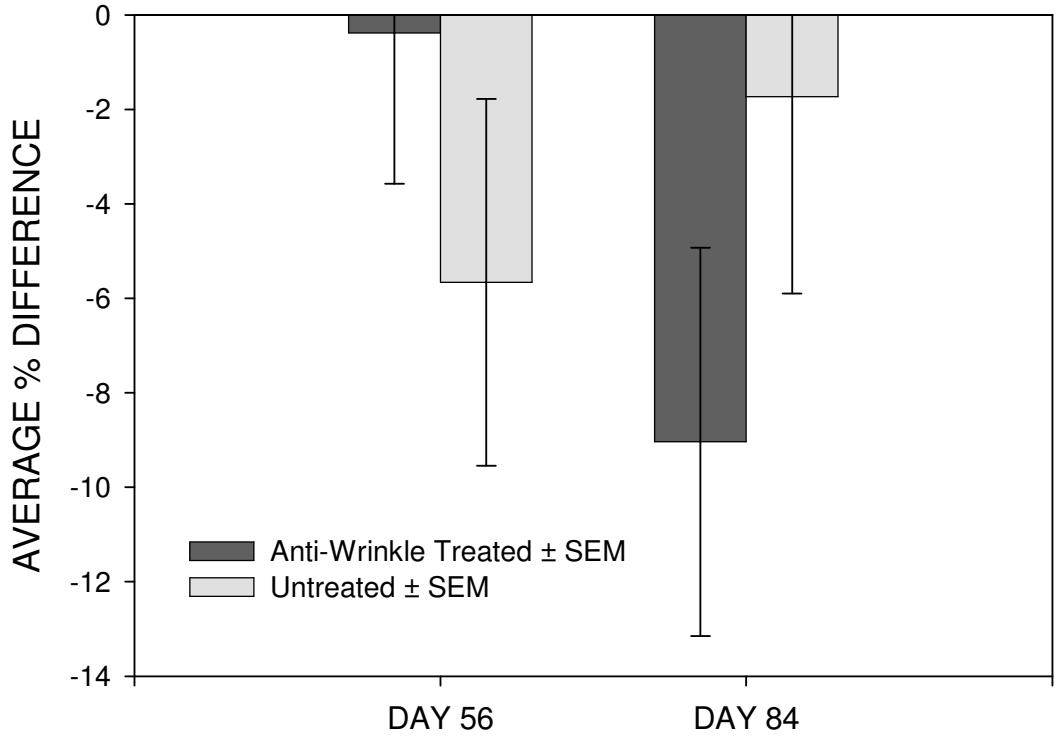

Figure 9. Wrinkle number results for "AWP-N" treated compared to untreated skin. Each point represents the mean percentage difference in the number of measured peaks in the analysis area of each participant compared to their baseline ± SEM, n = 10.

No significant difference was seen between the "AWP-N" and skin left untreated, overall. However, at day 84 the wrinkle number was significantly reduced from day 0, p = 0.023, for skin treated with the "AWP-N", which was observed for untreated skin.

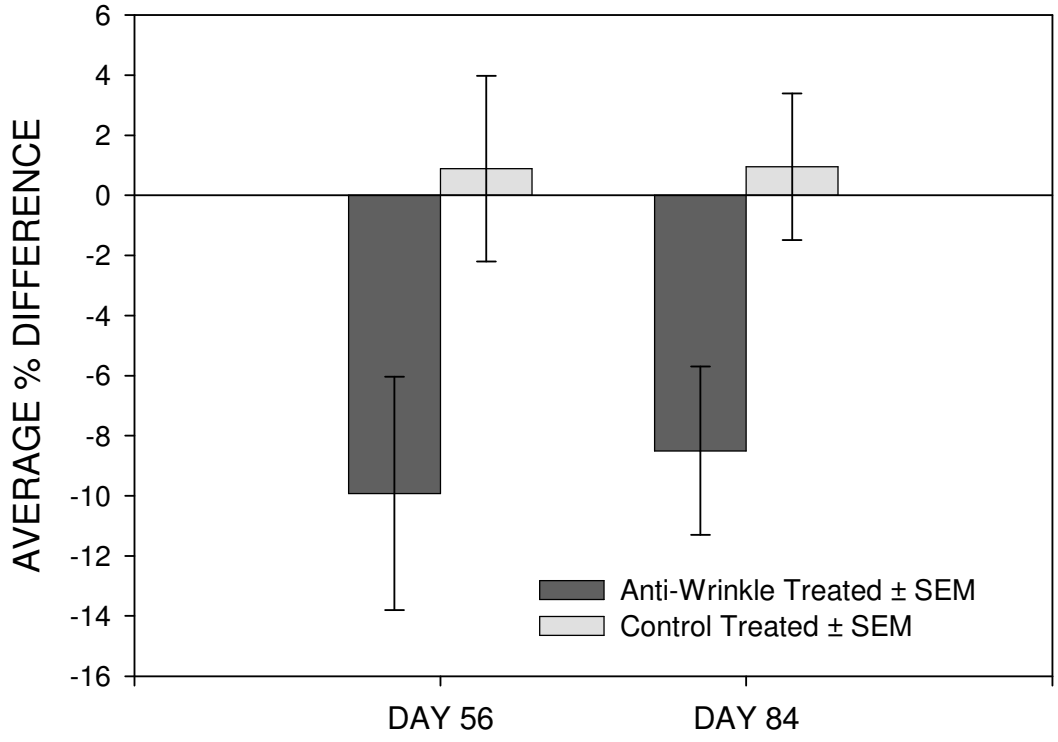

Figure 10. Wrinkle number results for "AWP-N" treated compared to control treated skin. Each point represents the mean percentage difference in the number of measured peaks in the analysis area of each participant compared to their baseline ± SEM, n = 11.

Overall, skin treated with the "AWP-N" was found to significantly reduce the number of wrinkles at both 56 and 84 days, compared to skin treated with the control formulation, p = 0.005 and 0.006 respectively. When compared to day 0, skin treated with the "AWP-N" was also shown to significantly reduce the number of wrinkles, this was not observed with skin treated with the control, p = 0.009 and 0.0035 respectively.

The average number of wrinkles in the analysis area was reduced by 9.92 ± 3.88 and 8.50 ± 2.80 % at day 56 and 84 respectively.

The Silicone Skin Replication technique revealed that skin treated with the "AWP-N" significantly reduced skin roughness compared to skin left untreated and treated with the control. "AWP-N" treated skin was also found to significantly reduce the number of wrinkles compared to control treated skin. The niacinamide clinical study results, therefore, indicate that the "AWP-N" containing 5 % niacinamide reduces the visible appearance and size of wrinkles compared to untreated skin. There is no indication that niacinamide causes improvements in wrinkle appearance over the vehicle. However, at a microscopic level niacinamide has been shown to reduce the size and number of wrinkles compared to the vehicle. The microscopic effect may, over a longer treatment period, translate to a visible improvement.

6.4.2 Palmitoyl tripeptide-5 Cream Split Face Clinical Study

6.4.2.1 Safety Assessment

On each evaluation day, days 14, 28, 42 and 56, the participants were asked if they had experienced any adverse skin reactions for the preceding fortnight, if so they were required to complete a safety evaluation form (Chapter Five Section 5.7.5 Appendix E).

Over the entire 56 day study period, out of the 20 participants none recorded any adverse skin reactions using the "AWP-P" (anti-wrinkle cream containing palmitoyl tripepide-5) or control product.

To further quantitatively assess skin irritation that may have been caused by the "AWP-P" or palmitoyl tripeptide-5 itself, digital photographs were assessed for a change in skin redness over the study period. Assessment was conducted as per the method described in Chapter Five Section 5.3.3.1.5. Skin redness was recorded as a percentage change in mean intensity of the red histogram at day X, compared to day 0. The skin redness results are presented below in Figures 11 and 12, for groups one and two respectively.

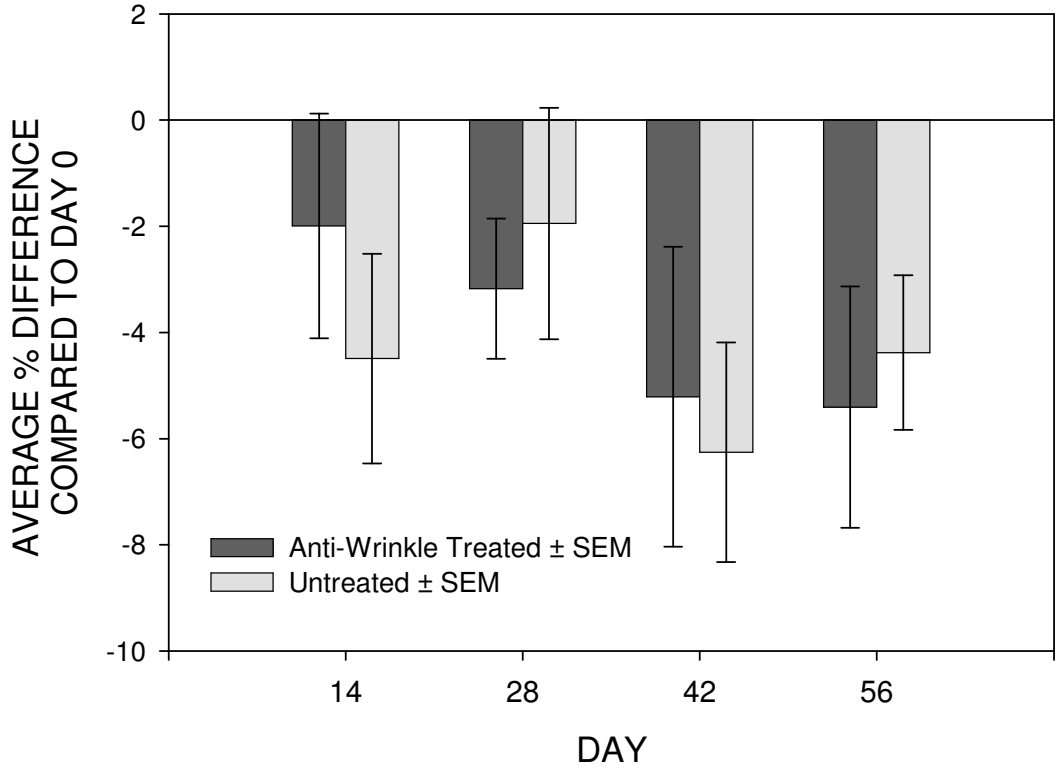

Figure 11. Skin redness results for "AWP-P" treated versus untreated skin as a function of average % difference in redness intensity at each evaluation day compared to day 0 over 56 day ± SEM, n = 10.

