Formulation, in vitro release and transdermal diffusion of salicylic acid and topical niacinamide

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SARITA JACOBS

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Supervisor: Prof. J.L. du Preez

Co-Supervisor: Prof. J. du Plessis

Co-Assistant Supervisor: Dr. J.M Viljoen

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ABSTRACT

Acne affects as many as 80% of young adults and adolescents all over the world. This detrimental condition can be classified into four stages: (a) open comedo (blackhead), (b) closed comedo (whitehead), (c) papule and (d) pustule (Russell, 2000:357-366). There are various factors that can lead to acne outbreaks which include: (a) hormone level changes during the menstrual cycle in women, (b) certain drugs (i.e. lithium), (c) certain cosmetics and (d) environmental conditions such as humidity (University of Maryland, 2009:1).

The skin performs a variety of functions which include the two major functions: (a) the containment and (b) the protection of the internal organs of the body. The containment function relates specifically to the ability of the skin to confine the underlying tissues and restrain their movement from place to place. The protective function, on the other hand, relates to the ability of the skin to act as a microbiological barrier to most micro-organisms; a chemical barrier to exogenous chemical compounds; barrier to radiation and electrical shock; and mechanical barrier to impact (Danckwerts, 1991:315).

Niacinamide and salicylic acid were chosen in combination, due to the beneficial effects that they have on acne. Niacinamide has an anti-inflammatory action on acne; which reduces redness, dryness and irritation caused by *Propioni-bacterium acnes* that live in the clogged pores of pimples (Acnetreatmentlab, 2008:1). Salicylic acid is a keratolytic and keratoplastic agent. It is used in combination with other ingredients to enhance the shedding of corneocytes. This causes penetration into the skin to be very difficult (SAMF, 2005:177).

The solubility of niacinamide and salicylic acid in PBS (pH 7.4 at 32°C) were 212.95 mg/ml and 4.07 mg/ml, respectively. The log D values of niacinamide and salicylic acid were determined to be -0.32 and 0.33, respectively. According to the solubility of niacinamide and salicylic acid it was expected that both of the active ingredients would permeate through the skin. However, it is expected that niacinamide will depict enhanced permeation with respect to salicylic acid. The results of the log D for both of the active ingredients indicate that there would not be optimal permeation.

This study involved the formulation of four different acne preparations (Pheroid™cream, Pheroid™gel, cream and gel), combining niacinamide and salicylic acid. The evaluation of stability parameters for the different formulations indicated that none of the formulations was stable under the different storage conditions determined by the Medicines Control Council. Nevertheless, the cream and gel were the most stable of the four formulations. Visual assessment of the Pheroid™ formulations with the confocal laser scanning microscopy (CLMS) was conducted and inconclusive evidence to whether the active substances were entrapped within the Pheroids™, was obtained.

Franz cell diffusion studies indicated that the cream (in the case of niacinamide) and gel (in the case of salicylic acid) depicted the highest average and median flux from hours 6 to 12. Results of the tape stripping studies showed that with the gel formulation, concentrations of 2.060 µg/ml and 44.749 µg/ml niacinamide were obtained in the epidermis and dermis respectively. After the Pheroid™ gel was applied, tape stripping depicted only 1.587 µg/ml niacinamide in the epidermis with respect to 22.764 µg/ml niacinamide in the dermis. The cream formulation, on the other hand, showed niacinamide concentrations of 2.001 µg/ml in the epidermis and 13.363 µg/ml in the dermis, whereas with the Pheroid™ cream formulation, concentrations of 1.097 µg/ml and 18.061 µg/ml were obtained in the epidermis and dermis respectively.

Tape stripping results depicted that with the gel formulation, concentrations of 2.113 µg/ml and 49.519 µg/ml salicylic acid were obtained in the epidermis and dermis respectively, whereas the Pheroid™ gel formulation showed salicylic acid, concentrations of 1.114 µg/ml in the epidermis and 95.360 µg/ml in the dermis. The cream formulation, however, depicted salicylic acid concentrations of 0.758 µg/ml in the epidermis and 44.729 µg/ml in the dermis. Lastly, after the Pheroid™ cream was applied, salicylic acid concentrations of 0.411 µg/ml and 48.424 µg/ml in the epidermis and dermis respectively, were measured.

It could, therefore, be concluded that both niacinamide and salicylic acid tend to concentrate more in the dermis, irrespective of the formulation. This may be an advantage since acne is usually targeted in the dermis and epidermis.

Keywords: Niacinamide, Salicylic acid, Pheroid™, Acne, Stability testing, Topical delivery.

OPSOMMING

Aknee affekteer omtrent 80% van alle jong volwassenes en adolessente reg oor die wêreld. Aknee kan geklassifiseer word in vier fases: (a) swartkoppie, (b) witkoppie, (c) papule en (d) pastule (Russell, 2000:357-366). Daar is 'n verskeidenheid faktore wat kan aanleiding gee tot die uitbreek van aknee, dit sluit die volgende in: (a) verandering in hormoonvlakke gedurende die menstruele siklus in vroue, (b) sekere geneesmiddels (bv, litium), (c) sekere kosmetiek en (d) omgewings faktore (soos humiditeit) (University of Maryland, 2009:1).

Die vel verrig 'n verskeidenheid funksies, maar sluit die twee belangrikste funksies in: (a) die insluiting en (b) die beskerming van die interne organe van die liggaam. Die insluitingsfunksie vewys spesifiek na die vermoë van die vel om die onderliggende weefsel se beweging te beperk. Die beskermingfunksie sluit in 'n mikrobiologiese versperring van die meeste mikro-organismes, chemiese versperring van eksogene chemiese verbindings, versperring van radiasie en elektriese skok, en die meganiese versperring teen impak. Dit veroorsaak dat penetrasie in die vel bemoeilik word (Danckwerts, 1991:315).

Niasinamied en salisielsuur is in kombinasie gekies, as gevolg van die voordelige effek wat die twee geneesmiddels afsonderlik op aknee het. Niasinamied het 'n anti-inflammatoriese aksie op aknee, wat die rooiheid, droogheid en irritasie verminder wat deur *Propioni-bacterium acnes* veroorsaak word. Die bakterium veroorsaak die bogenoemde simptome, omdat dit in die geblokte porieë van puisies woon (Acnetreatmentlab, 2008:1). Salisielsuur is 'n keratoliese en keratoplastieke agens. Dit word gebruik in kombinasie met ander geneesmiddels om vervelling van korneosiete te verbeter (SAMF, 2005:177).

Die wateroplosbaarheid van niasienamied en salisielsuur is bepaal en het 212.95mg/ml en 4.07mg/ml onderskeidelik gelewer in fosfaatbuffer (pH 7.4) by 'n temperatuur van 32°C. Die log D - waardes vir niasinamied en salisielsuur was onderskeidelik -0.32 en 0.33. As gekyk word na die wateroplosbaarheid van die bogenoemde aktiewes, kan 'n mens aflei dat beide geneesmiddels goed deur die vel sal penetreer. Niasienamied sal beter penetreer as salisielsuur, omdat die wateroplosbaarheid hoër is. Na afleiding van die log D - waardes, sal niasienamied en salisielsuur nie optimaal deur die vel penetreer nie.

Die studie het die formulering van vier verskillende akneepreparasies ingesluit naamlik Pheroid™ room, Pheroid™jel, room en jel. Die evaluering van die vier verskillende produkte met die stabiliteitsparameters bepaal deur die medisynebeheerraad, het getoon dat geen een van die formulerings stabiel was nie. Maar nieteenstaande was die room en jel die stabielste van die vier formulerings. Visuele assessering van die Pheroid™ formulerings met die *konfokaal – laser -*

aftaster mikroskoop, was onoortuigend dat die aktiewe bestandele binne in Pheroid™ vasgevang was.

Die Franz sel diffusiestudie het getoon dat die room (in die geval van niasienamied) en die jel (in die geval van salisielsuur) die hoogste gemiddelde en mediaan vloede getoon tussen die 6 tot 12 uur interval. Kleefbandafstropingstudies het getoon dat die jelformule 2.060 µg/ml en 44.749 µg/ml konsentrasies onderskeidelik opgelewer het in die epidermis en dermis vir niasienamied. Nadat die Pheroid™ - jelformule aangewend is, het dit 1.587 µg/ml en 22.764 µg/ml konsentrasies onderskeidelik opgelewer vir niasienamied in die epidermis en dermis. Die roomformule, aan die anderkant het 2.001 µg/ml en 13.363 µg/ml konsentrasies vir niasienamied opgelewer, maar die Pheroid™ - roomformule het konsentrasies van onderskeidelik 1.097 µg/ml en 18.061 µg/ml konsentrasies opgelewer in die epidermis en dermis.

Kleefbandafstropingstudies het ook getoon dat die jelformule onderskeidelik 2.113 µg/ml en 49.519 µg/ml konsentrasies in die epidermis en dermis opgelewer het vir salisielsuur, maar dat die Pheroid™ - jelformule 1.144 µg/ml en 95.360 µg/ml konsentrasies in die epidermis en dermis onderskeidelik opgelewer het. As gekyk word na die roomformule het dit 0.758 µg/ml en 44.729 µg/ml konsentrasies onderskeidelik opgelewer in verband met salisielsuur. Die Pheroid™ - roomformule het konsentrasies van onderskeidelik 0.411 µg/ml en 48.428 µg/ml in die epidermis en die dermis opgelewer vir salisielsuur.

Daar kan dus vasgestel word dat beide niasienamied en salisielsuur meer in die dermis vasgevang was, vir al die formulerings. Dit kan van groot voordeel wees, want aknee word geteiken in die epidermis en die dermis.

Sleutelwoorde: Niasienamied, Salisielsuur, Pheroid™, Aknee, Stabiliteitsevaluering, Topikale aflewering.