No difference was observed over the study between skin treated with the "AWP-P" and skin left untreated, with regards to skin irritation. However, skin redness was significantly reduced, $p < 0.05$, for "AWP-P" treated skin, at days 42 and 56 and for

untreated skin, at days 14, 42 and 56 compared to baseline. Again as skin redness can vary in response to factors such as temperature, emotional state and exercise, as well as irritation, these other factors may be responsible for the reduction in skin redness for untreated skin. Nervousness on day 0 may have caused skin reddening, leading to a reduction in the response as the participants became more comfortable with the procedures over the study period.

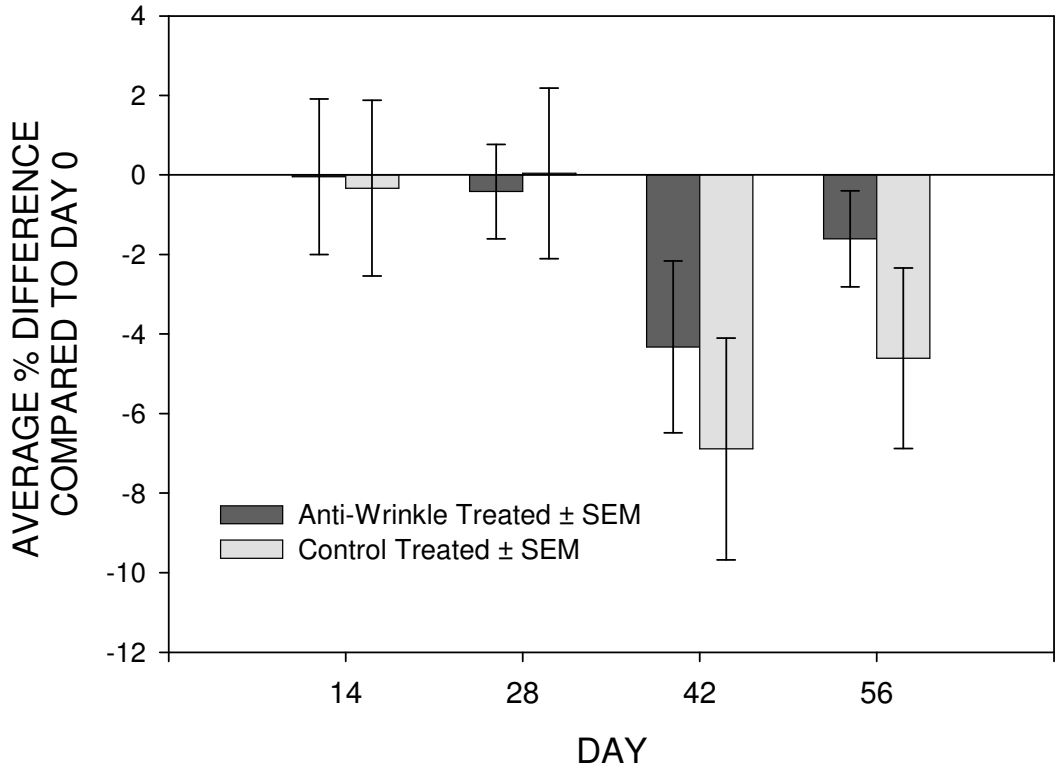

Figure 12. Skin redness results for "AWP-P" treated versus control treated skin as a function of average % difference in redness intensity at each evaluation day compared to day 0 over 56 day ± SEM, n = 10.

No difference was observed over the study between skin treated with the "AWP-P" and skin treated with the control with regards to skin irritation. "AWP-P" and control treated skin was found to significantly reduce skin redness, $p < 0.05$, at days 42 and for controlled treated skin at day 56 compared to day 0. As a significant difference was not

observed between skin treated with the control and skin treated with the "AWP-P" this demonstrates palmitoyl tripeptide-5 did not increase skin irritation over the vehicle.

Based on participants perceived safety evaluations and quantitative skin redness results it was found that neither the "AWP-P" vehicle nor palmitoyl tripeptide-5 itself caused any adverse skin reactions.

6.4.2.2 Participant Compliance

On each evaluation day participants were asked whether they had forgotten to apply the "AWP-P" and / or the control cream (if applicable) over the previous fortnight. The information was recorded in a compliance diary. At the completion of the study any unused creams were returned to the investigator and the bottle weights were measured.

The bottle weights indicated there were no compliance issues over the study period. It was discovered from the compliance diary that the participants' applied the formulation(s) 96.74 % of all application points. The largest percentage of non compliance was found to be at days 28 and 42. Lack of compliance, especially over the first fortnight of the clinical study is likely to be related to the time required for the participants to form a habitual behaviour of applying the product(s) twice daily. Compliance may also have been altered by the outcome of the clinical trial, associated with a participant's perceived improvement in their wrinkle's appearance.

6.4.2.3 Participant Perception

On each evaluation day the participants were questioned on whether they had noticed a change in the visible appearance of their dynamic forehead and crow's feet wrinkles on one side of their face compared to the other. A change was recorded on a 7-point scale, where: -3 = much worse, -2 = moderately worse, -1 = slightly worse, 0 = no change, 1 = slightly improved, 2 = moderately improved or 3 = much improved. Results, shown below in Figures 13 and 14, are represented as the mean of the perceived efficacy assessment for each group, for groups one and two respectively.

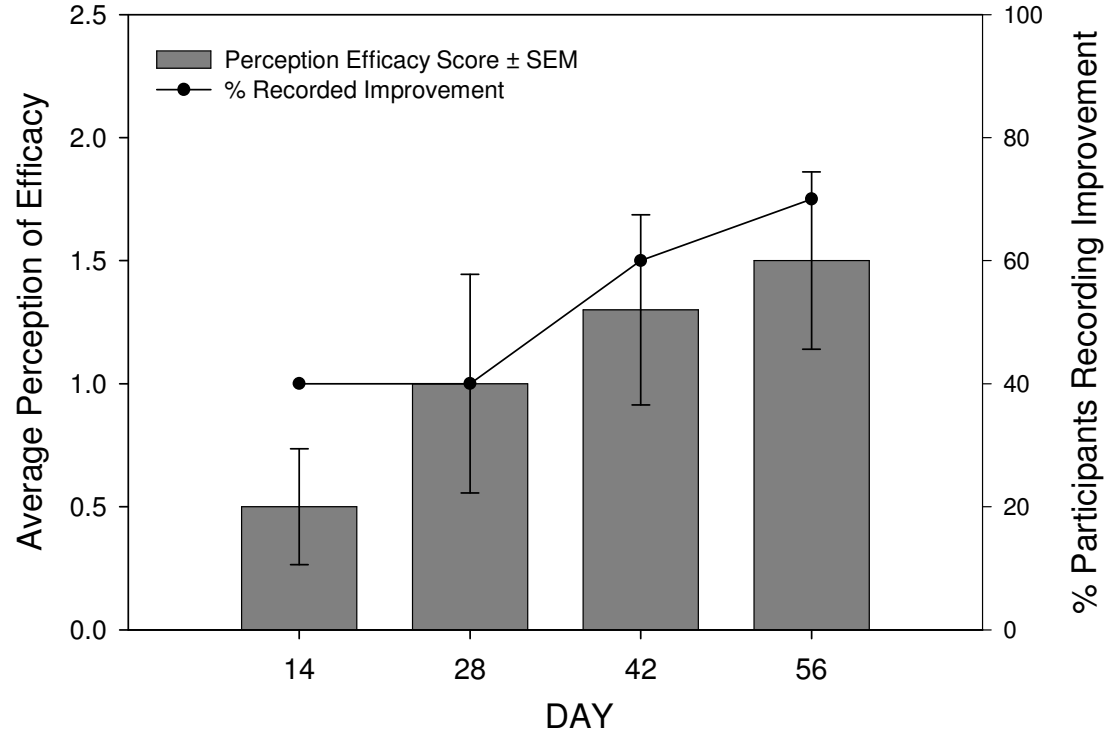

Figure 13. Participant perceived efficacy assessment results for "AWP-P" treated versus untreated skin. Each bar represents the mean of the perceived improvement of the participants ± SEM, n = 10. The line represents the percentage of participants at each time point that recorded any perceived improvement.

Over the entire study period 90 % of the participants recorded an improvement in skin treated with the "AWP-P" over skin left untreated. Of those 90 % of participants, 56 % recorded moderate and 22 % much improvement to the visible appearance of their dynamic forehead and crow's feet wrinkles compared to untreated skin.

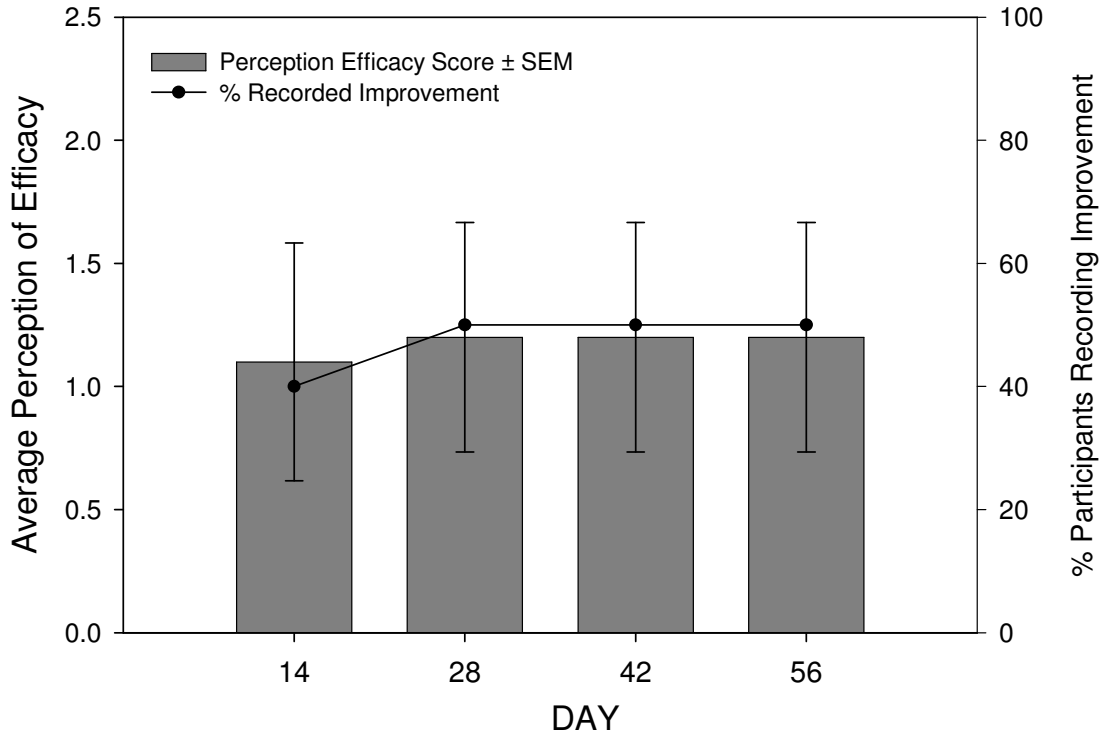

Figure 14. Participant perceived efficacy results for "AWP-P" treated versus control treated skin. Each bar represents the mean of the perceived improvement of the participants ± SEM, n = 10. The line represents the percentage of participants at each time point that recorded any perceived improvement.

Over the study, 50 % of the participants in group two recorded an improvement in skin treated with the "AWP-P" compared to skin treated with the control. Of that, 20 % recorded moderate improvement and 60 % much improvement. The average perceived improvement by the participants for skin treated with the "AWP-P" compared to both skin left untreated and skin treated with the control was regarded as a slight improvement. The perceived improvement for "AWP-P" treated skin compared to

untreated skin was less than that recorded for "AWP-N" treated skin (on average recorded a moderate degree of improvement from day 56 for the remainder of the study period), as seen below in Figure 15. There was, however, a higher perceived improvement for the "AWP-P" treated skin over the control compared to "AWP-N" treatment. Participant perception of slight improvement may be the cause of the reduction in participant compliance in the middle of the study, due to a limited satisfaction in the efficacy of the "AWP-P". There is a link between a satisfactory experience and an enhanced tendency to repeat the action [47]. Therefore, participants are more inclined to comply with the method protocol if they receive positive reinforcements by either noticing an improvement themselves and / or associates commenting on a noticed improvement.

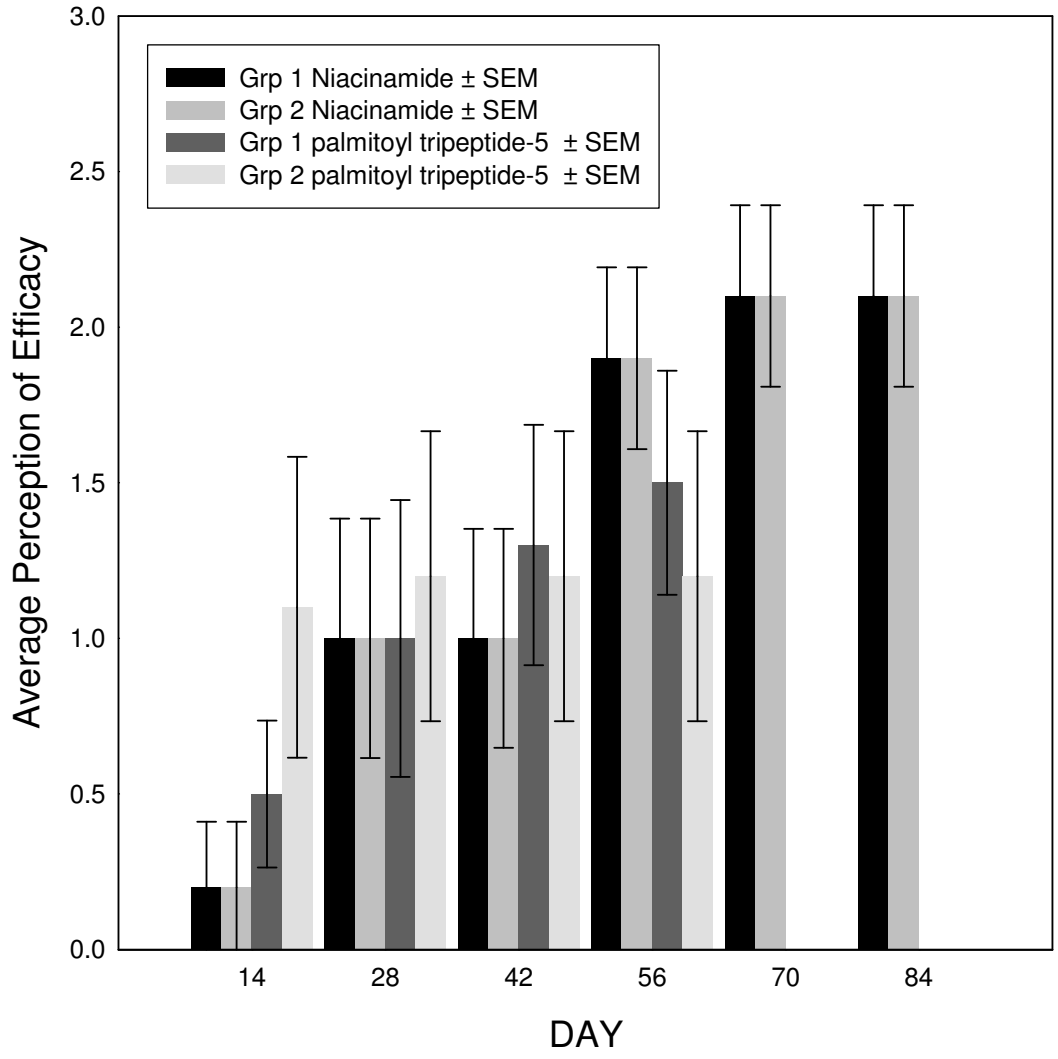

Figure 15. Participant perceived efficacy results for "AWP-P" treated versus "AWP-N" treated. Each bar represents the mean of the perceived improvement of the participants ± SEM.

6.4.2.4 Digital Photography

The assessment of change in wrinkle appearance caused by the anti-wrinkle formulation or the anti-wrinkle agent was conducted by capturing and analysing digital photographs for change over time, as per method described in Chapter Five Section 5.3.3.1. The photographs were graded on a 7-point scale at each evaluation day

compared to day 0, by the investigator and an independent assessor. The results are shown below in Figures 16 and 17, for groups one and two respectively.

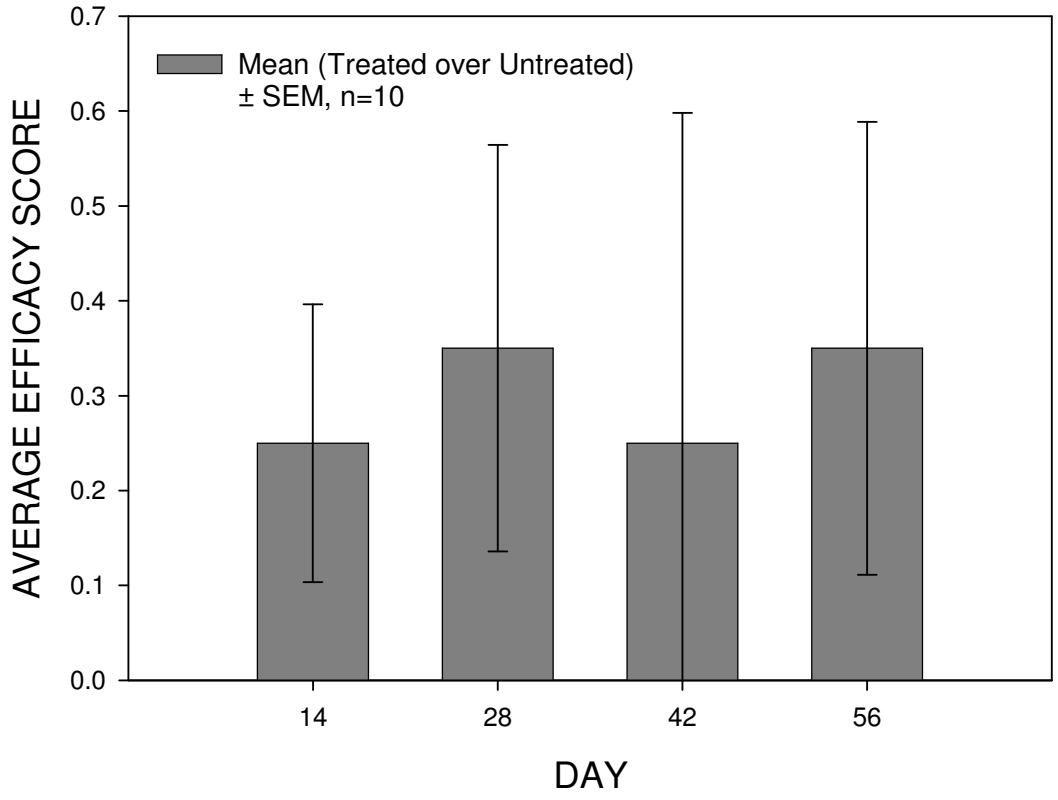

Figure 16. Photographic assessment results for "AWP-P" treated versus untreated skin. Each bar represents the mean efficacy score of the treated skin minus the untreated skin, therefore, results given are the improvement of the treatment over skin left untreated ± SEM, n = 10.