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TABLE OF CONTENTS

ABSTRACT i

OPSOMMING iii

REFERENCES v

ACKNOWLEDGEMENTS vi

TABLE OF CONTENTS vii

LIST OF FIGURES xiv

LIST OF TABLES xv

CHAPTER 1: INTRODUCTION AND STATEMENT OF THE PROBLEM 1

REFERENCES 3

CHAPTER 2: SALICYLIC ACID AND NIACINAMIDE IN THE TREATMENT OF ACNE 4

2.1 INTRODUCTION 4

2.2 PATHOGENESIS OF ACNE 4

2.3 THE CAUSE OF ACNE 5

2.4 TOPICAL ACNE MEDICATION 5

2.4.1 TOPICAL BENZOYL PEROXIDE 6

2.4.2 TOPICAL SALICYLIC ACID 6

2.4.3 TOPICAL SULPHUR PREPARATIONS 6

2.4.4 TOPICAL AZELAIC ACID 7

2.4.5 TOPICAL RETINOIDS 7

2.4.5.1 TRETINOIN 7

2.4.5.2 ADAPALENE 7

2.4.5.3 TAZAROTENE 7

2.4.6 TOPICAL ANTIBIOTICS 8

2.4.7 OTHER TOPICAL THERAPIES 8

2.4.7.1 NIACINAMIDE 8

2.5 SYSTEMIC THERAPY 8

2.5.1 ORAL ANTIBIOTICS 8

2.5.2 ORAL RETINOIDS 9

2.5.3 HORMONAL THERAPY 9

2.6 NIACINAMIDE AND THE EFFECTS ON THE SKIN 9

2.6.1 INTRODUCTION 9

2.6.2 TREATMENT OF ACNE WITH TOPICAL NIACINAMIDE 10

2.6.3 RISKS OR SIDE-EFFECTS OF TOPICAL NIACINAMIDE 11

2.6.4 PHYSICO-CHEMICAL CHARACTERISTICS OF NIACINAMIDE 11

2.6.5 TRANSDERMAL DELIVERY OF NIACINAMIDE 12

2.7 SALICYLIC ACID AND THE EFFECTS ON THE SKIN 12

2.7.1 INTRODUCTION 12

2.7.2 TREATMENT OF ACNE WITH SALICYLIC ACID 13

2.7.3 RISKS OR SIDE-EFFECTS OF SALICYLIC ACID 13

2.7.4 *PHYSICO*-CHEMICAL CHARACTERISTICS OF SALICYLIC ACID 13

2.7.5 TRANSDERMAL DELIVERY OF SALICYLIC ACID 14

2.8 TRANSDERMAL DRUG DELIVERY 17

2.8.1 STRUCTURE AND BARRIER FUNCTION OF THE SKIN 18

2.8.1.1 ANATOMY AND PHYSIOLOGY 18

A) THE EPIDERMIS 19

B) THE DERMIS 19

C) THE SUBCUTANEOUS TISSUE 20

2.8.1.2 FUNCTION OF THE SKIN 20

A) CONTAINMENT FUNCTION 21

B) PROTECTIVE FUNCTION 21

2.8.2 PERCUTANEOUS ABSORPTION 21

2.8.3 ROUTES OF DRUG PERMEATION ACROSS THE SKIN 22

2.8.4 PROPERTIES THAT INFLUENCE TRANSDERMAL DELIVERY 23

2.8.4.1 BIOLOGICAL FACTORS 25

A) SKIN AGE 25

B) SKIN CONDITION AND SITES 25

C) SKIN METABOLISM AND CIRCULATORY EFFECTS 25

D) BLOOD FLOW 26

2.8.4.2 PHYSICOCHEMICAL FACTORS 26

A) SKIN HYDRATION 26

B) DRUG-TO-SKIN BINDING 26

C) TEMPERATURE 26

D) DRUG-TO-VEHICLE INTERACTION 26

E) DIFFUSANT SOLUBILITY 27

F) PARTITION CO-EFFICIENT 27

G) PH VARIATION 27

H) COSOLVENTS 27

L) SURFACE ACTIVITY AND MICELLISATION 27

J) COMPLEXATION 27

K) DIFFUSIVITY 27

2.8.4.3 IDEAL MOLECULAR PROPERTIES OF DRUG PENETRATION 28

2.8.4.4 MATHEMATICS OF SKIN PERMEATION 28

A) THE DIFFUSION PROCESS 28

B) COMPLEX DIFFUSING BARRIERS 31

B.1) BARRIERS IN SERIES 31

B.2) BARRIERS IN PARALLEL 31

2.8.4.5 PENETRATION ENHANCERS 31

A) PHYSICAL PENETRATION ENHANCERS 32

B) BIOLOGICAL PENETRATION ENHANCERS 33

C) CHEMICAL PENETRATION ENHANCERS 34

2.9 PHEROID™ TECHNOLOGY IN AID OF OPTIMAL DELIVERY OF SALICYLIC ACID AND NIACINAMIDE. 38

2.9.1 STRUCTURAL CHARACTERISTICS OF PHEROIDS™ 38

2.9.2 FUNCTIONAL CHARACTERISTICS OF PHEROIDS™ 39

2.9.3 PLIABILITY 39

2.9.4 ENTRAPMENT EFFICIENCY (EE) 40

2.9.5 PENETRATION EFFICIENCY 40

2.9.6 THE UPTAKE OF PHEROIDS™ AND ENTRAPPED COMPOUNDS BY CELLS 40

2.9.7 TARGETING, METABOLISM AND DISTRIBUTION 41

2.9.8 THERAPEUTIC EFFICACY 41

2.10 SUMMARY 41

REFERENCES 43

CHAPTER 3: ARTICLE FOR PUBLICATION IN THE INTERNATIONAL JOURNAL OF PHARMACEUTICS 47

ABSTRACT 48

1 INTRODUCTION 49

2 MATERIALS AND METHODS 51

2.1 MATERIALS 51

2.2 METHODS 51

2.2.1 FORMULATIONS PREPARED 51

2.2.2 STABILITY TESTING 51

2.2.2.1 HPLC METHOD FOR STABILITY TESTING (ASSAY) 52

2.2.2.1.1 SAMPLE PREPARATION 52

2.2.2.1.2 STANDARD SOLUTION 52

2.2.2.2 PH DETERMINATION 53

2.2.2.3 RHEOLOGICAL MEASUREMENTS 53

2.2.2.4 VISUAL ASSESSMENT 53

2.2.2.5 CONFOCAL LASER SCANNING MICROSCOPY (CLSM) 53

2.2.2.5.1 SAMPLE PREPARATION 54

2.2.2.6 MASS VARIATION 54

2.2.3 PHYSICOCHEMICAL PROPERTIES 54

2.2.3.1 HPLC METHOD FOR PHYSICOCHEMICAL PROPERTIES AND DIFFUSION STUDIES 54

2.2.3.1.1 STANDARD PREPARATION 55

2.2.3.2 SOLUBILITY DETERMINATION 55

2.2.3.3 EXPERIMENTAL OCTANOL-BUFFER PARTITION COEFFICIENT (LOG D) 55

2.2.3.4 PERMEATION EXPERIMENTS 55

2.2.3.4.1 PREPARATION OF DONOR SOLUTION 55

2.2.3.4.2 SKIN PREPARATION 56

2.2.3.4.3 METHOD OF SKIN PERMEATION 56

2.2.3.4.4 TAPE STRIPPING 57

2.2.3.4.5 TRANSDERMAL AND STATISTICAL DATA ANALYSIS 57

3 RESULTS 59

3.1 STABILITY TESTING 59

3.1.1 ASSAY RESULTS 59

3.1.2 PH RESULTS 59

3.1.3 THE RHEOLOGICAL MEASUREMENTS 60

3.1.4 VISUAL ASSESSMENT 60

3.1.5 CONFOCAL LASER SCANNING MICROSCOPY (CLSM 61

3.1.6 MASS VARIATION 61

3.2 PHYSICOCHEMICAL PROPERTIES 61

3.2.1 AQUEOUS SOLUBILITY AND EXPERIMENTAL LOG D VALUES OF NIACINAMIDE AND SALICYLIC ACID 61

3.3 DIFFUSION STUDY 61

3.3.1 *IN VITRO* PERMEATION STUDIES 61

3.3.2 TAPE STRIPPING 63

3.3.3 TRANSDERMAL AND STATISTICAL DATA ANALYSIS 64

4 CONCLUSION 66

ACKNOWLEDGEMENTS 67

REFERENCES 68

FIGURE LEGENDS 71

FIGURES 72

CHAPTER 4: FINAL CONCLUSION AND FUTURE PROSPECTS 74

REFERENCES 77

APPENDIX A: INTERNATIONAL JOURNAL OF PHARMACEUTICS – GUIDE FOR AUTHORS 78

APPENDIX B: FORMULATION OF ACNE PREPERATIONS CONTAINING NIACINAMIDE AND SALICYLIC ACID 89

APPENDIX C: VALIDATION OF THE HPLC EXPERIMENTAL METHOD FOR NIACINAMIDE AND SALICYLIC ACID 99

APPENDIX D: VALIDATION OF THE HPLC EXPERIMENTAL METHOD FOR A PHEROID™ GEL CONTAINING NIACINAMIDE AND SALICYLIC ACID 111

APPENDIX E: STABILITY TESTING 139

APPENDIX F: DIFFUSION STUDY 163

APPENDIX G: PHOTOS OF APPARATUS USED DURING THE DIFFUSION STUDY, SAMPLE ANALYSIS AND STABILITY TESTING 168

List of Figures

Chapter 2

Figure 1: (**A**) Mild acne and (**B**) Moderate acne 4

Figure 2: Stages of acne. (**A**) Normal follicle, (**B**) Open comedo (blackhead), (**C**) Closed comedo (whitehead), (**D**) Papule, (**E**) Pustule 5

Figure 3: Treatment pathways 6

Figure 4: Chemical structure of niacinamide 11

Figure 5: The bark of the willow tree (Salix Alba) from which salicylic acid is derived 13

Figure 6: Chemical structure of salicylic acid 14

Figure 7: Schematic diagram of human skin broken down into three major layers 19

Figure 8: The 3 major routes of penetration through human skin. (1 – Sweat glands, 2 – Trans epidermal, 3 – Hair follicle) 22

Figure 9: Stages in drug delivery from a transdermal patch 24

Figure 10: The time course of absorption for the simple zero-order flux case obtained by plotting *m*, the cumulative amount of diffusant crossing the unit area of the membrane, as a function of time. Steady state is achieved when the plot becomes linear, extrapolation of the linear portion to the time axis yields the lag time *L* 30

CHAPTER 3

Figure 1: Box-plots of the flux values for **a)** niacinamide and **b)** salicylic acid to illustrate the median flux and red lines to illustrate the average flux after application of Pheroid™ gel, Pheroid™ cream, gel and cream 72

Figure 2: Cumulative concentration (µg/ml) of the epidermis and dermis for **a)** niacinamide and **b)** salicylic acid in the four different formulations 73

LIST OF TABLES

CHAPTER 2

Table 1: Hormonal therapy 9

Table 2: The physico-chemical characteristics of niacinamide 11

Table 3: The physico-chemical characteristics of salicylic acid 14

Table 4: *In vivo* urinary excretion of salicylic acid in humans 15

Table 5: Physical methods of penetration enhancement 32

Table 6: The classification of penetration enhancers 34

CHAPTER 1: INTRODUCTION AND STATEMENT OF THE PROBLEM

Acne can more often be defined as a chronic disease and not just a self-limiting disorder of teenagers. Many patients with symptoms relating to chronic acne have the following characteristics that have been used to define the chronic implications: a prolonged course of acne (more than 6 months), a pattern of relapse or recurrence, manifestation as acute outbreaks or slow onset, as well as a social and psychological impact that affect the individual's quality of life (Thiboutot *et al.*, 2009:S3). These patients have a need for an effective solution to clear the symptoms and to reduce the emotional stress related to acne.

Cosmeceuticals are referred to as the marriage between cosmetics and pharmaceuticals. Cosmeceuticals are topically applied, but they consist of active ingredients that influence the biological function of the skin (Schwartz, 2008:1). Since the late 1980's, consumer demand for more effective products that substantially enhance personal appearance has led to increased scientific research and product development in the cosmeceutical industry (Lupo, 2001:472).

In this study salicylic acid and niacinamide were incorporated into cosmeceutical formulations for the treatment of acne, in order to determine whether the 2 active ingredients can target the four treatment pathways of acne in the epidermis and dermis. Salicylic acid acts by softening keratin; a protein that forms part of the skin structure. This helps to remove the dry scaly skin easier so that niacinamide can penetrate into the skin more effectively. Salicylic acid also decreases or slows the shedding of the cells inside the follicles, preventing clogging (DermNet, 2009:1). Niacinamide, on the other hand, has been shown to have anti-inflammatory properties when applied to the skin. Additional studies conducted on the anti-inflammatory effects of niacinamide presented the suppression of antigen-induced lymphocyte transformation and inhibition of potassium-iodide-induced inflammation. Human keratinocytes incubated with niacinamide depicted improved barrier function and decreased trans-epidermal water loss due to stimulation of ceramide synthesis (Lupo, 2001:473).

According to Danckwerts (1991:314), topical and transdermal drug delivery have increased dramatically over the last decade due to the non-invasive and painless delivery of drugs, as well as increased patient compliance. The target site for the treatment of *acne vulgaris* lies within the epidermis and dermis of the skin. Topical formulations are placed on the skin to deliver drugs to the local tissues directly under the application site, or within the tissues under, and around the site of application. These formulations are intended to treat acne, whilst keeping the pharmacological effects of salicylic acid and niacinamide restricted to the intracutaneous regions of drug penetration and deposition (Ghosh & Pfister, 1997:7).

During this study the Pheroid™ delivery system was used to entrap salicylic acid and niacinamide into microsponges, to determine whether the Pheroid™ delivery system can deliver the active ingredients in the skin more effectively. The Pheroid™ consists of the *cis*-formation of fatty acids that are highly compatible with fatty acids in the human body (Grobler *et al.*, 2008:285). Fluidity of the Pheroid™ membrane contributes to the efficient dermal and transdermal delivery (Grobler *et al.*, 2008:297).

The aims and objectives of this study were to:

- Determine the saturated solubility and partition coefficient of salicylic acid and niacinamide.
- Develop dosage forms with and without the Pheroid™ such as a cream and gel.
- Develop and validate a high-performance liquid chromatographic (HPLC) method for each model drug as well as for the ingredients within the formulations in order to determine their stability.
- Evaluate the transdermal delivery of the selected drugs with and without Pheroids™ by means of Franz cell diffusion studies and tape stripping.
- Perform stability testing (6 months) of each formulation. The properties which were determined included:
  - pH levels,
  - Rheology determination,
  - Mass variation,
  - Visual assessment,
  - Assay of ingredients and active ingredients and
  - The use of the confocal laser scanning microscope (CLMS) in order to determine whether there are Pheroids™ present in the Pheroid™ formulations; to establish the size of the particle in each formulation; and to determine whether the specific formulation is homogenous and without crystals.

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CHAPTER 2: SALICYLIC ACID AND NIACINAMIDE IN THE TREATMENT OF ACNE

2.1 INTRODUCTION

Acne (**Figure 1**) and its associated problems with self-esteem and social inhibition affect as many as 80% of young adults and adolescents (Russell, 2000:357-366). The objective of this study is to formulate acne products containing salicylic acid and niacinamide. This is a fairly new combination on which little research has been done to date.

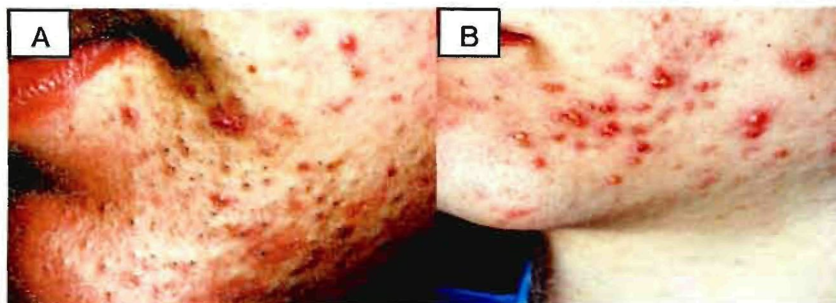

Figure 1: (**A**) Mild acne and (**B**) Moderate acne (James, 2005:1464).

2.2 PATHOGENESIS OF ACNE

Acne vulgaris is a multifactorial disease (Manela-Azulay & Bagatin, 2009:470). During the onset of puberty, elevated androgen levels stimulate the sebaceous glands to enlarge and produce increased amounts of sebum in the sebaceous follicle. Later, abnormal keratinisation with hyperkeratosis of the follicular epithelium leads to obstruction of the duct by a horny plug. The blocked duct becomes clogged with a dense material composed of keratinous debris and sebum, forming a microcomedo, the originator of all acne lesions. Further distension leads to open and closed comedones (**Figure 2** – B & C) that are non-inflammatory lesions (Berson & Shalita, 1995:531). An anaerobic growth medium for *Propioni-bacterium acnes* is provided by the excess sebum in microcomedo. Lipases from the bacteria hydrolyse sebum triglycerides into free fatty acids that are both pro-inflammatory and comedogenic. *Propioni-bacterium acnes* also secrete chemotactic factors that attract neutrophils, lymphocytes and macrophages (Manela-Azulay & Bagatin, 2009:470). Lysosomal enzymes released from the neutrophils break the follicle wall and release pro-inflammatory mediators that include lipids and keratin such as interleukin IL-1, IL-8, IL-12 and tumour necrosis factor α, into the surrounding dermis (Berson & Shalita, 1995:531; Manela-Azulay & Bagatin, 2009:470). This results in the formation of inflammatory papules and pustules (**Figure 2** – D & E). Further inflammation with macrophages and foreign-body reactions lead to cysts and nodules (Berson & Shalita,

pathogenesis of acne are, therefore, increased sebum production; abnormal follicular keratinisation; increased *P.acnes*; and inflammation. The stages of acne are illustrated in **Figure 2.**

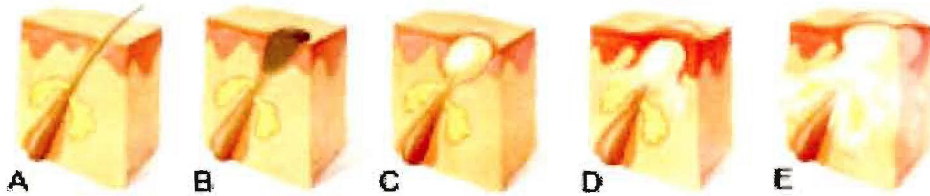

Figure 2: Stages of acne. (**A**) Normal follicle, (**B**) Open comedo (blackhead), (**C**) Closed comedo (whitehead), (**D**) Papule, (**E**) Pustule (Russell, 2000:358).