Skin treated with the "AWP-P" was shown to have a significant improvement over untreated skin over the entire study period, $p = 0.013$, using a Wilcoxon Signed Rank Test. Results at day 42 are lower than expected when examining the trend of the data as a whole. This may be caused by a higher degree of variation as demonstrated by the large error bars at this time point. The variation may be a reflection of the climatic variations over that fortnight i.e. high temperatures and strong winds coupled with air-conditioning effects, which can cause the skin to dry out and result in wrinkle variations

[48]. As there is only a small change in the average efficacy scores recorded over the study period, variability makes it more difficult to detect real effects caused by the "AWP-P".

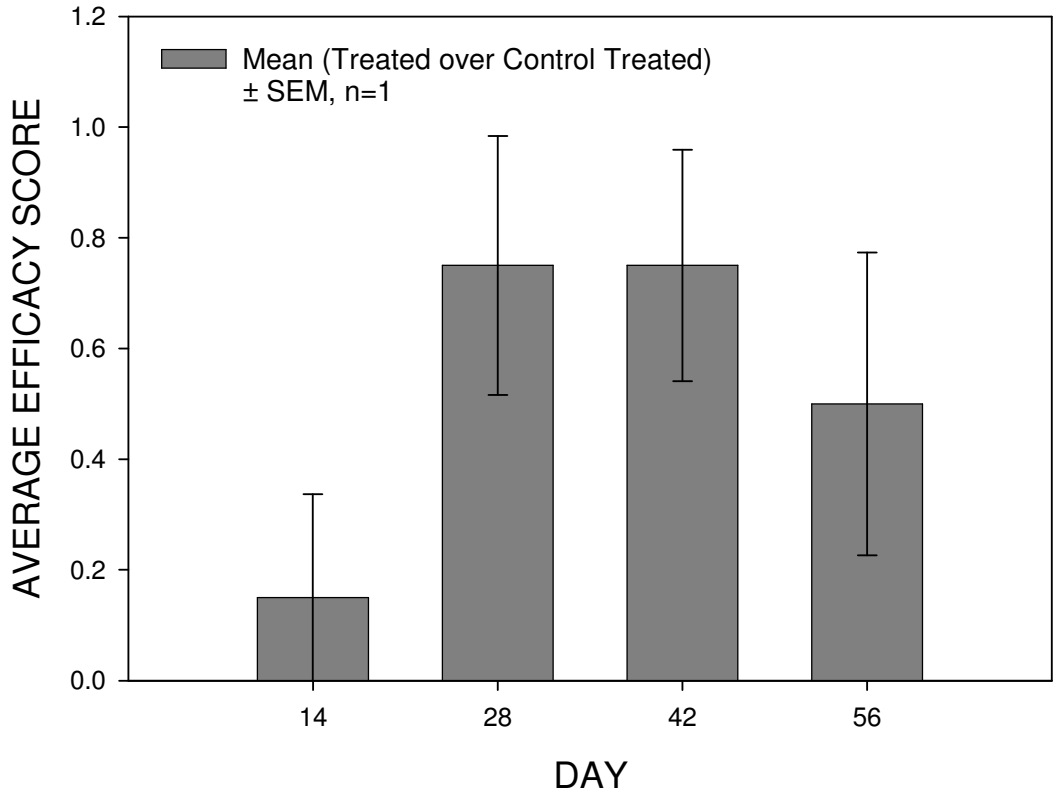

Figure 17. Photographic assessment results for "AWP-P" treated versus control treated skin. Each bar represents the mean efficacy score of the treated skin minus skin treated with the control, therefore, results given are the improvement of the treatment over control treatment ± SEM, n = 10.

Overall treatment with the "AWP-P" resulted in a significant improvement compared to skin treated with the control product, $p = 0.000$. Statistical significance in "AWP-P" treated skin over the control treated skin was recorded on day 42, $p = 0.04$. Figure 18 is an example of a participant in the clinical study exhibiting visible improvement in wrinkle appearance caused by the "AWP-P" over the study period.

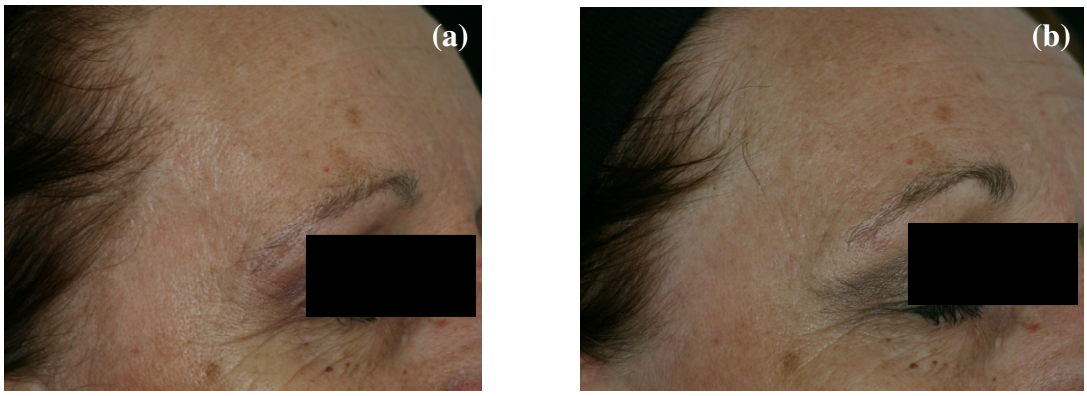

Figure 18. Participant right side from group two at (a) baseline and (b) after 5 weeks of treatment with the "AWP-P" containing 1 % palmitoyl tripeptide-5.

A slight improvement was seen in the photographic assessment of "AWP-P" treated skin compared to both skin left untreated or skin treated with the control product. Individual participants responded well to the anti-wrinkle benefits of this "AWP-P", however, this was not consistent over the study population. The collated results demonstrate a large degree of variability. It would, therefore, be beneficial to increase the sample size of the study population.

6.4.2.5 Silicone Skin Replication Technique

As stated previously changes in wrinkle size and number of wrinkles was determined as a comparison of each individual, compared to their baseline (day 0) for each side of the participants face.

6.4.2.5.1 Roughness

The average roughness for each side of each participant's face, at each evaluation day, is presented as a percentage different compared to day 0. The results are

shown below in Figures 19 and 20 for groups one and two respectively, are a function of the average percentage difference of all participants at each evaluation day.

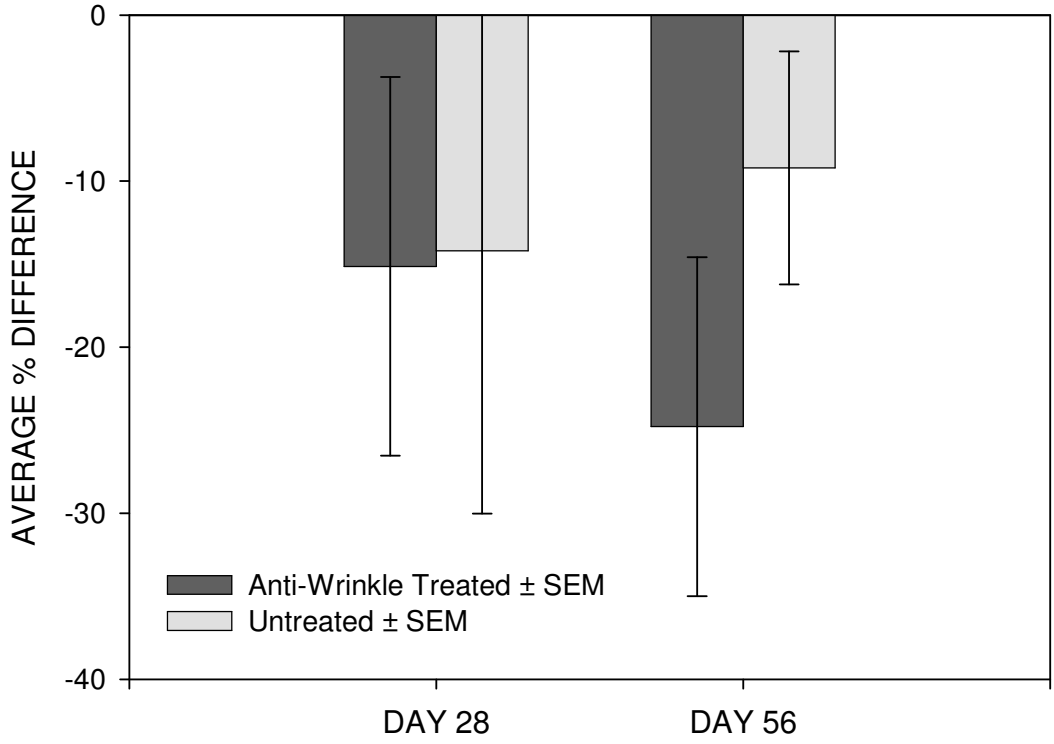

Figure 19. Skin roughness results for "AWP-P" treated compared to untreated skin. Each point represents the mean percentage difference of each participant compared to their baseline ± SEM, n = 10.