2.3 CAUSES OF ACNE

There are various factors that can lead to acne outbreaks which include:

- Hormone level changes during the menstrual cycle in women,
- Certain drugs (such as corticosteroids, lithium and barbiturates),
- Oil and grease from the scalp; mineral or cooking oil; certain cosmetics,
- Friction or pressure from helmets, backpacks or tight collars, and
- Environmental conditions (such as pollution and humid conditions) (University of Maryland, 2009:1).

2.4 TOPICAL ACNE MEDICATION

The study conducted, involved the four topical treatment pathways that included *P.acnes*, hyperkeratosis, sebum production and inflammation. Acne may quickly and successfully be resolved if a product is developed which acts on all of the above topical pathways **(Figure 3)** (Berson & Shalita, 1995:533). Before formulating an effective acne product, it is important to gain extensive knowledge of the variety of therapies, with different mechanisms, available to treat acne. For patients with non-inflammatory comedones or mild-to-moderate inflammatory acne, topical therapy is usually primarily attempted. Use of topical therapy minimises potential side effects associated with the use of systemic agents. Topical therapies include comedolytic agents such as tretinoin, benzoyl peroxide, salicylic acid, niacinamide, antibiotics and various anti-inflammatory drugs (Berson & Shalita, 1995:533).

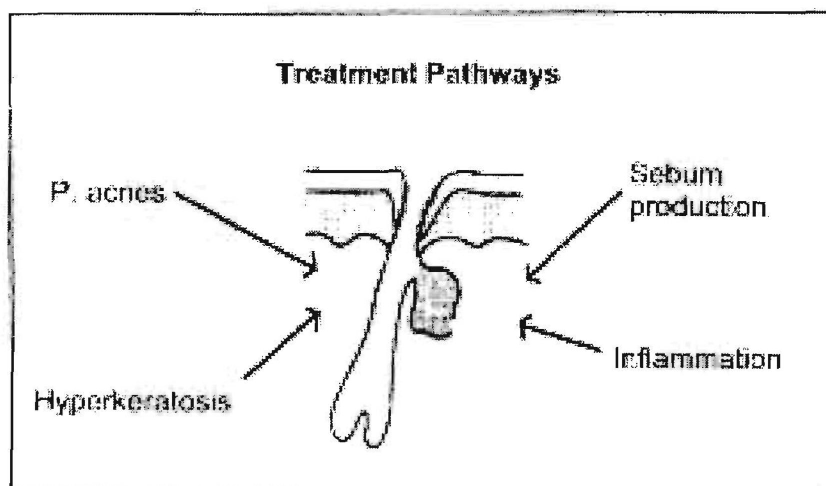

Figure 3: Treatment pathways (Berson & Shalita, 1995:533).

2.4.1 TOPICAL BENZOYL PEROXIDE

Benzoyl peroxide has been the foundation of acne treatment since 1950. It is available over the counter or through prescription. Benzoyl peroxide has bactericidal and comedolytic properties. It is a topical agent most effective against *P.acnes,* with bacteriostatic activity greater than of topical antibiotics. It also functions as a mild comedolytic agent by increasing epithelial cell turnover with desquamation (Russell, 2000:366).

2.4.2 TOPICAL SALICYLIC ACID

Salicylic acid is an ingredient in a variety of over-the-counter preparations. It is available in a number of creams and lotions at a concentration of 0.5 to 2%. The agent promotes desquamation of follicular epithelium and by so doing it inhibits comedogenesis. In various studies, it has been shown that salicylic acid is as effective as benzoyl peroxide in the treatment of comedonal acne. Salicylic acid is well tolerated and should be applied once or twice daily (Russell, 2000:366).

A detailed description of salicylic acid is given in section **2.7.**

2.4.3 TOPICAL SULPHUR PREPARATIONS

Sulphur preparations have been used to treat acne since the beginning of time. Sulphur is combined with other acne medications in many over-the-counter washes and cleansing bars. Sulphur is effective in the treatment of inflammatory acne lesions, most likely as a result of keratolysis. The efficacy may be related to the irritative effect (Russell, 2000:366).

2.4.4 TOPICAL AZELAIC ACID

Azelaic acid is a dicarboxylic acid. It was coincidentally found to be effective in acne treatment after it was first investigated in the 1970's as a treatment for hyper pigmentation. In 1996, the U.S. Food and Drug Administration (FDA) labelled azelaic acid for the treatment of mild to moderate inflammatory acne. The exact mechanism of action is unknown; however, this agent has antibacterial and anti-keratinising activity. Azelaic acid appears to be as effective as benzoyl peroxide or tretinoin (Retin-A) in the treatment of mild to moderate acne (Russell, 2000:366).

2.4.5 TOPICAL RETINOIDS

Retinoids are derivates of vitamin A. The functions of these retinoids are the slowing down of the desquamation process, thereby decreasing the number of comedones and microcomedones. Retinoids are the most effective comedolytic agents in use. They have been used to treat acne during the last 25 years (Russell, 2000:366).

2.4.5.1 TRETINOIN

Until recently, tretinoin was the only available topical retinoid. Tretinoin is effective in the monotherapy of patients with non-inflammatory or mild to moderate inflammatory acne (Russell, 2000:366).

2.4.5.2 ADAPALENE

In 1997 the FDA approved adapalene as a topical retinoid. Its mechanism of action is similar to that of tretinoin. Adapalene is formulated in a 0.1% gel or solution for application once daily, in the evening (Russell, 2000:366).

Studies have shown that 0.1% adapalene gel is at least as effective as 0.025% tretinoin gel and considerably less irritating. Adapalene may, however, cause initial exacerbation of acne lesions and skin irritation (Russell, 2000:366).

2.4.5.3 TAZAROTENE

Tazarotene has been approved by the FDA for the treatment of psoriasis and mild to moderate acne. It is formulated in a 0.05 or 0.1% gel for once daily application. Studies comparing tazarotene with a placebo gel, cream or lotion alone have shown that the medication is effective in treating non-inflammatory acne lesions (Russell, 2000:366).

2.4.6 TOPICAL ANTIBIOTICS

Topical antibiotics act directly by killing *P.acnes*. Topical antibiotics have bactericidal activity as well as a mild indirect effect on comedogenesis. These agents are available in a variety of forms and are applied once or twice daily.

Topical clindamycin and erythromycin are the most commonly used agents and have a similar effect on patients with acne. Clindamycin is shown to be significantly more effective than topical tetracycline (Russell, 2000:366).

Most of the topical antibiotics are, however, associated with some minor skin irritation (Russell, 2000:366).

2.4.7 OTHER TOPICAL THERAPIES

2.4.7.1 NIACINAMIDE

The main action of niacinamide on acne, is that it provides an anti-inflammatory action. Niacinamide anti-inflammatory action reduces the redness, dryness and irritation caused by the bacteria that live in clogged pores of pimples (Acnetreatmentlab, 2008:1).

A detailed description of niacinamide is given in section **2.6**.

2.5 SYSTEMIC THERAPY

Oral antibiotics are widely used in clinical practice and are highly effective for inflammatory acne. Oral retinoids are usually prescribed in cases of severe acne or to patients suffering from inflammatory or pustular acne which is non-responsive to other approaches (Leyden, 2003:S201). The long-term use of these drugs has potential side-effects such as hypertension and the development of antibiotic resistance (Grange *et al.*, 2009:110).

2.5.1 ORAL ANTIBIOTICS

Antibiotics such as tetracycline, erythromycin, doxycycline, and trimethoprim-sulfamethoxazole are indicated for the treatment of acne on the chest, back or shoulders; in patients with inflammatory disease. It may also be used for moderate-to-severe-disease in which topical combinations have failed or are not tolerated (James, 2005:1468).

2.5.2 ORAL RETINOIDS

Isotretinoin is the only drug that both directly inhibits the sebaceous gland function and influences abnormal follicular keratinisation. A distinct decrease in sebum production occurs within 2 weeks of onset therapy. As a result, the growth of *P.acnes* and its ability to generate pro-inflammatory mediators are reduced. Thus, isotretinoin is the only therapy that affects all four of the pathogenic factors for *acne vulgaris* (Berson & Shalita, 1995:S37).

2.5.3 HORMONAL THERAPY

Systemic hormonal treatment reduces sebum production by counteracting the effects of androgens on the sebaceous gland. Hormonal therapy is currently limited to systemic treatment of acne for women. The three choices are estrogens (which suppress ovarian androgen), glucocorticoids (which suppress adrenal androgen) and systemic anti-androgens (which act primarily at the peripheral level) (Berson & Shalita, 1995:S39). The different hormonal therapy is listed with their benefits and side effects in **Table 1**.

Table 1: Hormonal therapy (Berson & Shalita, 1995:S39; Katsambas & Papakonstantinou, 2004:412-418; Wyatt, 2001:1800).

Hormonal therapy	Benefits	Side effects
Estrogens	Suppress ovarian androgen; Decreased sebum production.	Vascular thrombosis; Melasma; Weight gain.
Glucocorticoids	Anti-inflammatory and androgen-inhibiting.	Skin atrophy; Striae; Peptic ulcer and Purpura.
Spironolactone	Suppress sebum production; Inhibits androgen production in the ovaries and adrenals.	Menstrual irregularities; Breast tenderness; Headache; Fatigue and possible hyperkalemia.

2.6 NIACINAMIDE AND THE EFFECTS ON THE SKIN

2.6.1 INTRODUCTION

Vitamin B_3, also called nicotinamide or niacinamide, is a derivative of niacin obtained through a diet from meat, fish, milk, egg and nuts. Its deficiency is one of the causing factors of pellagra. Niacin has been used in medicine, most commonly to lower cholesterol. Nicotinamide is part of the coenzymes nicotinamide adenine dinucleotide (NAD^+), NAD phosphate ($NADP^+$), and its

reduced forms of nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH). These molecules are important in many cellular metabolic enzyme reactions and the reduced factors may act as antioxidants (Manela-Azulay & Bagatin, 2009:472). Niacinamide is a water-soluble, B complex vitamin (Bethesda, 2007:1) and functions in cosmetic formulations primarily as a hair and skin conditioning agent due to its existence as a heterocyclic aromatic compound (Anderson, 2005:1). Niacinamide is used in approximately 30 cosmetic formulations, including shampoos, hair tonics, skin moisturisers and cleansing formulations (Anderson, 2005:1). The possible utility of topical niacinamide in the improvement of skin appearance may be related to its action in the synthesis of sphingolipids, free fatty acids, cholesterol and ceramides, thus decreasing transdermal water loss (Manela-Azulay & Bagatin, 2009:472).

2.6.2 TREATMENT OF ACNE WITH TOPICAL NIACINAMIDE

According to the Acnelabtreatment (2008:1), the main action of niacinamide on acne is that it provides an anti-inflammatory action. The anti-inflammatory action of niacinamide reduces the redness, dryness and irritation caused by the bacteria that live in clogged pores of pimples. According to a study conducted by Draelos *et al.*, (2006:96-101), niacinamide was able to reduce the sebum excretion rate (SER) or the amount of oil that the glands pump out. The reduction in the sebum excretion rate reduces the formation of clogged pores which causes blackheads and white heads. It furthermore keeps the skin hydrated by preventing water loss, thus acting as a moisturiser as well. Niacinamide is a mild keratolytic agent. This means it promotes mild shedding of the skin which prevents pore blockage.

According to Manela-Azulay and Bagatin (2009:106), nicotinamide significantly decreases IL-8 production in a dose-dependent manner, decreasing both the mRNA and protein levels for this chemokine in immortalised human keratinocyte cells line (HaCat) and primary keratinocytes. *P.acnes*-induced IL-8 promotor activation seemed to be down regulated by nicotinamide, which inhibited I-kappa-B protein (IκB) degradation and the phosphorylation of extra-cellular-signal-regulated-kinase (ERK) and Jun-N-terminal-kinase (JNK) Mitogen-activade-protein (MAP) kinases in a study that was conducted on the effects of nicotinamide on the production of IL-8 by the HaCat human immortalised keratinocyte cell line and on primary keratinocytes stimulated by *P.acnes.*

Topical application of niacinamide has a stabilising effect on epidermal barrier function, seen as reducing transepidermal water loss and the improvement in moisture content of the horny layer. Niacinamide leads to the increase in protein synthesis (ie, keratin); has a stimulating effect on ceramide synthesis; speeds up the differentiation of keratinocytes; and raises intracellular

NADP levels. On ageing of the skin, topical application of niacinamide improves the surface structure; smoothes out wrinkles; and inhibits photocarcinogenesis (Gehring, 2004: 88-93).

2.6.3 RISKS OR SIDE-EFFECTS OF TOPICAL NIACINAMIDE

According to a study conducted by Carraway (2004:84) on using aldara, copper peptide, and niacinamide for skin care, there were no significant complications of the topical application of niacinamide except for mild flushing and itching of the skin.

2.6.4 PHYSICO-CHEMICAL CHARACTERISTICS OF NIACINAMIDE

The physico-chemical characteristics of niacinamide are depicted in **table 2** and the chemical structure is shown in **figure 4**.

Table 2: The physico-chemical characteristics of niacinamide (Moety *et al.,* 1991:497-499).