At day 56, "AWP-P" treated skin was shown to significantly reduce skin roughness compared to skin left untreated, p=0.034, using a Paired Sample T Test, which was also significantly reduced compared to day 0, using a One Sample T Test. No difference was recorded for skin treated with the "AWP-P" at day 28 compared to either untreated skin or day 0. The average roughness after 56 days was reduced by 24.78 ± 10.21 %.

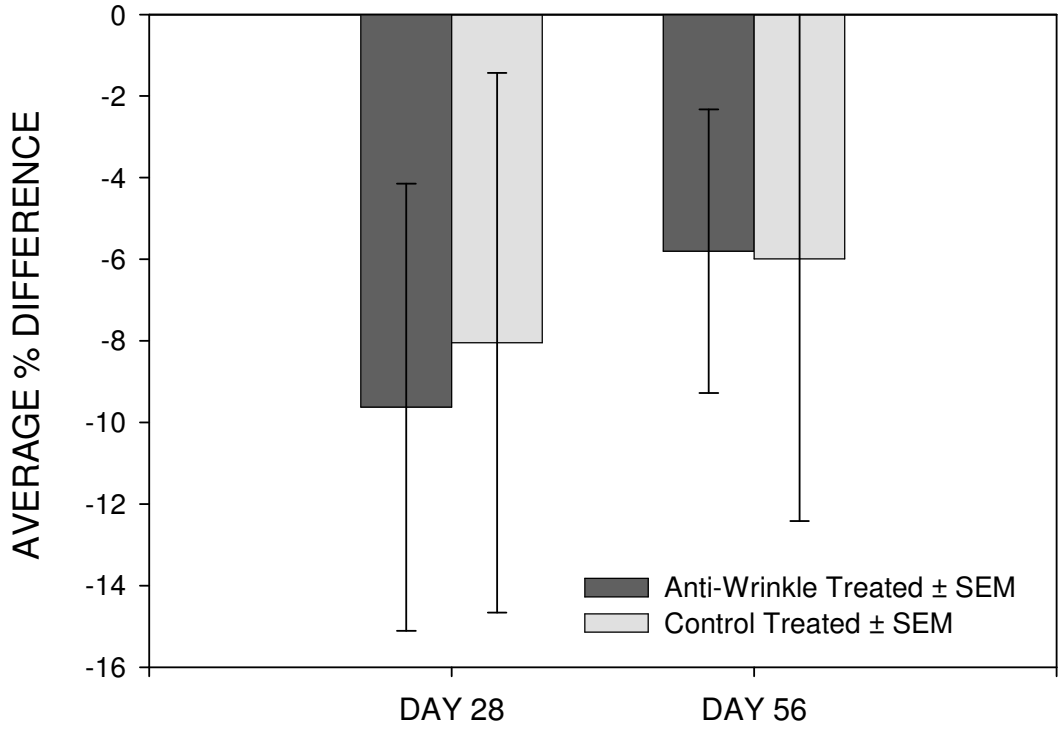

Figure 20. Skin roughness results for "AWP-P" treated compared to control treated skin mean percentage difference of each participant compared to their baseline ± SEM, n = 10.

Overall at day 28, "AWP-P" treated skin showed significant improvement from day 0, $p = 0.048$. There was, however, no significant difference between treated and control treated skin at day 28 or day 56.

Microscopic assessment of skin roughness, over the 56 day study period, indicated the "AWP-P" has the potential to reduce the size of facial wrinkles. It is highly likely, however, that this anti-wrinkle behaviour was not specifically caused by palmitoyl tripeptide-5 itself but the vehicle.

6.4.2.5.2 Wrinkle Number

Wrinkle number was calculated from the total number of peaks analysed per slice for the same side of each participant's face. The results, presented below in Figures

21 and 22 for groups one and two respectively, are expressed as an average of all participants in each group as a percentage difference compared to day 0 of the average number of wrinkles analysed at each evaluation day.

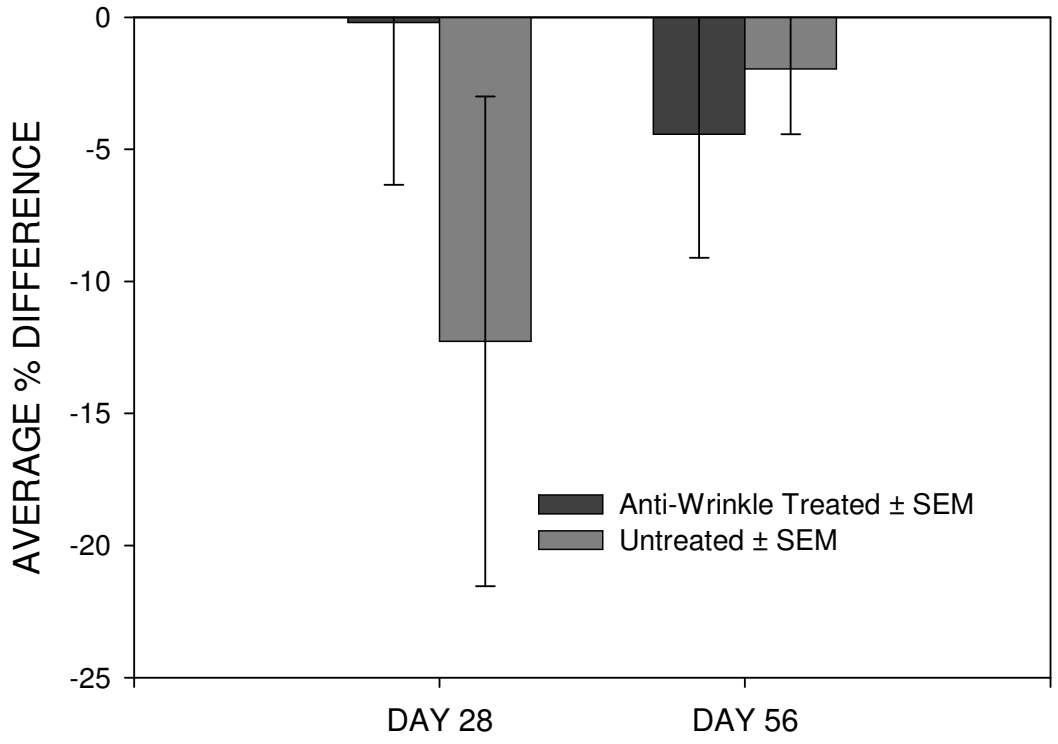

Figure 21. Wrinkle number results for "AWP-P" treated compared to untreated skin, the mean percentage difference in the number of measured peaks in the analysis area of each participant compared to their baseline ± SEM, n = 10.

Overall both the "AWP-P" treated skin and skin left untreated showed no difference in the number of wrinkles compared to day 0 at both day 28 and day 56.

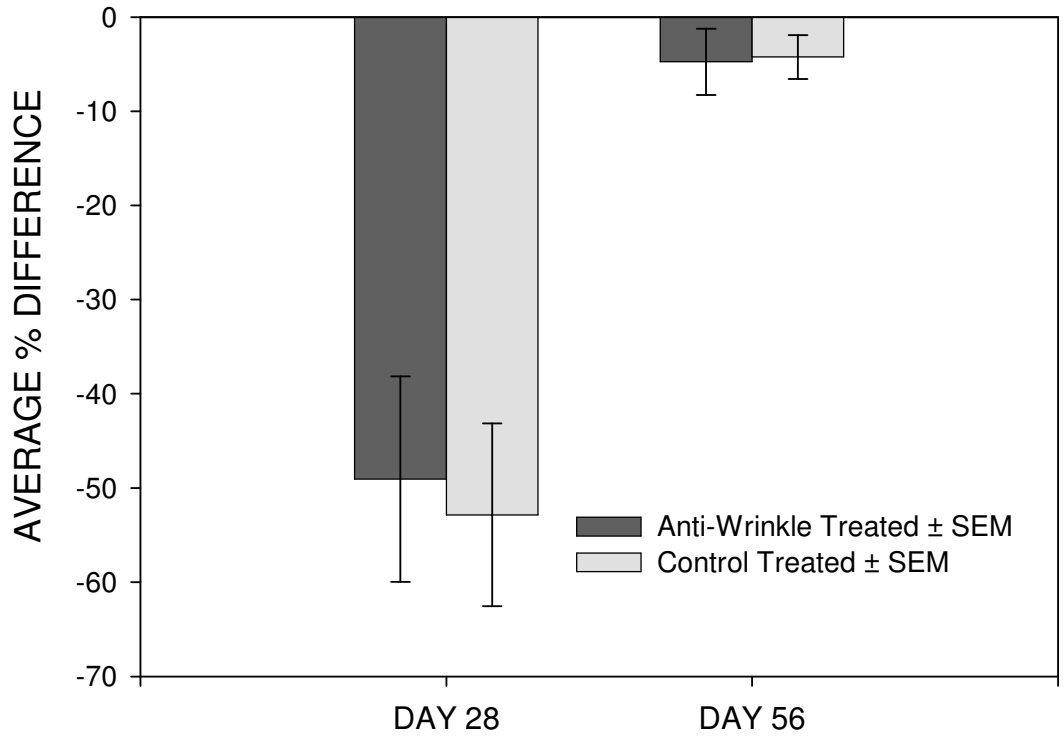

Figure 22. Wrinkle number results for "AWP-P" treated compared to control treated skin, the mean percentage difference in the number of measured peaks in the analysis area of each participant compared to their baseline ± SEM, n = 11.

Both the skin treated with the "AWP-P" and the control product showed a significant improvement in the number of wrinkles over day 0, at days 28 and 56, $p = 0.004$ and $p = 0.000$ respectively. There was, however, no notable difference between "AWP-P" treated and control treated skin at day 28 or day 56. The large variation in the number of wrinkles in group two at day 28 compared to day 56 is likely to be a reflection of the numerous factors that might alter the appearance of dynamic facial wrinkles to varying degrees on a daily basis. Such factors may include: sun exposure, extremes in weather (e.g. wind and cold), anxiety, pain, headache and smoking [49-52], which could not be controlled throughout the study. It has been shown that the size of wrinkles is significantly increased by the end of the day compared to the morning. This is caused by repeated facial movements from various expressions during the day, which

may increase the severity of the wrinkles [50]. To reduce the effect of this problem the impression moulds were taken at the same time of day. Participant's eyes were closed while the moulds were taken to relax their face, allowing their wrinkles and their facial expression to retain comparability to baseline.

Change in the number of wrinkles over the study period for skin treated with the "AWP-P" compared to skin left untreated or treated with the control product suggests that, although the "AWP-P" has been shown to reduce the size of facial wrinkles *in vivo*, it has little to no effect on reducing the number of wrinkles for the participant.

It is notable that the Visual Image Analysis results for group two were not consistent with the Silicon Skin Replication results. A significant improvement was demonstrated with regards to the visible improvement in wrinkle appearance for skin treated with the "AWP-P" compared to skin treated with the control. However, this was not confirmed in the skin roughness results. The inconsistency between these results is likely to be a function of the different analysis techniques. The Visual Image Analysis method can take into account factors not measured by the Silicone Skin Replication technique. Even though the depth of wrinkles and the number of wrinkles may not have changed, the skin may be smoother and therefore this would result in a visual improvement, even if this was not objectively measured.

6.5 Conclusion

The "AWP" containing 5 % niacinamide was found to:

- Significantly improve the visual appearance of dynamic wrinkles compared to skin left untreated, however, no difference was seen over control treated skin.

The visible improvement in the appearance in skin wrinkles was, therefore, likely to be caused by the "AWP-N" vehicle rather than by the niacinamide.

- Significantly reduce skin roughness compared to untreated skin, an effect caused by the "AWP-N" vehicle.

- Significantly reduce skin roughness and the number of wrinkles compared to the control. Niacinamide was found to reduce the size and number of wrinkles compared to the vehicle.

While niacinamide was found not to cause skin irritation, the results suggest it may be responsible for reducing irritation caused by other components in the "AWP-N". Participant irritation forms revealed the "AWP-N" did cause some skin irritation. This irritation may have been caused by alcohol and pineapple extract [2]. The presence of retinyl palmitate also raised safety concerns as it has been shown to cause reproductive and developmental issues at low doses in animal studies [39], therefore pregnant women were excluded from this clinical study.

The niacinamide clinical study results, with support from the niacinamide finite permeation study results of the formulation used i*n vivo* (results shown in Chapter Three Section 3.4.1.3) suggest it is possible niacinamide is penetrating the stratum corneum, reaching its site of action (the dermis) and exerting its anti-wrinkle effect through the proposed mechanisms of action (i.e. collagen regeneration and antioxidant behaviour).

The "AWP-P" containing 1 % palmitoyl tripeptide-5 solution was found to:

- Not cause any skin irritation

- Significantly improve the visual appearance of dynamic wrinkles compared to skin left untreated and also skin treated with the control.

- Significantly reduce skin roughness compared to skin left untreated but not compared to control treated skin. The improvement in the size of wrinkles was, therefore, caused by the "AWP-P" vehicle and not the palmitoyl tripeptide-5.

The "AWP-P" split face clinical study demonstrated that the "AWP-P" caused a reduction in the visible appearance and to a minor degree, size of dynamic facial wrinkles. Palmitoyl tripeptide-5 was shown to cause a visible improvement in wrinkle appearance. However, the improvement was not translated into a quantitative reduction in the size and number of facial wrinkles. Based on efficacy and finite dose permeation studies, shown in Chapter Three Section 3.4.3.1, it is highly likely the anti-wrinkle efficacy is caused by superficial surface effects rather than the claimed collagen regeneration mechanism reported.

The anti-wrinkle products including both niacinamide and palmitoyl tripeptide-5 contain ingredients, other than those extensively examined in this research, that may be responsible for causing an anti-wrinkle effect. Ingredients include: moisturisers, which add water to hydrate the skin; humectants, which absorb water and hold it in the skin with one example being glycerin that is in both anti-wrinkle products; and emollients, which lubricate and slow down the loss of water from the skin, such as almond, crambe, olive and carrot oils for "AWP-N" and caprylic / capric triglyceride, dicaprylyl carbonate and stearic acid for "AWP-P" [1].

Both anti-wrinkle products also include a number of active ingredients claiming more substantial anti-wrinkle mechanisms of action. For the "AWP-N" other active ingredients include: retinyl palmitate (antioxidant and skin cell regulator [2, 7-10]),

tocopheryl acetate (antioxidant and skin conditioning agent [2-3, 10-12]), ascorbyl palmitate (antioxidant and collagen regeneration [13-14]) and pea extract (skin tension, moisturising and firming [20]). For the "AWP-P" other active ingredients include: sodium ascorbyl phosphate (antioxidant and collagen regeneration [14, 21-22]), punica granatum extract (antioxidant when taken orally [23-24]), crithmum maritimum extract (antioxidant [25]), mushroom extract (antioxidant [26]), ceramide 2 (restore skin's barrier function [27-29]), palmitoyl oligopeptide (stimulate collagen production and encourage production of intercellular matrix substances [2]), glycine soja (soybean) sterols (mimics estrogen [2]) and copper gluconate (antioxidant, collagen and elastin synthesiser [31-35]). It can be assumed these ingredients are responsible for the improvement recorded for both the niacinamide and the palmitoyl tripeptide-5 control products with respect to visible appearance, size and number of wrinkles examined over this research.

6.6 References

1. Urquhart, N., (2002) Formulation of Cosmetic Preparations, Undergraduate Lecture Formulation Science. Parkville, Australia. Personal Communication.
2. Paula's Choice, Cosmetic Ingredients Dictionary, Wausa, USA. Available At: http://www.cosmeticscop.com/cosmetic-ingredient-dictionary.aspx. Accessed March 2010.
3. Specialchem Inci Directory. Available At: http://www.specialchem4cosmetics.com/services/inci/index.aspx. Accessed: March 2010.
4. Bissonnette, R., (1997) Update on Sunscreens. Skin Therapy Letter **2**.
5. Henz, B.M., et al., (1999) Double-Blind, Multicentre Analysis of the Efficacy of Borage Oil in Patients with Atopic Eczema. *Br J Derm,* **140**: 685-688.
6. Worm, M. et al., (2000) Novel Unconventional Therapeutic Approaches to Atopic Eczema. *Derm*, **201**: 191-195.
7. Duell, E.A., et al., (1997) Unoccluded Retinol Penetrates Human Skin *In Vivo* more Effectively than Unoccluded Retinyl Palmitate or Retinoic Acid. *J Invest Derm*, **109**: 301-305.
8. Ellis, C.N. et al., (2001) Uses and Complications of Isotretinoin Therapy. *J Am Acad Derm*, **45**: S150-S157.
9. Maddin, S., et al., (2000) Isotretinoin Improves the Appearance of Photodamaged Skin: Results 36-Week, Multicenter, Double-Blind, Placebo-Controlled Trial. *J Am Acad Derm*, **42**: 56-63.

10. Kerscher, M., (2003) Dermocosmetics: Cosmetics and Skin Care from Dermatological View- Current Trends in Dermocosmetics. Skin Care Forum **35**. Available at http://www.scf-online.com. Internet Access Jan 2010

11. Baumann, L., (2005) How to Prevent Photoaging? *J Invest Derm*, **125**: xii-xiii.

12. Chiu, A. et al., (2003) Topical Vitamins, Minerals and Botanical Ingredients as Modulators of Environmental and Chronological Skin Damage. *Br J Derm*, **149**: 681-691.

13. Perricone, N., et al., (1999) The Hydroxyl Free Radical Reactions of Ascorbyl Palmitate as Measured in Various *In Vitro* Models. *Biochem Biophys Res Comm*, **262**: 661-665.

14. Davidson, J.M., et al., (1997) Ascorbate Differentially Regulates Elastin and Collagen Biosynthesis in Vascular Smooth Muscle Cells and Skin Fibroblasts by Pretranslational Mechanisms. *J Biol Chem*, **272**: 345-352.

15. Bissett, D.L., et al., (2004) Topical Niacinamide Reduces Yellowing, Wrinkling, Red Blotchiness, and Hyperpigmented Spots in Aging Facial Skin. *Int J Cosmet Sci*, **26**: 231-238.

16. Bissett, D.L., et al., (2005) Niacinamide: A B Vitamin that Improves Aging Facial Skin Appearance. *Derm Surg*, **31**: 860-865.

17. Tanno, O., et al., (2000) Nicotinamide Increases Biosynthesis of Ceramides as well as other Stratum Corneum Lipids to Improve the Epidermal Permeability Barrier. *Br J Derm*, **143**: 524-531.

18. Bulk Actives Ingredients for Skin Care, Keelung City, Taiwan Available At: http://www.bulkactives.com. Accessed: March 2010.

19. Environmental Working Group, Skin Deep Cosmetic Safety Database. Available At: http://www.cosmeticsdatabase.com/ingredient. Accessed March 2010.

20. Active Ingredients in Skin Care Cosmetics, Essential Day Spa, Vancouver Canada. http://www.essentialdayspa.com. Accessed: March 2010.

21. Savini, I., et al., (2002) Characterization of Keratinocyte Differentiation Induced by Ascorbic Acid: Protein Kinase C Involvement and Vitamin C Homeostasis. *J Invest Derm,* **118**: 372-379.