Niacinamide Property	Physico-chemical Characteristics
Chemical name	3-Pyridine carboxamide; Nicotinic acid amide
Generic name	Niacinamide; Nicotinamide; Vitamin B3; Vitamin PP
Molecular weight	122.13 g/mole
pKa	pKa 3.3 (20°C)
Melting point	128-131°C
Appearance	White crystalline powder or colourless crystals
Odour	Faint characteristic odour
Taste	Salty and bitter taste
Density	1.44 $g.cm^{-3}$
pH	pH 3-3.5 (1.3% (w/v)) aqueous solution of nicotinamide

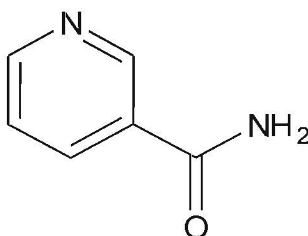

Figure 4: Chemical structure of niacinamide

2.6.5 TRANSDERMAL DELIVERY OF NIACINAMIDE

Barai *et al.* (2007:21-28) studied the barrier function in cultured skin substitutes prepared from human cell sources. He used non-invasive (surface hydration, transepidermal water loss) and invasive methods (water permeation, niacinamide flux) before and after grafting onto athymic mice. *In vitro* measurements were taken after 7 and 14 days. Although three of the four measurements of barrier function improved markedly from day 7 to 14, the values obtained were still significantly inferior from those obtained with native human skin controls. Additional cultured skin substitutes were grafted onto athymic mice on day 14 and skin was harvested 2 and 6 weeks after grafting. Grafting brought about a substantial decrease in all measurements after 2 weeks and almost completed normalisation of barrier function after 6 weeks. The most sensitive measurement of the recovery was niacinamide permeability, which decreased from (280 ± 40) x 10^{-4} cm/h *in vitro* to (17 ± 30) x 10^{-4} cm/h two weeks after grafting and (5 ± 2) x 10^{-4} cm/h six weeks after grafting; versus control values of (2 ± 2) x 10^{-4} cm/h in human cadaver skin and (0.6 ± 0.4) x 10^{-4} cm/h in human epidermal membrane prepared from freshly excised breast skin. These results demonstrated the reformation of epidermal barrier function after transplantation and provided insight into the development of functional epidermal barrier in cultured skin substitutes.

Feldmann and Maibach (1970:399-404) studied the percutaneous penetration of 21 organic chemicals of which nicotinamide and nicotinic acid were included. The method consisted of applying 4mg/cm^2 chemical to the human forearm and quantified its penetration through the skin by measuring its metabolites in urine. The chemical was dissolved in acetone and applied to a marked area with a microliter syringe on unprotected skin. Subjects were asked not to wash the area for approximately 24 hours. All urine were collected for 5 days. Studies were performed with radio labelled (^{14}C) tracer doses. The absorption was expressed as the percentage (%) of the applied dose over a period of 5 days. The total absorption value of nicotinamide was 11.08 ± 6.17% (% of dose ± SD) and for nicotinic acid was 0.34 ± 0.09% (% of dose ± SD). Nicotinic acid depicted minimal penetration whereas 10% of nicotinamide was absorbed.

2.7 SALICYLIC ACID AND ITS EFFECTS ON SKIN

2.7.1 INTRODUCTION

Salicylic acid (from salix, the Latin word for the willow tree) illustrated in **figure 5**, is a phytohormone, a plant product that acts as a hormone by regulating cell growth and differentiation. Salicylic acid is a beta-hydroxy acid that is chemically similar to the active component aspirin (Whitney & Shalita, 2008:172). Salicylic acid is found in different quantities in all fruit, vegetables, herbs and spices (Schier, 2001:1406-1408). Salicylic acid functions as a topical desquamating agent, dissolving the intracellular cement holding the cells of the stratum

and has a comedolytic effect. It is used to remove excess keratin in hyperkeratonic skin disorder such as common and plantar warts, psoriasis, calluses, and corns. Salicylic acid is also used to treat acne (Roberts II & Morrow, 2001:696-703).

Figure 5: The bark of the willow tree (Salix Alba) from which salicylic acid is derived from (Crosby, 2006).

2.7.2 TREATMENT OF ACNE WITH SALICYLIC ACID

According to the SAMF (2005:177), salicylic acid is a keratolytic and keratoplastic agent. It is used alone or in combination with other agents in a wide variety of preparations to enhance shedding of corneocytes. This is established by reducing intercellular stickiness and epithelial hyperplasia. Salicylic acid promotes penetration of certain drugs into the skin. It also assists in reducing the redness of pimples (Zander & Weisman, 1992:247). Salicylic acid is absorbed rapidly from intact skin; especially when applied in oily liniments or ointments (Roberts II & Morrow, 2001:696-703).

2.7.3 RISKS OR SIDE-EFFECTS OF SALICYLIC ACID

Skin peeling, dryness and irritation can occur when using salicylic acid in concentrations of 2% and higher. Possible adverse effects include salicylate toxicity, toxic inner ear damage and hypersensitivity. There may be increased skin sensitivity (photosensitisation). Salicylic acid changes the structure of the skin and allows other chemicals to seep deeper into the skin and even into the bloodstream (Zander & Weisman, 1994:247).

2.7.4 PHYSICO-CHEMICAL CHARACTERISTICS OF SALICYLIC ACID

The physico-chemical characteristics of salicylic acid are depicted in **table 3** whereas the structure of the compound is shown in **figure 6**.

Table 3: The physico-chemical characteristics of salicylic acid (Abounassif *et al.*, 1994:424-426).

Salicylic Acid Property	Physico-chemical Characteristics
Chemical name	2-Hydroxybenzoic acid
Generic name	Salicylic acid
Molecular weight	138.12 g/mole
pKa	2.98
Melting point	157-159°C
Appearance	White crystalline powder
Odour	Odourless
Taste	Sweetish acrid taste
Density	$1.443^{20}{}_{4}$ g/cm^3
pH	Saturated solution - 2.4

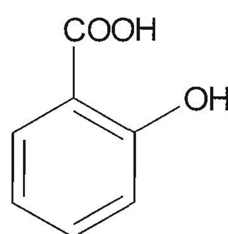

Figure 6: Chemical structure of salicylic acid

2.7.5 TRANSDERMAL DELIVERY OF SALICYLIC ACID

A study was conducted by Wester *et al.* (1999:571-585) to determine the percutaneous absorption of salicylic acid *in vivo* in humans. The study included five to six normal volunteer outpatients per group (males, ages 18-85 and postmenopausal women, ages 50-65 years), from whom informed consent had been obtained. The subjects were topically dosed with a (^{14}C)-labelled chemical that was salicylic acid, on the ventral forearm. Salicylic acid was solubilised in 50 µl ethanol and spread over 10 cm^2 of the skin surface. The ethanol vehicle was allowed to air-dry and the site was not occulated. Subjects were instructed not to touch or wash the study area for at least 24 hrs. The subjects were instructed to collect all urine in containers provided for that day and the subsequent 6 days (7 days total urine collection period). At 7 days post application, the skin dosing site was cellophane tape-stripped (Scotch Transparent; 3M Co., St Paul, MN) 10 times for the residual (^{14}C)-labelled chemical. Percutaneous absorption was determined from the (^{14}C) urinary excretion. **Table 4** provides the *in vivo* data of salicylic acid in humans. A total of 5.8 ± 4.5% (mean ± SD) of the applied dose

was excreted over 7 days. After 24 hrs approximately 53.4 ± 6.3% of dose could be recovered in the skin surface after it was washed with 50% liquid ivory soap and water. The greater part of the surface dose was recovered with the first soap application. After 7 days, no significant (0.22 ± 0.25) residual material was recovered with the stratum corneum tape stripping.

Table 4: *In vivo* urinary excretion of salicylic acid in humans (Wester *et al.*, 1999:571-585).

	Percent dose		
Subject	Urine excretion (a)	Skin surface wash (b)	Tape stripping recovery (c)
1	8,9	49,3	0,04
2	3,9	56,7	0,09
3	13,6	49,3	0,02
4	2,6	46,1	0,35
5	3,7	63,2	0,67
6	2,3	56,0	0,17
Mean	5,8	53,4	0,22
SD	4,5	6,3	0,25

(a) For 2.4 μg/cm² ethanol vehicle, unoccluded ventral forearm.
(b) For 24-h post application soap-and-water wash.
(c) For 7 days post application/10 consecutive tape strips.

Feldmann and Maibach (1970:399-404) studied the percutaneous penetration of 21 organic chemicals including salicylic acid. The method consisted of applying 4mg/cm^2 of the chemical to the human forearm and quantifying its penetration through the skin by measuring its metabolites in urine. The chemical was dissolved in acetone and applied to a marked area with a microliter syringe on unprotected skin. The subjects were asked not to wash the area for at least 24 hrs. All urine were collected for 5 days. Every study was performed with radio labelled (^{14}C) tracer doses. The absorption was expressed as the percentage (%) of the applied dose over a period of 5 days. They found the total absorption value of salicylic acid to be approximately 22.78 ± 13.25 (mean ± SD) percentage (%) of the applied dose.

Benfeldt *et al.* (1999:783–810) used microdialysis in the dermis for assessing penetration kinetics of salicylic acid in healthy volunteers (n = 18) following application on the volar aspect of the left forearm. Penetration was monitored at four locations: in normal (unmodified) skin and in skin with perturbed barrier function from (i) repeated tape stripping (ii) irritant dermatitis from 1 or 2% sodium lauryl sulphate (SLS) for 24 h, and (iii) delipidisation by acetone. The order of the treatments was randomised according to a latin square design. Epidermal barrier function and skin irritation were assessed in each location using evaporimetry and colorimetry. Transepidermal water loss (TEWL) values confirmed that mild (acetone), moderate (1% SLS)

and severe barrier damage (tape stripping and 2% SLS) had occurred. Microdialysis sampling with two parallel probes in the dermis was performed in each of the four treatment areas for every subject. Salicylic acid (5% in ethanol) was applied in a chamber glued to the skin overlying the microdialysis probes and sampling was continued for 4 hrs. Salicylic acid was detectable, and measurable in all samples from penetration through perturbed skin. Comparing the salicylic acid penetration in barrier-perturbed skin with the penetration in unmodified skin in the same subject, the mean salicylic acid penetration increase was 2.2-fold in acetone-treated skin ($P = 0.012$), 46-fold in mild dermatitis and 146- and 157-fold in severe dermatitis and tape stripped skin, respectively ($P < 0.001$). The penetration of salicylic acid depicted a significant correlation with the measurements of barrier perturbation through TEWL ($P=0.01$) and erythema ($P=0.02$) for each individual. Microdialysis sampling of salicylic acid penetration was more sensitive than non-invasive measuring techniques in detecting significant barrier perturbation in acetone-treated skin. A positive dose response relationship for the percutaneous penetration of salicylic acid in response to increasing SLS pre-treatment concentrations, and thus the degree of irritant dermatitis, was found. When analysing data by location on the forearm, a tendency towards an intraregional variation in the reactivity to barrier damage was found, with the most proximal location displaying higher reactivity scores than the most distal location in response to the same barrier perturbation procedures. The penetration of salicylic acid did not differ significantly between the locations. In conclusion, using microdialysis in the dermis to obtain real-time dermal pharmacokinetics in the target organ, this study demonstrated highly increased and differentiated cutaneous penetration of salicylic acid in barrier-perturbed skin. The measured drug penetration demonstrated a correlation with non-invasive quantification of barrier damage.

In another study, the effect of drug lipophilicity on *in vivo* iontophoretic transdermal absorption was evaluated. Non-steroidal anti-inflammatory drugs (NSAIDs), such as salicylic acid (SA), ketoprofen (KP), naproxen (NP) and indomethacin (IM) were selected as model drugs with a wide range of lipophilicity. Cathodal iontophoresis of NSAIDs was conducted in rats (0.625 mA/cm^2; 90 min), and drug concentrations in skin, cutaneous veins and systemic veins were determined. Skin concentrations of NSAIDs were higher in the case of lipophilic drugs ($SA = KP = NP < IM$), whereas cutaneous plasma concentrations decreased with an increase in lipophilicity ($SA > KP = NP > IM$). Additionally, the dependence of drug lipophilicity on systemic plasma concentration was similar to the cutaneous plasma concentration. The transfer rate from skin to cutaneous veins (R(SC)) was calculated from the arterio-venous plasma concentration difference of drug in the skin. Normalised R(SC) by skin concentration (R(SC)/X(S)) yielded a negative correlation with the logarithm of n-octanol/buffer partition coefficient (Log P at pH 7.4), suggesting that transfer of NSAIDs from the skin to cutaneous veins decreased with increasing lipophilicity ($SA > KP = NP > IM$). This correlation means that

drug partitioning between the stratum corneum and viable epidermis might be a dominant step (Tashiro *et al.*, 2001:278–283).

In a study conducted by Asman *et al.* (2007:3291-3299), on the *in vitro* release of salicylic acid through poly(vinyl alcohol-g-itaconic acid) membranes; itaconic acid (IA) was grafted on poly(vinyl alcohol) (PVA) at two different grafting percentages, 7.0% (w/w) and 14.0% (w/w). Membranes were prepared from the grafted copolymer (PVA-*g*-IA). Performances of PVA and PVA-*g*-IA membranes for transdermal release of salicylic acid at *in vitro* conditions were investigated by using 2.0 mg/ml salicylic acid solutions. Effect of the pH on the release of salicylic acid was studied by keeping the pH of donor and acceptor solutions in a range between 2.1-7.4. Permeation studies were conducted with different salicylic acid concentrations. The effect of temperature on the release of salicylic acid was investigated in the temperature range between 32-39 ± 1°C. Results showed that the presence of IA decreased the release of salicylic acid from the PVA membranes, and 73% salicylic acid was released at the end of 48 hrs at 32 ± 1°C from the IA-1 membranes. Variation in pH affected the release of salicylic acid through the grafted membranes and studies showed that release of salicylic acid was high in a donor solution with a pH of 2.1. When the pH of the donor and receiver solutions were kept at the same value, the overall percentage of salicylic acid that permeated increased. Increase in concentration of salicylic acid decreased the release of salicylic acid for the studied membranes. Release of salicylic acid from PVA-*g*-IA membranes was temperature sensitive and increase in temperature from 32 ± 1°C to 39 ± 1°C increased the release percentage of salicylic acid by 24%. The overall activation energy for the permeation of salicylic acid through IA-1 membrane was found to be 22.97 kJ/mol (Asman *et al.*, 2007:3291–3299).