22. Nusgens, B.V., et al., (2001) Topically Applied Vitamin C Enhances the MRNA Level of Collagens I and III, their Processing Enzymes and Tissue Inhibitor of Matrix Metalloproteinase 1 in the Human Dermis, **116**: 853-859.

23. Singh, R.P., et al., (2001) Studies on the Antioxidant Activity of Pomegranate (Punica Granatum) Peel and Seed Extracts Using *In Vitro* Models. *J Agr Food Chem*, **50**: 81-86.

24. Noda, Y., et al., (2001) Antioxidant Activities of Pomegranate Fruit Extract and Its Anthocyanidins: Delphinidin, Cyanidin, and Pelargonidin. *J Agr Food Chem*, **50**: 166-171.

25. Cunsolo, F., et al., (1993) Bioactive Metabolites from Sicilian Marine Fennel, Crithmum Maritimum. *J Natural Products*, **56**: 1598-1600.

26. Mau, J-L., et al., (2002) Antioxidant Properties of Several Medicinal Mushrooms. *J Agr Food Chem*, **50**: 6072-6077.

27. Choi, M.J., et al., (2005) Role of Ceramides in Barrier Function of Healthy and Diseased Skin. *Am J Clin Derm*, **6**: 215-219.

28. Proksch, E., et al., (2006) Skin Barrier Function, Epidermal Proliferation and Differentiation in Eczema. *J Derm Sci*, **43**: 159-169.

29. Geilen, C.C., et al., (2001) Sphingolipid Signaling in Epidermal Homeostasis. *Skin Pharmacol Physiol*, **14**: 261-271.

30. Nielsen, N.S., et al., (2002) Different Effects of Diets Rich in Olive Oil, Rapeseed Oil and Sunflower-Seed Oil on Postprandial Lipid and Lipoprotein Concentrations and on Lipoprotein Oxidation Susceptibility. *Br J Nutrition*, **87**: 489-499.

31. Tenaud, I., et al., (1999) *In Vitro* Modulation of Keratinocyte Wound Healing Integrins by Zinc, Copper and Manganese. *Br J Derm*, **140**: 26-34.

32. Maquart, F.X., et al., (1993) *In Vivo* Stimulation of Connective Tissue Accumulation by the Tripeptide-Copper Complex Glycyl-L-Histidyl-L-Lysine-Cu2+ in Rat Experimental Wounds. *J Clin Invest*, **92**: 2368-2376.

33. Yasuhara, Y., et al., (2005) Protective Effect of Metallogluconates against UVA-Induced Cutaneous Lesion in HR-1 Hairless Mice. *Biomed Res Trace Elements*, **16**: 302-305

34. Wegrowski, Y., et al., (1992) Stimulation of Sulfated Glycosaminoglycan Synthesis by the Tripeptide-Copper Complex Glycyl-L-Histidyl-L-Lysine-$Cu2^{+}$. *Life Sciences*, **51**: 1049-1056.

35. Pickart, L. (2002) Copper-Peptides for Tissue Regeneration. Speciality Chemicals Magazine Vol. 1347: Bellevue, USA

36. Parish, W.E., et al., (2005) Changes in Basal Cell Mitosis and Transepidermal Water Loss in Skin Cultures Treated with Vitamins C and E. *Exp Derm*, **14**: 684-691.

37. Ekanayake-Mudiyanselage, S., et al., (2005) Vitamin E Delivery to Human Skin by a Rinse-Off Product: Penetration of α-Tocopherol Versus Wash-out Effects of Skin Surface Lipids. *Skin Pharmacol Physiol*, **18**: 20-26.

38. Eberlein-König, B. et al., (2005) Relevance of Vitamins C and E in Cutaneous Photoprotection. *J Cosmet Derm*, **4**: 4-9.

39. Gellert, R.J., (1977) Inhibition of Cyclic Ovarian Activity in Rats Treated Chronically with Vitamin A. *J Reprod Fertil*, **50**: 223-229.

40. Fox, S.I., (2002) Cardiac Output, Blood Flow, and Blood Pressure, in Human Physiology, S.I. Fox, Editor., McGraw-Hill: New York. 429-431.

41. Gehring, W., (2004) Nicotinic Acid/ Niacinamide and the Skin. *J Cosmet Derm*, **3**: 88-93.

42. Bruno, L., et al., (2003) Recessive X-Linked Ichthyosis Associated with Hypertrophic Pyloric Stenosis: A Chance Occurrence? *Clinical & Exper Derm*, **28**: 74-76

43. Ronis, D.L., et al., (1989) Attitudes, Decisions and Habits, in Attitute Structure and Function. Editors: Pratkanis, A.R., Breckler, S.J., and Greenwald, A.G., Lawrence Erlbaun Associates Inc. : New Jersey, USA 48-49.

44. De Deyn, P.P., et al., (1996) Placebos in Clinical Practice and Research. *J Med Ethics*, **22**: 140-146.

45. Foddy, B., (2009) A Duty to Deceive: Placebos in Clinical Practice. *Am J Bioethics*, **9**: 4-12.

46. Vernillo, A., (2009) Placebos in Clinical Practice and the Power of Suggestion. *Am J Bioethics*, **9**: 32-33.

47. Aarts, H., et al., (1997) Physical Exercise Habit: On the Conceptualization and Formation of Habitual Health Behaviours. *Health Educ Res*, **12**: 363-374.

48. Rohr, M., et al., (2005) Climatic Influence on Cosmetic Skin Parameters, in Textbook of Cosmetic Dermatology. 3rd Edition, Editors: Baran, R. and Maibach, H.I., Taylor & Francis: Oxon, United Kingdom.

49. Hatzis, J., (2004) The Wrinkle and its Measurement: A Skin Surface Profilometric Method. *Micron*, **35**: 201-219.

50. Tsukahara, K., et al., (2004) A Study of Diurnal Variation in Wrinkles on the Human Face. *Arch Derm Res*, **296**: 169-174.

51. Aizen, E. et al., (2001) Smoking Effect on Skin Wrinkling in the Aged Population. *Int J Derm*, **40**: 431-433.

52. Kang, S., et al., (2005) A Multicenter, Randomized, Double-Blind Trial of Tazarotene 0.1 % Cream in the Treatment of Photodamage. .*J Am Acad Derm*, **52**: 268-274.

Chapter Seven

General Discussion

7.1 Overall Discussion

The core objectives of this research were to: evaluate the penetration of a number of commonly used anti-wrinkle ingredients through skin; based on the penetration studies, select commercially available products that contained one or more of the ingredients tested to investigate their anti-wrinkle efficacy *in vivo*; and finally, evaluate the correlation between *in vitro* penetration studies and effects seen *in vivo.*

Three anti-wrinkle ingredients were selected for penetration investigation using modified flow-through diffusion cells these included niacinamide, genistein and palmitoyl tripeptide-5. Ingredient selection was based on: extensive commercial use of the ingredient in cosmetic products as an anti-wrinkle ingredient, variety in the mechanism of action at which the ingredients cause an anti-wrinkle effect, the ability to develop a RP-HPLC detection method for quantitative analysis of the ingredient (due to equipment availability) and, where possible, the ingredient was to have favourable physicochemical properties for percutaneous penetration.

Penetration studies, presented in Chapter Three, demonstrate that genistein penetrates the stratum corneum slowly under the conditions used. An average maximum of 0.4163 ± 0.07029 % of the applied dose of a simple cream containing 0.5 % genistein penetrated in 24 hrs. Whether this will result in the desired anti-aging effect will depend heavily on the potency of genistein. The estrogenic potency of genistein has been shown to be ~1/10,000 and 1/50,000 that of estriol and estradiol respectively [1]. Also, 0.3 % estriol and 0.01 % estradiol has been shown to reduce wrinkle depth and increase the moisture, elasticity and firmness of the skin [2]. Based on the low potency of genistein, and compared to the effective concentrations of estriol and estradiol, it is unlikely genistein would produce a reduction in the appearance, size and number of facial

wrinkles at a concentration commonly used in commercial products. The use of higher concentrations of genistein would also become cost prohibitive.

Niacinamide was found to permeate the skin well, resulting in a flux from propylene glycol of 3.773 ± 0.5138 µg/cm^2/hr across human skin *in vitro*, indicating it has the potential to reach its proposed site of action in significant concentrations. Therefore, niacinamide has the potential to elicit its anti-wrinkle mechanisms of action such as generating an antioxidant effect and stimulating: collagen synthesis, epidermal cell growth and epidermal lipid production [3-5]. Niacinamide was selected for further investigation of anti-wrinkle efficacy *in vivo* due to the positive results with regards to its penetration potential. Penetration from the cream used for the *in vivo* clinical study of niacinamide resulted in 10.147 ± 1.0845 % of the applied amount penetrating after 24 hrs.

In vivo clinical investigation, shown in Chapter Six Section 6.4.1, demonstrated the cream ("AWP-N") containing 5 % niacinamide significantly improved the visual appearance of dynamic wrinkles compared to skin left untreated, no difference, however, was seen over skin treated with the base cream without niacinamide. It was, therefore, concluded that visible improvement in the appearance of wrinkles was caused by the "AWP-N" vehicle rather than by the niacinamide. The "AWP-N" significantly reduced skin roughness compared to untreated skin, an effect caused by the "AWP-N" vehicle. Finally, inclusion of niacinamide was found to reduce the size and number of wrinkles compared to the control product, the "AWP-N" excluding niacinamide. The niacinamide clinical study results, with support from the niacinamide finite permeation study results of the *in vivo* formulation, suggests it is possible that niacinamide is penetrating the stratum corneum, reaching its site of action (the dermis) and causing an anti-wrinkle effect such as collagen regeneration.

Palmitoyl tripeptide-5 is reported to have an anti-wrinkle mechanism of action by inducing a release of active TGF-β, which can lead to the production of collagen [6], *in vivo* this must occur within the dermis. Based on the chemical structure of palmitoyl tripeptide-5 it is unlikely to penetrate the stratum corneum. Palmitoyl tripeptide-5 possesses a MW of 611.90 Da [7], which is larger than the optimum MW for penetration, less than 500 Da [8]. It also contains many hydrogen bonding groups, which is generally inhibitory to penetration. While the tripeptide is predicted not to penetrate the skin well a definitive answer can only be obtained by testing the penetration experimentally. Compounds that possess unfavourable physico-chemical properties for percutaneous penetration have been shown to penetrate the stratum corneum; for example tacrolimus, which is used to treat eczema. Tacrolimus possesses a MW of 822.05 Da and is highly loaded with hydrogen bonding groups. It is has been shown, however, to penetrate both normal and dermatitis effected human skin [9-10] and is effective at treating eczema [11]. Based on this evidence it is, therefore, worth investigating the potential permeation of ingredients claiming mechanisms within the dermis. Under permeation investigation in this research, palmitoyl tripeptide-5, demonstrated an inability to penetrate or permeate the stratum corneum. It is, therefore, unlikely palmitoyl tripeptide-5 will reach its site of action within the dermis and is highly unlikely to be able to activate the production of collagen. Although palmitoyl tripeptide-5 showed poor penetration potential it was still used for further *in vivo* anti-wrinkle efficacy investigation. Rather than the reported mechanism of action, collagen regeneration, *in vivo* efficacy studies were used to investigate the potential for palmitoyl tripeptide-5 to superficially reduce the appearance, size and number of facial wrinkles.

The palmitoyl tripeptide-5 split face clinical study shown in Chapter Six Section 6.4.2 demonstrated the "AWP-P", containing 1 % palmitoyl tripeptide-5 solution and

other potential anti-wrinkle ingredients, was found to improve the visual appearance of dynamic wrinkles compared to skin left untreated and skin treated with the control. It was, therefore, concluded that the "AWP-P" vehicle caused a reduction in the visible appearance and to a minor extent size of dynamic facial wrinkles, while palmitoyl tripeptide-5 caused a visible improvement in wrinkle appearance. Based on finite dose permeation studies, shown in Chapter Three Section 3.4.3.1, it is highly likely the anti-wrinkle efficacy observed for palmitoyl tripeptide-5 was caused by superficial surface effects rather than the claimed collagen regeneration mechanism. However, the improvement in wrinkle appearance is not a consequence of a quantitative reduction in size and number of facial wrinkles.

7.2 Suggestions for Future Research

Research presented in Chapter Four has comprehensively characterised the autofluorescence of the junctions between corneocytes for a number of skin thicknesses. The research demonstrates the potential of fluorescence lifetime imaging by TCSPC as a method for monitoring the transdermal diffusion pathway and diffusion rate of topically applied compounds in human skin. The method utilises a change in the fluorescence lifetime within intracorneocyte junctions to determine the presence of topically applied non fluorescent compounds, such as genistein, niacinamide or palmitoyl tripeptide-5. Based on this preliminary research further investigation is worth pursuing. As this method is able to observe what is happening within the skin throughout the penetration period, we would be able to gain a better understanding of the penetration process and may also gain a more accurate method of monitoring diffusion rates through the skin. The study also indicated the potential for further *in*

vitro and *in vivo* investigation on compounds claiming collagen regeneration. As this highly sensitive method was able to provide well detailed images of collagen ribbons, a change in the mass of the ribbons could be used to identify what effect a test formulation may have on collagen regeneration over time.

In vivo investigation of a 'AWP-P" containing 1 % palmitoyl tripeptide-5 solution demonstrated that while palmitoyl tripeptide-5 causes a visible reduction in wrinkle appearance it is highly unlikely this is caused by stimulation of collagen production. Further research could be conducted to determine the mechanism(s) responsible for this anti-wrinkle effect. Possible mechanisms of action responsible for visible improvement in facial wrinkles caused by palmitoyl tripeptide-5 could include: improving the barrier function of the skin, preventing water loss from the skin surface; acting as a humectant (possessing the ability to absorb water and hold it in the skin – this is common for high MW hydrophilic polymers) [12]; increasing skin surface lipids [13]; and acting as an emollient (providing partial occlusion causing lubrication and moisturisation) [14]. The following methods could be utilised to assess these mechanisms of action: the use of a hydrometer (i.e. Corneometer®) or a Nova Dermal Phase Meter® for measuring the relative state of skin hydration *in vivo* through electrical measurements (i.e. capacitance and conductance [14-16]); measuring the amount of superficial skin lipids [13]; and measuring the transepidermal water loss (TEWL) with an evaporimeter, indicating the barrier function capacity of the stratum corneum *in vivo* [17]. An improvement in the barrier function of the skin caused by palmitoyl tripeptide-5 will result in a reduced amount of water eliminated by evaporation leading to an increase moisturisation of the skin surface.

The *in vivo* clinical studies presented in this research are small pilot studies with only 20 participants per study. Although the results presented provided an indication of

the likely efficacy of the two test products containing niacinamide and palmitoyl tripeptide-5, larger scale clinical studies would allow for a more accurate investigation of the ability of the test products at reducing the appearance, size and number of facial wrinkles. With a larger sample group there is greater confidence that the results are a true indication of the product efficacy for the general consumer rather than participant variability. The use of a more automated quantitative analysis system, i.e. optical, mechanical, laser, transmission, interference fringe and electrical profilometry [18-21], would increase the number of participants and also the number of sample points per study able to be analysed.

7.3 References

1. Shemesh, M., et al., (1972) Affinity of Rabbit Uterine Oestradiol Receptor for Phytoestragens and its use in a Competitive Protein Binding Radioassay for Plasma Coumestrol. *J Reprod Fertil*, **29**: 1-9.
2. Schmidt, J.B., et al., (1994) Treatment of Skin Ageing Symptoms in Perimenopausal Females with Estrogen Compounds. A Pilot Study. *Maturitas*, **20**: 25-30.
3. Bissett, D.L., et al., (2004) Topical Niacinamide Reduces Yellowing, Wrinkling, Red Blotchiness, and Hyperpigmented Spots in Aging Facial Skin. *Int J Cosmet Sci*, **26**: 231-238.
4. Bissett, D.L., et al., (2005) Niacinamide: A B Vitamin that Improves Aging Facial Skin Appearance. *Derm Surg*, **31**: 860-865.
5. Tanno, O., et al., (2000) Nicotinamide Increases Biosynthesis of Ceramides as well as other Stratum Corneum Lipids to Improve the Epidermal Permeability Barrier. *Br J Derm*, **143**: 524-531.
6. DSM Nutritional Products Ltd a Branch of Pentapharm Syn®Coll Boost and Protect Collagen to Iron out Wrinkles. Available At: www.pentapharm.com. Accessed Dec 2008
7. Xiamen Bj Chem. Technology Co. Ltd. China. Cosmetic Ingredients. Available At: http://www.bjchemicals.com. Accessed: Feb 2010.
8. Lipinski, C.A., et al., (1997) Experimental and Computational Approaches to Estimate Solubility and Permeability in Drug Discovery and Development Settings. *Adv Drug Del Reviews*, **23**: 3-25.

9. Billich, A., et al., (2004) Percutaneous Absorption of Drugs used in Atopic Eczema: Pimecrolimus Permeates Less through Skin than Corticosteroids and Tacrolimus. *Int J Pharm*, **269**: 29-35.

10. Meingassner, J.G., et al., (2005) Pimecrolimus Permeates Less than Tacrolimus through Normal, Inflamed, or Corticosteroid-Pretreated Skin. *Exp Derm*, **14**: 752-757.

11. Ashcroft, D.M., et al., (2005) Efficacy and Tolerability of Topical Pimecrolimus and Tacrolimus in the Treatment of Atopic Dermatitis: Meta-Analysis of Randomised Controlled Trials. *BMJ*, **330**: 516-.

12. Xhauflaire-Uhoda, E., et al., (2008) Kinetics of Moisturizing and Firming Effects of Cosmetic Formulations. *Int J Cosmet Sci*, **30**: 131-138.

13. Cardillo, A., et al., (1994) A Fast Non-Invasive Method for Skin Hydration Control. *J Appl Cosmet*, **12**: 11-13.

14. Rawlings, A.V., et al., (2004) Moisturizer Technology Versus Clinical Performance. *Derm Therapy*, **17**: 49-56.

15. Berardesca, E., (1997) Eemco Guidance for the Assessment of Stratum Corneum Hydration: Electrical Methods. *Skin Res Technol*, **3**: 126-132.

16. Heinrich, U., et al., (2003) Multicentre Comparison of Skin Hydration in Terms of Physical, Physiological and Product-Dependent Parameters by the Capacitive Method (Corneometer Cm 825). *Int J Cosmet Sci*, **25**: 45-53.

17. Gioia, F., et al., (2002) The Dynamics of Transepidermal Water Loss (TEWL) from Hydrated Skin. *Skin Res Technol*, **8**: 178-186.

18. Lagarde, J.M., et al., (2001) Skin Topography Measurement by Interference Fringe Projection: A Technical Validation. *Skin Res Technol*, **7**: 112-121.

19. Leveque, J.L., et al., (2003) Skinchip®, a New Tool for Investigating the Skin Surface *In Vivo. Skin Res Technol*, **9**: 343-347.

20. Jacobi, U., et al., (2004) *In Vivo* Determination of Skin Surface Topography Using an Optical 3d Device. *Skin Res Technol*, **10**: 207-214.

21. Sauermann, K., et al., (2002) Age Related Changes of Human Skin Investigated with Histometric Measurements by Confocal Laser Scanning Microscopy *In Vivo. Skin Res Technol*, **8**: 52-56.