2.8 TRANSDERMAL DRUG DELIVERY

Topical drug delivery is a non-invasive and painless way of administering active drugs and leads to greater patient compliance. Over the last decade, topical drug delivery systems such as transdermal patches, liposomes and enhancement of drug delivery across the skin via penetration enhancers and iontophoretic techniques, have opened the way for the administration of many more existing compounds that were previously unable to be delivered via the topical route. These new generation topical drug delivery systems also have the ability to release the active drug closer to, and at a more controlled rate of delivery, to the active *therapeutic site* (Danckwerts, 1991:314). The last ten years of research conducted on topical drug delivery have produced more breakthroughs than any other decade. It has resulted in the marketing of many new delivery systems such as nitro-glycerine, scopolamine, steroids, and cardiovascular drugs. The advances in the understanding of drug delivery through the skin, the development of controlled release patches and liposome formulations, as well as the use of

iontophoresis and penetration enhancers, have opened up the range of drugs that can be effectively delivered via topical application (Danckwerts, 1991:315).

Topical transdermal drug delivery systems have the following advantages over other traditional drug delivery systems:

- Transdermal patches provide constant and controlled levels of drug in the plasma for long periods of time.
- Drugs, sensitive to the hepatic first-pass metabolic degradation, can be applied via the topical route to prevent first-pass metabolic degradation.
- As it is a painless, non-invasive means of taking medicine, patient compliance is significantly improved.
- Therapy can be discontinued in a simple and rapid manner if problematic situations of adverse drug reactions occur.
- Drugs that are sensitive to acid hydrolysis in the stomach, when delivered orally, may possibly be reformulated as a stable and effective transdermal drug delivery system.
- Drugs can be delivered much closer to their therapeutically active site/s.
- Side-effects are reduced due to the number and concentration of doses which are reduced with controlled release transdermal drug delivery systems (Danckwerts, 1991:315).

The disadvantages associated with transdermal drug delivery relate primarily to the possible activation of allergic responses on and in the skin, and to the excellent barrier properties of the skin (Danckwerts, 1991:315).

2.8.1 STRUCTURE AND BARRIER FUNCTION OF THE SKIN

2.8.1.1 ANATOMY AND PHYSIOLOGY

The human skin consists of three tissue layers: the epidermis, the underlying dermis and subcutaneous tissue. Hairy skin contains hair follicles and sebaceous glands. The hairless skin of the soles and palms produce a thick epidermis with a compact stratum corneum, but there are no hair follicles or sebaceous glands (Barry, 2002:501-502). **Figure 7** illustrates a schematic diagram of the skin and its three layers.

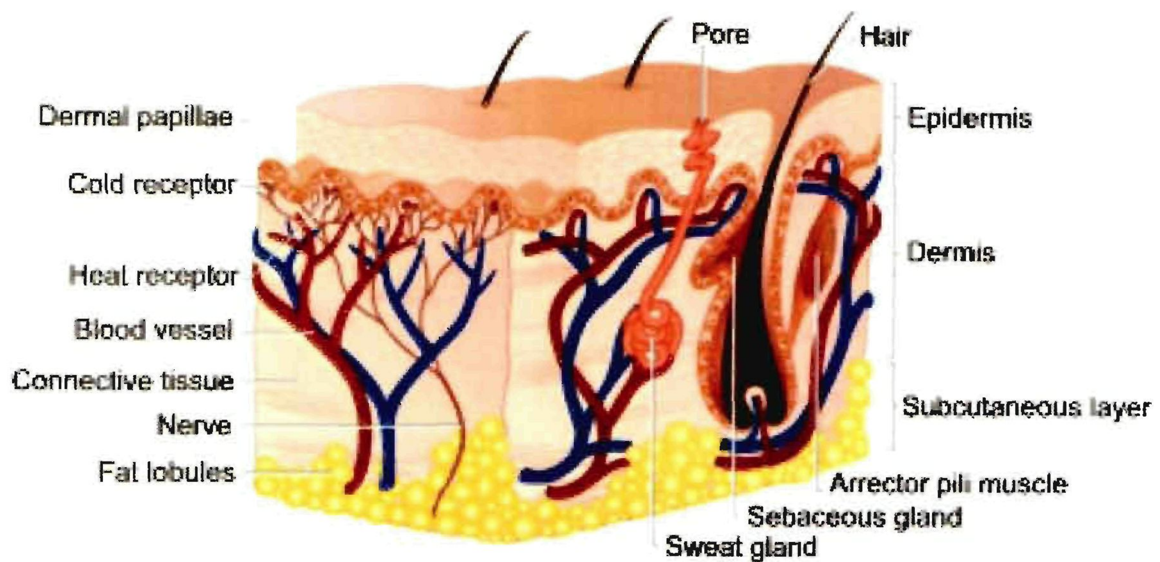

Figure 7: Schematic diagram of human skin broken down into three major layers (Edelson, 2006).

A) THE EPIDERMIS

The multilayered epidermis varies in thickness, ranging from 0.8 mm on the palms and soles to 0.006 mm on the eyelids (Barry, 2002:502; Danckwerts, 1991:315). The cells of the basal layer (stratum germinativum) divide and migrate upwards to produce the stratum corneum or horny layer. Humans survive in a non-aqueous environment because of the almost resistant nature of this dead, dense layer, which is crucially important in controlling the percutaneous absorption of drugs and other chemicals. The stratum corneum may be only 10 µm thick when dry but swells several fold in water. There are two main types of horny layer: the pads of the palms and soles, which are adapted for weight bearing and friction; and the remaining flexible, rather impermeable membranous layer. The basal cell layer also includes melanocytes, which produce and distributes melanin granules to the keratinocytes. Langerhans cells (important in defence mechanisms operated by the immune system) are prominent in the epidermis (Barry, 2002:502).

B) THE DERMIS

The dermis consists of a matrix of connective tissue which in turn consists of fibrous proteins embedded in an amorphous ground substance consisting mainly of mucopolysaccharides. It is approximately 3 - 5 mm in thickness. Many nerves, blood vessels and lymphatic glands span the matrix (Danckwerts, 1991:315). The dermis needs an efficient blood supply to transfer nutrients, remove waste products, regulate pressure and temperature, mobilise defence forces, and contribute to the colour of the skin. Branches from the arterial plexus deliver blood to sweat

glands, hair follicles, subcutaneous fat, and the dermis itself. This supply reaches within 0.2 mm of the skin surface so that it quickly absorbs and systematically dilutes most compounds passing the epidermis. The generous blood volume in the skin usually acts as a 'sink' for diffusing molecules reaching the capillaries, keeping penetrant concentrations in the dermis very low, maximising epidermal concentration gradients, and thus, promoting percutaneous absorption (Barry, 2002:502).

C) THE SUBCUTANEOUS TISSUE

The subcutaneous fat layer of the skin provides a mechanical cushion from external blows and a thermal barrier from external variations in temperature. It also synthesises and stores readily available high-energy chemicals.

In addition to the above three major layers of the skin, the skin has many other appendages including:

- Eccrine sweat glands produce sweat and may also secrete proteins, antibodies, drugs and antigens. The main function is to aid heat control.
- An apocrine sweat gland is a pilobaceous follicle that provides distribution in the adult armpit, breast areola and the perianal region. The oily/milky secretion may be coloured and contains lipoproteins, lipids and saccharides. Bacteria metabolise odourless liquid and they produce body odour.
- Hair follicles are all over the skin except on the red part of the lips, palms and soles, and parts of the genitalia.
- Sebaceous glands are the most and largest on the face, forehead, inside the ear, and on the midline of the back. It produces sebum from cell disintegration. The main components are glycerides, cholesterol, cholesterol esters, free fatty acids, wax esters, and squalene.
- Nails like hair consist of hard keratin with a high sulphur content, mainly as cysteine that affect the transdermal delivery of drug compounds (Barry, 2002:502).

2.8.1.2 FUNCTIONS OF THE SKIN

The skin performs many varied functions. The two major functions of the skin are: (a) the containment and (b) the protection of the internal organs of the body (Danckwerts, 1991:315).

A) CONTAINMENT FUNCTION

The containment function relates specifically to the ability of the skin to confine the underlying tissues and restrain their movement from place to place. The strength of the skin fabric requisite to performance of the mechanical role is mostly derived from the collagen and elastin fibrous matrix found in the dermis. The skin is tight even when under a resting tension, and it stretches elastically during normal body movements. The elastic character provides for a return to normal contours and condition when stretching ceases, much as a rubber band returns to its original shape after it has been stretched and relaxed. The extensibility of the skin is attributable to the alignment of rigid dermal collagen fibers under tension and in the nature of the dermal structural fibers and changes in their organisation. As a consequence, the skin looses its elasticity and experiences an increase in tensile strength. Eventually, it is stretched beyond its ability to restore itself in wrinkles. Loss of elastically is accelerated through protracted exposure to ultraviolet radiation (sunlight) (Flynn, 1990:273).

B) PROTECTIVE FUNCTION

The skin performs many protective functions of which the following are the most vital for sustaining human life (Danckwerts, 1991:315):

- Microbiological barrier to most micro-organisms,
- Chemical barrier to exogenous chemical compounds,
- Barrier to radiation and electrical shock, and
- Mechanical barrier to impact (Danckwerts, 1991:315).

2.8.2 PERCUTANEOUS ABSORPTION

Percutaneous absorption may be defined as the movement of surface-applied agents through the assorted layers of the skin into systemic circulation. This process is the sum of permeation and penetration of the active ingredient into, and through the layers of the skin (Barry, 1983:531; Schaefer *et al.*, 1982:739-740).

The factors that influence the percutaneous absorption of a drug via the skin are:

- Physico - chemical nature of the drug,
- Timescale of observation,
- Site and condition of the skin,

- Formulation and
- How the vehicle components temporarily change the properties of the stratum corneum (Barry, 2002: 508).

2.8.3 ROUTES OF DRUG PERMEATION ACROSS THE SKIN

When molecules access the intact skin, the diffusant then has three potential entry routes to the viable tissue (Barry, 2002:507). The three potential entry routes for drugs to penetrate through the skin are described as:

- Into the hair follicles with their associated sebaceous glands (transcellular route),
- Into the sweat ducts (intercellular route) or
- Across the stratum corneum between these appendages (appendageal route) (Barry, 2002: 507; Guy & Hadgraft, 1989: 95-96).

The following figure (**figure 8)** displays the three different permeation routes into the human skin (Barry, 2002: S32).

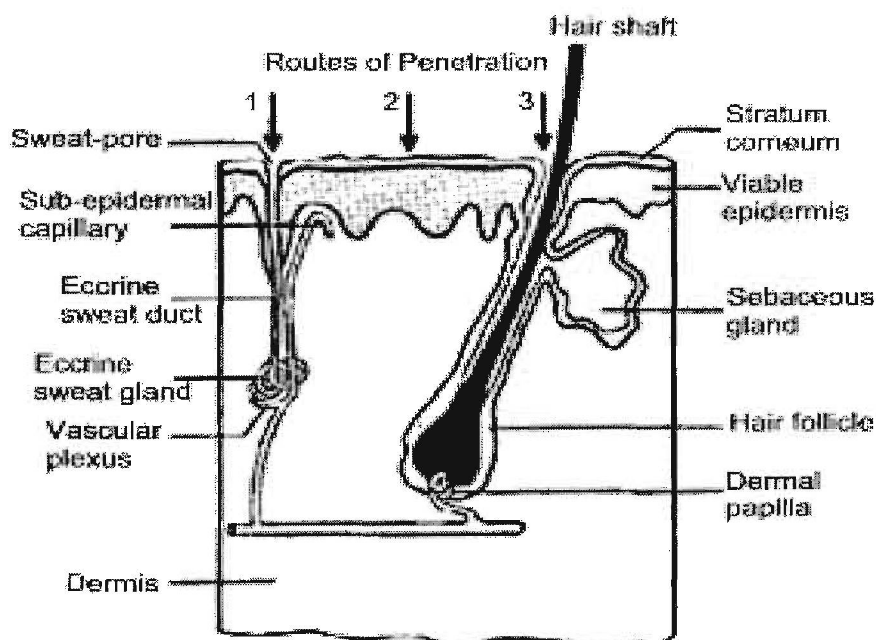

Figure 8: The 3 major routes of penetration through human skin. (1 – Sweat glands, 2 – Trans epidermal, 3 – Hair follicle) (Barry, 2002: S32).

2.8.4 PROPERTIES THAT INFLUENCE TRANSDERMAL DELIVERY

When a preparation is applied to diseased skin the clinical result arises from a sequence of processes:

- Release of the medicament from the vehicle,
- Penetration through the skin barriers and
- Activation of the pharmacological response (Barry, 2002:508).

Effective therapy optimises these steps as they are affected by three components – the drug, the vehicle and the skin. **Figure 9**, which represents the movement of a drug through a rate-controlling membrane, illustrates the complexity of percutaneous absorption. Drug particles must first dissolve so that molecules may diffuse towards the membrane within the patch. The penetrant partitions into the membrane, diffuses across the polymer, and partitions into the skin adhesive. The molecules diffuse towards the vehicle/stratum corneum interface. They then partition into the stratum corneum and diffuse through it. Some drug may bind at a depot site. The remaindering drug permeates further, meets a second interface and partitions into the viable epidermis. For a lipophilic species, this partition coefficient may be unfavourable, i.e. less than 1. Within the epidermis, enzymes may metabolise the drug or may interact at a receptor site (Barry, 2002:509). After passing into the dermis, additional depot regions and metabolic sites may interfere as the drug moves to a capillary, partition into blood for systemic removal. A fraction of the diffusant may partition into the subcutaneous fat to form a further depot. A portion of the drug may furthermore reach deep muscle layers, as illustrated by, for example, the efficacy of non-steroidal anti-inflammatory drugs (Barry, 2002:509). According to Barry (2002:509), the following factors may be important:

- The non-homogeneity of the tissue,
- The presence of lymphatics,
- Interstitial fluid,
- Hair follicles and sweat glands,
- Cell division,
- Cell transport to, and through the stratum corneum, and
- Cell service loss (Barry, 2002:509).

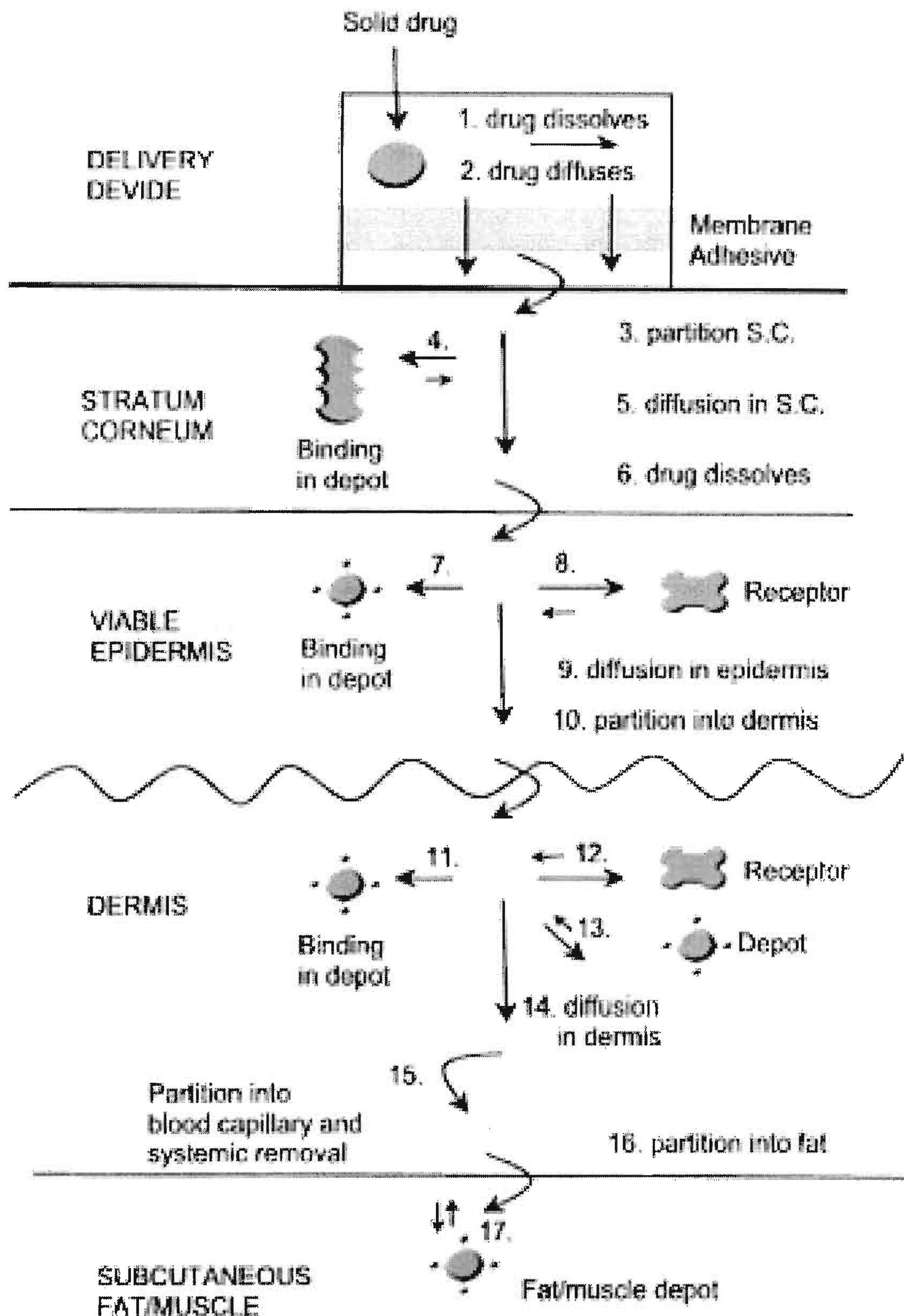

Figure 9: Stages in drug delivery from a transdermal patch (Barry, 2002:509).

The skin barrier may be modified by disease states, the healing processes of these disease states, and drug and vehicle components. As vehicle ingredients diffuse into the skin, cellular debris, sweat, sebum and surface contaminants pass into the dermis, changing its physiochemical characteristics (Barry, 2002:509; Flynn, 2002:202).

2.8.4.1 BIOLOGICAL FACTORS

A) SKIN AGE

The design of a topical drug delivery system must be adapted to suit the physiological conditions under which it may be used. With increasing age, the elasticity and hence, the permeability of skin decrease. Care must be taken when administering topical preparations to young children as their skin are more permeable, and one runs the risk on the one hand of overdosing paediatric patients and underdosing geriatric ones. This means that the concentration of the active ingredient in a paediatric formulation may need to be less than that used for an elderly patient (Pefile & Smith, 1997:147).

Premature infants may be born with no stratum corneum. This can be changed into an advantage when treating breathing difficulties with caffeine; or pain with buprenorphine, via simple topical applications instead of intravenous injection through tiny, delicate veins (Barry, 2002:510)

B) SKIN CONDITION AND SITES

The undamaged, healthy skin provides a tough barrier to absorption, but the function of the barrier can be decreased if the skin is damaged or diseased. The permeability may be increased by damaged, broken or inflamed skin. If the skin is thickened by calluses and corns, it has a decreasing effect on the permeability. Transdermal absorption is also influenced by the area to which the topical preparation is applied and not just by the physical state of the skin. Transdermal drug delivery will be affected by the size of the cells and bilayer lipid composition in the stratum corneum. Diffusion of a substance across the skin is inversely proportional to the thickness of the stratum corneum. Thus, the thinner the epidermal layer, the greater the permeability of the drug through the skin's surface (Pefile & Smith, 1997:147).

C) SKIN METABOLISM AND CIRCULATORY EFFECTS

Skin has the ability to metabolise many drugs, and therefore, reduce their absorption and therapeutic effects. The skin is a metabolically active organ. Below the stratum corneum lies the viable epidermis, which is the most metabolically active layer in the skin. The pharmacological potential of the active drug through cutaneous first-pass effects may be reduced through the percutaneous metabolism in the viable dermis. It is essential to inquire into

the extent of percutaneous absorption that a topical drug undergoes as this will determine the deposition of the substance in other parts of the skin and delivery to the capillaries in the dermis (Pefile & Smith, 1997:147).

D) BLOOD FLOW

Drug absorption from the dermis into the systemic circulation is enhanced by excellent circulation in the dermis. Enhanced blood flow at the absorption site increases the concentration gradient to the dermis through constant removal of the drug from the site. Topical steroids have a vasoconstriction effect on the skin. They reduce their own clearance from the skin due to decreased blood flow at the absorption site (Pefile & Smith, 1997:147).

2.8.4.2 PHYSICOCHEMICAL FACTORS

A) SKIN HYDRATION

Compounds such as water, which hydrate the skin, cause it to swell and, therefore, increase its permeability. Water-in-oil emulsions and lipids in ointments are examples of formulations that prevent water loss and thus keep the skin hydrated (Pefile & Smith, 1997:147).

B) DRUG-TO-SKIN BINDING

The obvious interaction desired between drug and skin is for the drug to act on (or in) the skin to produce a desired pharmacologic action for example sunscreen, topical corticosteroids (Danckwerts, 1991:317; Katz & Poulson, 1971:118).

C) TEMPERATURE

If there is a large temperature variation, the penetration rate of compounds through the human skin can change by tenfold. When the skin increases in temperature it swells and increases in permeability (Danckwerts, 1991:317).

D) DRUG-TO-VEHICLE INTERACTION

The drug-to-vehicle interactions decrease the rate of percutaneous absorption or totally prevent it, for example, the uptake of pentachlorophenol (PCP) was drastically reduced, due to the surfactant in the vehicle that interacted with the skin surface and preventing absorption of the PCP (Baynes *et al.*, 2002:295; Danckwerts, 1991:317;).

E) DIFFUSANT SOLUBILITY

Diffusion is proportional to the concentration gradient across the skin. The topical drug delivery system must therefore be a saturated solution. The level of saturation will depend on the solubility of the active ingredient in the drug delivery system base (Danckwerts, 1991:317).

F) PARTITION CO-EFFICIENT

The higher the partition propensity of the drug for the membrane that it is to diffuse through, the greater the rate of diffusion through the membrane. The stratum corneum to vehicle partitioning is in most cases the rate limiting step in percutaneous diffusion (Danckwerts, 1991:317).

G) PH VARIATION

According to Danckwerts (1991:317), it is usually only the unionised molecules that passes readily across the lipid membrane. Ionised molecules are more aqueous soluble whereas unionised molecules are more lipid soluble. The ionisation of drugs depend on the pH of the vehicle in which the drug is placed, because it will determine whether the drug is ionised or unionised. Buffer solutions can then be added to the formulation to keep most of the active ingredient at a pH in which it is mainly in the unionised form.

H) COSOLVENTS

The solubility of the active ingredient may be increased by cosolvents that produce saturated solutions. However, as the solubility of the drug in the solvent increases, the partitioning of the drug between the membrane and the solvent decreases. It is necessary to keep the solubility of the drug in the vehicle as near to the saturation solubility as possible, but still soluble in the vehicle (Danckwerts, 1991:317).

L) SURFACE ACTIVITY AND MICELLISATION

Micelles are drugs entrapped in a sphere of surface active agents, and can be formed around the active ingredient by the surface active agents that are present in the topical formulation such as creams. This reduces the effective concentration of the drug as well as the concentration gradient that is very important for optimal percutaneous absorption (Danckwerts, 1991:317).

J) COMPLEXATION

The effective concentration of the active ingredient can also be influenced when complexes of drug with other excipients are formed in the formulation. Some of these complexes decrease the partition coefficient of the drug and therefore decrease the percutaneous diffusion. However,

some of the complexes increase the partition coefficient which results in an increase in percutaneous diffusion (Danckwerts, 1991:317).

K) DIFFUSIVITY

Diffusivity depends mostly on the state of matter in which the drug delivery system is in. For example, in gases the diffusion coefficient are large due to the large empty space available to the active drug molecules as compared to their size and the distance the molecule travels to collide with another. Therefore, it can be said that the diffusivity of the active ingredient(s) decreases from gaseous formulations > lotions > creams > powdered solid formulations (Danckwerts, 1991:317).

2.8.4.3 IDEAL MOLECULAR PROPERTIES OF DRUG PENETRATION

From the above physiological properties it can be determined that the ideal properties for a molecule to optimally penetrate the stratum corneum are:

- A low molecular mass (lower than 600 Da), when the diffusion coefficient tends to be high,
- An adequate solubility in oil and water, so that the concentration gradient in the membrane can be high,
- A balanced partition coefficient, and
- A low melting point. This correlates with ideal solubility (Barry, 2002:513).

2.8.4.4 MATHEMATICS OF SKIN PERMEATION

A) THE DIFFUSION PROCESS

Passive diffusion can be defined as the movement of matter from one region of a system to another, following random molecular motions. The basic hypothesis underlying the mathematical theory for isotropic materials is that the rate of transfer of diffusing substances per unit area of a section is proportional to the concentration gradient measured to the section. This is expressed as Fick's First Law of diffusion (Barry, 2002: 506). **Equation 1** describes it as:

$$J = -D\frac{\partial C}{\partial x}$$

Equation 1: Ficks's First Law (Barry, 2002:506).

J is the flux (the rate of transfer per unit area of surface),

C is the concentration of diffusing substance,

x is the space coordinate measured normal to the section and

D is the diffusion coefficient (Barry, 2002:506).

The negative sign indicates that the flux is in the direction of decreasing concentration. In many situations D is constant, but in more complex materials D is dependent on the concentration. D consists of two components namely $(\text{length})^2$ and $(\text{time})^{-1}$; and it is specified as $cm^2.s^{-1}$ (Barry, 2002:506).

Fick's First law contains three variables, J, C and x, where, J is additionally a multiple variable (dm/dt), m is amount and t is time (Barry, 2002:506).

Therefore, Fick's second law employed, which reduces the number of variables by one. For the common experimental situation in which diffusion is unidirectional, Fick's second law is expressed in **Equation 2** as (Barry, 2002:506):

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial x^2}$$

Equation 2: Fick's Second Law (Barry, 2002: 506)

Many experimental designs employ a membrane separating two compartments, with a concentration gradient operating during a run and 'sink' conditions prevailing in the receptor compartment. **Figure 10** can be obtained if the cumulative mass of the diffusant (m) which passes per unit area through the membrane is measured as a function of time (t). The plot approaches a straight line as time runs on and from its slope we obtain a steady flux dm/dt (**Equation 3**) (Barry, 2002:506):

$$\frac{dm}{dt} = \frac{DC_0K}{h}$$

Equation 3: Flux values (Barry, 2002:506)

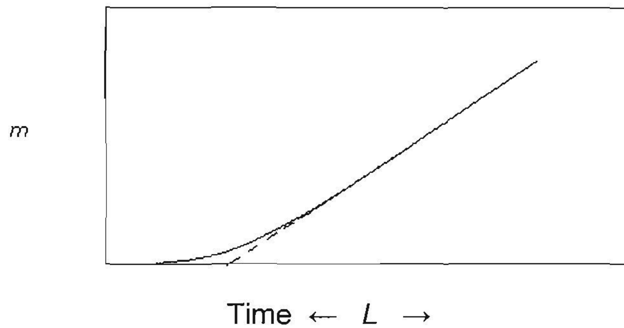

Figure 10: The time course of absorption for the simple zero-order flux case obtained by plotting *m*, the cumulative amount of diffusant crossing the unit area of the membrane, as a function of time. Steady state is achieved when the plot becomes linear, extrapolation of the linear portion to the time axis yields the lag time *L*.

In equation 3 C_0 is the constant concentration of drug in the donor solution. *K* is the partition coefficient of the solute between the membrane and the bathing solution and *h* is the thickness of the membrane (Barry, 2002:506).

If a steady-state plot is extrapolated to the time axis, the intercept obtained at $m = 0$, is the lag time, *L* (**Equation 4**) (Barry, 2002: 506):

$$L = \frac{h^2}{6D}$$

Equation 4: Lag time (Barry, 2002:506).

From the above equation, D is estimated, provided that the membrane thickness, h, is available. Knowing these parameters, C_0 and measuring *dm/dt*, equation 3 depicts one way of assessing K. Equation 3 depicts why this permeation procedure may be referred to as a zero order process. By analogy with chemical kinetic operations, equation 3 represents a zero-order process with a rate constant of *DK/h* (Barry, 2002:506).

With biological membranes (such as the skin) it is difficult to separate the value of *D* from that of *K*. A composite parameter is therefore used, the permeability coefficient, *P*, where $P = KD$ or $P = KD/h$. The last equation is used when h is uncertain (Barry, 2002:506).

B) COMPLEX DIFFUSING BARRIERS

B.1) BARRIERS IN SERIES

The equations above only deal with simple situations in which diffusion occurs in a single medium of identical structural and diffusing properties in all directions. Nevertheless, the skin is a diverse multilayered tissue and in percutaneous absorption the concentration gradient evolves over the many layers of the skin. Each layer confers a diffusing resistance (R). The total resistance through a three layered heterogeneous membrane (skin) (**Equation 5**) is (Danckwerts, 1991:316):

$$Rt = \frac{1}{Pt} = \frac{h_1}{D_1K_1} + \frac{h_2}{D_2K_2} + \frac{h_3}{D_3K_3}$$

Equation 5: The total resistance (Barry, 2002:507).

Rt = total diffusing resistance of the layers and Pt = thickness-weighted permeability co-efficient of the various layers (Danckwerts, 1991:316).

B.2) BARRIERS IN PARALLEL

If a molecule reaches unbroken skin, it contacts cellular debris, sebum, other exogenous materials and micro-organisms. There are three routes to the viable tissue that the drug is likely to enter:

- Down and through the hair follicles,
- Down and through the sweat ducts, and
- Across the stratum corneum (Danckwerts, 1991:316).

Other glands and hair follicles transverse human skin and can be considered as different diffusing pathways all linked in parallel. For calculation objectives the diffusion rates through these barriers are treated each on their own, due to the fact that they are not mutually dependent on each other's diffusion rates. When the drug is preferred to reach the sub dermal layers all at once, the barriers in parallel are preferred (Danckwerts, 1991:316).

2.8.4.5 PENETRATION ENHANCERS

A large amount of research went into finding materials to increase the penetration rate of drugs, due to the significant slow transdermal absorption. These materials are called penetration

enhancers (Washington *et al.*, 2001:191). Several technological advances have been made to overcome the skin barrier properties. This includes iontophoresis, sonophoresis, micro needles, penetration enhancers, liposomal vesicles and enzyme inhibition (Thong *et al.*, 2007:272).

A) PHYSICAL PENETRATION ENHANCERS

The mechanism of the physical penetration enhancers is mainly to transiently circumvent the normal barrier function of the stratum corneum and to allow passage of macromolecules. In **Table 5** the different physical methods of penetration enhancement are listed (Thong *et al.*, 2007:273).

Table 5: Physical methods of penetration enhancement (Thong *et al.*, 2007:273).

Method	Definition	Mechanism(s)	Example of drugs
Ionotophoresis	The electrical driving of charged molecules into tissue by passing a small direct current through a drug-containing electrode in contact with the skin	(1) Electrical repulsion from the driving electrode drives charged molecules (2) The flow of electric current enhances skin permeability (3) Electro-osmosis affects uncharged and large polar molecules	Calcitonin, trans-nail delivery of salicylic acid, transdermal delivery of peptides, proteins and oligonucleotides
Electroporation	A method of reversibility permeabilising lipid bilayers by the application of an electric pulse	Application of short (micro- to millisecond) electrical pulses of ~100 – 1,000 V/cm creates transient aqueous pores in the lipid bilayers	Methotrexate, timolol, fentanyl, tetracaine, nalbuphine, cyclosporin-A
Sonoporation	Ultrasound-mediated delivery of therapeutic agents into biological cells	(1) low energy frequency disturbs the lipid packing in the stratum corneum by cavitation (2) shock waves increase free volume space in bimolecular leaflets, thus enhancing permeation	Insulin, cutaneous vaccination, transdermal heparin delivery, transdermal glucose monitoring, delivery of acetyl cholinesterase inhibitors for the treatment of Alzheimer's disease, treatment of bone disease and Peyronie's disease and dermal exposure assessment
Micro needle-enhanced delivery	A method using arrays of microscopic	Bypasses the stratum corneum and delivers drugs	Oligonucleatide, insulin, protein vaccine, DNA vaccine, methyl nicotinate

system	needles to open pores in the stratum corneum, thus facilitating drug permeation	directly to the skin capillaries, also has the advantage of being too short to stimulate the pain fibres	

B) BIOLOGICAL PENETRATION ENHANCEMENT

The biological penetration enhancers include chemical modification, prodrug molecules, enzyme inhibition and the usage of a vesicular system or colloidal particles. Liposomes (phospholipids-based artificial vesicles) and niosomes (non-ionic surfactant vesicles) are broadly used to enhance drug delivery across the skin. Proliposomes and proniosomes are converted with simple hydration to liposomes and niosomes and also used in topical drug delivery (Thong *et al.*, 2007:273-275).

A new type of liposomes has been introduced into the transdermal drug delivery system; namely transferosomes. It consists out of phospholipids, cholesterol, and additional activators such as sodium cholate (surfactant molecules). The inventors claim that 200 to 300 nm-sized transferosomes are ultra deformable and can fit through pores less than one tenth of diameter. They are furthermore able to penetrate through intact skin (Thong *et al.*, 2007:273-275).

Ethosomes are liposomes with high ethanol content of up to 45%. They penetrate the skin and enhance the delivery of the active ingredient to the deep skin strata. The mechanism suggested, is that the ethanol fluidises both ethosomal lipids and lipid bilayers in the stratum corneum, allowing the soft vesicles to penetrate through the disorganised lipid bilayers (Thong *et al.*, 2007:273-275).

According to Thong *et al.* (2007:273-275), six potential mechanisms of action for these colloidal carriers have been proposed:

- Penetration of the stratum corneum by a free drug process – drug release from vesicle and then penetrate skin independently,
- Penetration of the stratum corneum by intact liposomes,
- Enhancement due to release of lipids from carriers and interaction with stratum corneum lipids,
- Improved drug uptake by skin,
- Different enhancement efficiencies control drug input, and

- The role of protein requires elaboration.

C) CHEMICAL PENETRATION ENHANCERS

These are substances that help promoting drug diffusion through the stratum corneum and the epidermis. They are referred to as penetration enhancers. Drug transport is improved by reducing the resistance of the stratum corneum to drug permeation (Thong *et al.*, 2007:275). Lambert *et al.* (1993:181), grouped most penetration enhancers into three classes:

- Solvents and hydrogen bond acceptors,
- Simple fatty acids and alcohols, and
- Weak surfactants containing a moderately sized polar group.

In **Table 6** the different chemical penetration enhancers are listed (Thong *et al.*, 2007:276).

Table 6: The classification of penetration enhancers (Thong *et al.*, 2007:276).

Category and examples	**Mechanism**	**Examples of drugs**
Sulphoxides		
Dimethylsulphoxide (DMSO)	(1) Increase lipid fluidity (2) Promote drug partitioning	DMSO: theophylline, salicylic acid, testosterone, scopolamine, antimycotics, fluocinolone acetonide, flufenamic acid
Decylmethylsulphoxide (DCMS)	Protein – DCMS interactions, resulting in a change in protein conformation, creating aqueous channels	DCMS: methotrexate, naloxone, pyridostigmine bromide, hydrocortisone, progesterone
Alkanones N-heptane, N-octane, N-nonane, N-decane, N-undecane, N-dodecane, N-tridecane, N-tetradecane, N-hexadecane	Extensive barrier alteration of stratum corneum	Propanolol, diazepam
Alcohols		
Alkanol Ethanol, propanol, butanol, 2-butanol, pentanol, 2-pentanol, hexanol, octanol, nonanol, decanol, benzyl alcohol	(1) Low-molecular weight alkanols (C≤ 6) may act as solubilising agents. (2) More hydrophobic alkanols may extract lipids from the stratum corneum, leading to increase diffusion.	E: tacrine, metrifonate, dichlorvos, ketolorac, nitroglycerin, tazifylline, betahistine, cyclosporine A

Fatty alcohol Caprylic, decyl, lauryl (LA), 2-lauryl, myristyl, cetyl, stearyl, oleyl, linoleyl, linolenyl alcohol		LA: buprenorphine
Polyols Propylene glycol(PG), Polyethylene glycol (PEG), ethylene glycol, diethylene glycol, triethylene glycol, dipropylene glycol, glycerol (G), propanediol, butanediol, pentanediol, hexanetriol	PG may solvate α-keratin and occupy hydrogen bonding sites, reducing drug-tissue binding	PG: 5-fluorouracil, tacrine, ketorolac, isosorbide dinitrate, clonazepem, albuterol, verapamil, betahistine, estradiol, dihydroergotamine, methotrexate, steroids, midazolam maleate, diazepam PEG: terbutaline G: diazepam, terbutaline, 5-fluorouracil
Amides		
Urea, dimethylacetamide (DMA), diethyltoluamide, dimethylformamide (DMF), dimethylactamide, dimethyldecamide	Urea: hydration of stratum corneum, keratolytic, creating hydrophilic diffusion channels DMA/DMF: low concentration: partition to keratin, High concentration: increases lipid fluidity, disrupt lipidpackaging	Urea: ketoprofrn, 5-fluorouracil DMA/DMF: griseofluvin, betamethasone 17-benzoate, caffeine
Pyrrolidone derivates 1-methyl-2-pyrrolidone (1M2P), 2-pyrrolidone, 1-lauryl-2-pyrrolidone, 1-methyl-4-carboxy-2-pyrrolidone, 1-hexyl-4-carboxy-2-pyrrolidone, 1-lauryl-4-carboxy-2-pyrrolidone, 1-methyl-4-methoxycarbonyl-2-pyrrolidone, 1-hexyl-4-methoxycarbonyl-2-pyrrolidone ect.	Interact with both keratin in the stratum corneum and with lipids in the skin structure	1M2P: griseofulvin, theophylline, tetracycline, ibuprofen, betamethasone 17-benzoate NMP: prazosin
Cyclic amides 1-dodecylazacycloheptane-2-one (Azone), 1-geranylazacycloheptan-2-one, 1-farnesylazacycloheptan-2-one ect.	Azone: (1) affects lipid structure of SC (2) increases partitioning (3) increases membrane fluidity	Azone: 5-fluorouracil, antibiotics, glucorticoids, peptides, clonazepam, albuterol, estradiol, levonorgestrel, HIV protease inhibitor (LB-71148), betahistine, dihroergotamine
Fatty acids		

Linear Linoleic (LIA), valeric, heptanoic, pelagonic, caproic, capric (CA), lauric (LAA), myristic, stearic, oleic (OA), caprylic	Selective perturbation of the intercellular lipid bilayers OA: decrease the phase transition temperatures of lipid, increasing motional freedom or fluidity of lipids	Naloxone, mannitol, betamethasone 17-benzoate, hydrocortisone, acyclovir, nitroglycerin OA: galanthamine, estradiol, levonorgestrel CA: buprenorphine, albuterol LAA: buprenorphine, betahistine
Fatty acid esters		
Aliphatic Isopropyl n-butyrate, isopropyl n-hexanoate, isopropyl n-decanoate, isopropyl myristate (IPM), isopropyl palmitate, octyldodecyl myristate	IPM: direct action on the SC, permeating into liposome bilayers, increasing fluidity Aliphatic: increases diffusivity in the stratum corneum and/or the partition coefficient	IPM: galanthamine, ketorolac, chlorpheniramine, dexbrompheniramine, diphenhydramine, theophylline, pilocarpine, verapamil
Alkyl Ethyl acetate (EA), butyl acetate, methyl acetate ect.	Alkyl : increases lipid fluidity (similar to DMSO)	EA: levonorgestrel, 17β – estradiol, hydrocortisone, 5-fluorouracil, nefedipine
Surfactants		
Anionic Sodium laurate, sodium lauryl sulfate, sodium octyl sulfate	Alter the barrier function of stratum corneum, allowing removal of water-soluble agents that normally act as plasticisers	
Cationic Cetyltrimethylammonium bromide, tetradecyltrimethylammonium bromide ect.	Adsorb at interfaces and interact with biological membranes, causing damage to the skin	
Non-ionic's Polyxamer (231, 182, 184), Polysorbate (20, 60), Brij (30, 93, 96, 99), Span (20, 40, 60, 80, 85), Tween (20, 40, 60, 80), Myrj (45, 51, 52), Miglyol 840	Emulsify sebum, enhancing the thermodynamic activity of coefficients of drugs	Tween 80: ketoprofen Polysorbate 20, 60: lidocaine
Terpenes Hydrocarbons • D-limonene, α-pinene, β-carene Alcohols • α -Terpineol,	(1) Increases diffusivity of drugs within stratum corneum due to disruption of intercellular lipid barrier (2) Opens new polar	5-Fluorouracil, aspirin, haloperidol

terpinen-4-ol, carvol Ketones • Carvone, pulegone, piperitone, menthone Oxides • Cyclohexene oxide, limonene oxide α-pinene oxide, cyclopentene oxide, 1,8-cineole Oils • Ylang ylang, anise, chenopodium, eucalyptus	pathways within and across the stratum corneum	
Cyclodextrins 2-hydroxypropyl-β-cyclodextrin (HPβCD), 2,6-dimethyl-β-cyclodextrin (DIMEB)	Forms inclusion complexes with lipophilic drugs and increase their solubility in aqueous solution	Liarzole

According to Thong *et al.* (2007:280), the ideal characteristics for penetration enhancers include the following:

- Chemically and pharmacologically inert.
- Chemically stable.
- High degree of potency with specific activity, rapid onset, predictable duration of activity and reversible effects on skin properties.
- Chemical and physical compatibility with formulation and system components.
- Non-irritant, non-allergenic, non-phototoxic and non-comedogenic.
- Odourless, tasteless, colourless, cosmetically acceptable, and inexpensive.
- Readily formulated into dermatological preparations, transdermal patches, and skin adhesives, and
- A solubility parameter approximating that of the skin.

2.9 PHEROID™ TECHNOLOGY IN AID OF OPTIMAL DELIVERY OF SALICYLIC ACID AND NIACINAMIDE

There are several delivery technologies available, but most of them have disadvantages such as stability problems, low solubility and bioavailability, high cost to the market, and a limited field of application (Grobler *et al.*, 2008:284).

The Pheroid™ technology is based on the previously called Emzaloid™ technology. Piet Meyer was the initial developer of the technology for the treatment of his own psoriasis and the intellectual property on which Pheroid™ technology is based was purchased by the North-West University of South-Africa in December 2003 (Grobler *et al.*, 2008:284).

The Pheroid™ is a stable structure within a system which can be manipulated in terms of morphology, structure, function and size. It consists mainly of plant and essential fatty acids and has the ability to entrap, transport and deliver pharmacologically active compounds as well as other useful molecules (Grobler, 2004:6).

2.9.1 STRUCTURAL CHARACTERISTICS OF PHEROIDS™

The Pheroid™ delivery system is a colloidal system that contains stable and unique lipid-based submicron- and micron-sized structures, also known as Pheroids™, which are evenly distributed in a dispersion medium that may be adapted to a specific indication. Pheroids™ are typically formulated to have a diameter of between 200nm and 2µm (Grobler *et al.*, 2008:285).

The parameters of the Pheroid™, i.e. the type and diameter, may be determined by the amount and size of the active ingredient to be entrapped; the rate of delivery; and the administration route (Grobler *et al.*, 2008:285).

Pheroids™ primarily consist of ethylated and pegylated polyunsaturated fatty acids, including omega-6 and omega-3 fatty acids (Grobler *et al.*, 2008:285), linoleic acid, linolenic acid as well as oleic acid (Sanders *et* al., 1999:99), however, arachidonic acid is excluded (Grobler *et al.*, 2008:285). Due to *cis*-formation of the fatty acids in the Pheroid™, it is compatible with the orientation of fatty acids in the human body (Grobler *et al.*, 2008:285).

Nitrous oxide, (N_2O), is a unique component of the Pheroid™. Nitrous oxide forms the dispersed gas phase to the oil and water phase; adding another dimension to the basic Pheroid™ (Grobler *et al.*, 2008:289). There are 3 functions of the nitrous oxide, namely:

- Contributing to the miscibility of the fatty acids in the dispersal medium.
- Contributing to the self-assembly process of the Pheroids™, and

- Contributing to the stability of formed Pheroids™ (Grobler *et al.*, 2008:289).

All Pheroid™ formulations contain a tocopherol or tocopherol-based molecule, which is also known as vitamin E or vitamin E derivatives. These molecules are used as emulsion stabilisers as well as anti-oxidants. They are also used to prevent oxidation of unsaturated fatty acyl residues of membrane lipids (Grobler *et al.*, 2008:293).

Various molecular modellings indicate that there is some interaction between the fatty acids and the nitrous oxide, resulting in stable vesicular Pheroid™ structures. This matrix provides a functional model for the transport of hydrophobic and hydrophilic drugs (Grobler *et al.*, 2008:289). In controlled experiments on various formulations, it was established that if either nitrous oxide or essential fatty acids were absent in the formulations, the stability and efficacy of the formulation were decreased significantly (Grobler *et al.*, 2008:289).

A diverse investigation into the different Pheroid™ formulations has shown that the structural and functional characteristics of Pheroids™ can be manipulated by:

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

2.9.2 FUNCTIONAL CHARACTERISTICS OF PHEROIDS™

Delivery and efficacy of an active compound to the skin involve different processes and depend on the target of delivery – both the tissue and the cell type (Grobler *et al.*, 2008:293).

2.9.3 PLIABILITY

Due to the use of pliable pegylated tails, as well as gas added to the fatty acids, extremely elastic structures are formed. Pheroids™ are not shattered under moderate pressure or extravascation (Grobler *et al.*, 2008:294).

2.9.4 ENTRAPMENT EFFICIENCY (EE)

The entrapment efficiency of Pheroid™-based products is generally determined by the confocal laser scanning microscopy (CLSM) (Grobler, 2004:6). The Pheroid™ as well as the active compounds are visualised through fluorescence labelling. An entrapment efficiency of more than 90% was set for all products in the development phase (Grobler *et al.*, 2008:294).

The size, charge and solubility of the active compound determine the number of molecules trapped within one Pheroid™ (Grobler *et al.*, 2008:294-295). Drugs that have different solubilities, or insoluble drugs, can be entrapped by the polyphillic Pheroid™ (Grobler, 2004:11).

2.9.5 PENETRATION EFFICIENCY

Recent studies have shown that the percentage of active compound delivered to the skin is enhanced by the entrapment of the Pheroids™. The efficiency of penetration can be measured in a number of ways; the comparative investigation is the easiest of all, were the new formulations are compared to existing commercial products (Grobler *et al.*, 2008:296). The Pheroid™ can easily transverse the stratum corneum with its entrapped compound (Grobler *et al.*, 2008:297).

2.9.6 THE UPTAKE OF PHEROIDS™ AND ENTRAPPED COMPOUNDS BY CELLS

Pheroids™ have been shown to cross capillary walls. The dense stratum corneum offers a similar challenge and it is thought that fluidity of the Pheroid™ membrane contributes to efficient dermal and transdermal delivery (Grobler *et al.*, 2008:297).

Uptake of Pheroids™ by cells may be influenced by the Pheroid™ formulation as well as by the mechanism of uptake by the cells. The permeation of the Pheroid™ formulation is determined by one or more of the following factors:

- The molecular geometry of the fatty acids themselves.
- The concentration and ratios of various fatty acids.
- The hydration medium.
- The pH of the preparation.
- The presence of charge-charging molecules.
- The presence of molecules that influence the electrostatic milieu.
- The character and concentration of the active or drug.

- The state of the Pheroids™.
- The size of the Pheroids™, and
- The morphology of the Pheroids™ (Grobler *et al.*, 2008:298-299).

Since the Pheroid™ consists of fatty acids, there is an affinity between the Pheroid™ and the cell membranes. A specific family of proteins in a cell membrane is responsible for the binding and uptake of essential fatty acids. This causes the Pheroid™ to interact with the cell membrane and follow the endosome sorting mechanism which results in penetration and delivery (Grobler, 2004:10).

2.9.7 TARGETING, METABOLISM AND DISTRIBUTION

As opposed to small molecule drug compounds that have large volumes of distribution on administration; the entrapment of active compounds in the Pheroid™, reduces the volumes of distribution, and as a result, the concentration at the target site is increased. An enhanced, but narrow therapeutic index, may be achieved with a decrease in a specific toxicity (Grobler, 2004: 12).

Pheroids™ can be metabolised in either the mitochondria or the peroxisomes of the cell depending on the composition. This results in the release of the active compound (Grobler *et al.*, 2008:300).

Through the use of different combinations of fatty acids, or through adding molecules, Pheroids™ at subcellular levels are targeted (Grobler, 2004:10).

The Pheroid™ protects the drugs from metabolism, opsonisation (make susceptible to destruction), and inactivation in the plasma or other body fluids (Grobler, 2004:12).

2.9.8 THERAPEUTIC EFFICACY

Formulations of active compounds in Pheroids™ have shown to increase the efficacy of a number of such active compounds. Even the effect of essential oils on the skin is enhanced by entrapment in Pheroids™ (Grobler *et al.*, 2008:300).

2.10 SUMMARY

Acne affects as many as 80% of young adults and adolescents all over the world. This detrimental is condition classified into four stages: (a) open comedo (blackhead), (c) closed comedo (whitehead), (c) papule and (d) pustule (Russell, 2000:357-358). There are various factors that can lead to acne outbreaks which include: (a) hormone level changes during the

menstrual cycle in women, (b) certain drugs (ie, lithium), (c) certain cosmetics and (d) environmental conditions such as humidity (University of Maryland, 2009:1)

In this study niacinamide and salicylic acid were chosen in combination, due to the beneficial effects that they have on acne. Niacinamide has an anti-inflammatory action on acne; which reduces redness, dryness and irritation caused by *Propioni-bacterium acnes* that live in the clogged pores of pimples (Acnetreatmentlab, 2008:1). Niacinamide is highly soluble in PBS (212.95 mg/ml at pH 7.4), with a low molecular weight, and a low melting point. According to the log D value (-0.32), niacinamide will not be able to permeate optimally through the skin.

Salicylic acid on the other hand is a keratolytic and keratoplastic agent (SAMF, 2005:177). It is used in combination with other ingredients to enhance the shedding of corneocytes (Zander & Welsman, 1992:247). Salicylic acid is soluble in PBS (4.07 mg/ml at pH 7.4), with a low molecular weight and a low melting point. The log D value (0.33) of this active ingredient also indicates that it will not permeate optimally.

Since acne treatment is generally targeted in the dermis and epidermis, the fact that both niacinamide and salicylic acid do not permeate the skin well, may be an advantage in treating acne successfully.

The objective of this study was therefore to investigate the *in vitro* release and transdermal diffusion of salicylic acid and topical niacinamide, with the assistance of the Pheroid™ technology.

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CHAPTER 3: ARTICLE FOR PUBLICATION IN THE INTERNATIONAL JOURNAL OF PHARMACEUTICS

Release and diffusion of salicylic acid and niacinamide from various formulations

Sarita Jacobs, Jeanetta du Plessis, Joe M Viljoen, Minja Gerber, Jan L du Preez*

Unit for Drug Research and Development, North-West University, Private Bag X6001, Potchefstroom 2520, South Africa

*Corresponding author. Tel.: +27 18 299 2236; Fax: +27 18 299 2225. E-mail address: Jan.duPreez@nwu.ac.za (J du Preez)

Abstract

Acne affects as many as 85% of young adults and adolescents all over the world. This dermatological disorder causes reduced self-esteem and emotional distress to patients suffering from acne. The disorder involves abnormalities in sebum production, follicular epithelial desquamation, bacterial proliferation, and inflammation. Niacinamide and salicylic acid were chosen in combination for this study, due to the beneficial effects that they have on acne. For this study four preparations combining niacinamide and salicylic acid (Pheroid™ cream, Pheroid™ gel, cream and gel) were developed. These preparations were evaluated with respect to stability (assay, pH, rheology, visual assessment, mass variation, confocal laser scanning microscopy) and diffusion behaviour. Stability results indicated that the preparations were unstable with regard to pH and assay. None of the preparations exhibited a significant variation in mass. Entrapment of the active ingredients into the Pheroid™ system could not be established due to the small particle size of the Pheroid™. Confocal laser scanning microscopy could only confirm homogeneity of the formulation and the absence of crystals. No significant enhancement in the delivery of niacinamide and salicylic acid by the Pheroid™ system could be demonstrated with diffusion studies.

Keywords: Niacinamide, Salicylic acid, Pheroid™, Acne, Stability testing, Topical delivery

1. Introduction

Acne vulgaris is the most common dermatological disorder, affecting approximately 85% of individuals between the age of 12 and 24 years. However, 8% of adults between the age of 25 and 34 years, and 3% in the age group 35 to 44 years also suffer from this disease (Leyden, 2003). The disease can be psychologically and physically scarring. Patients presenting with acne may experience reduced self-esteem and emotional distress (Leyden, 2003).

Abnormal peeling of the follicular tissue which results in the obstruction of the pilosebaceous canal causes acne. This obstruction leads to the formation of non-inflammatory lesions (comedones). The comedones then become inflamed as a result of the overgrowth of *Propioni-bacterium acnes* (Russell, 2000). The problem of acne may be resolved if a preparation is formulated which acts on all four of the topical treatment paths that include – *P. acnes*; hyperkeratosis; sebum production and inflammation (Berson & Shalita, 1995).

In this study, salicylic acid and niacinamide were combined in different formulations in order to provide the best possible results during acne treatment. Niacinamide provides an anti-inflammatory action that reduces redness, dryness and irritation caused by the bacteria living in clogged pores of pimples. Niacinamide was also found to reduce the sebum excretion rate and is a mild keratolytic agent. It also keeps the skin hydrated by preventing water loss, and thus, acts as a moisturiser (Acnetreatmentlab, 2008). Salicylic acid on the other hand is a topical keratolytic agent with anti-inflammatory effects (SAMF, 2005). Hence a product containing both niacinamide and salicylic acid will be able to target several of the four topical treatment paths of acne.

The Pheroid™ technology is based on the previously called Emzaloid™ technology, which was purchased by the North-West University of South-Africa in 2003 (Grobler et al., 2008). The Pheroid™ is a stable structure within a system that can be manipulated in terms of morphology, structure, function and size. It consists mainly of plant and essential fatty acids such as omega-3 and -6 (Grobler et al., 2008) as well as linoleic acid and oleic acid (Sanders et al., 1999). Due to the *cis*-formation of fatty acids in the Pheroid™, it is very compatible with the orientation of the fatty acids in the human body (Grobler et al., 2008). Nitrous oxide (N_2O) is also a very important

component in the Pheroid™ system. It contributes to the miscibility of fatty acids in the dispersal medium; as well as to the self-assembly process of the Pheroid™ and to the stability of formed Pheroid™ (Grobler et al., 2008). Pheroid™ has the ability to entrap, transport and deliver pharmacologically active compounds as well as other useful molecules (Grobler, 2004). The formulation of active compounds in Pheroid™ has been shown to increase the efficacy of a number of such active compounds. Even the effect of essential oils on the skin is enhanced by the entrapment in Pheroid™ (Grobler et al., 2008).

The aim of the study was to formulate four different acne preparations (Pheroid™ cream, Pheroid™ gel, cream and gel); combining niacinamide and salicylic acid; evaluating some stability parameters for the different formulations; and comparing the diffusion ability of these formulations.

2. Materials and Methods

2.1 Materials

Salicylic acid and niacinamide were purchased from Sigma-Aldrich (Johannesburg) South-Africa Ltd. HPLC grade methanol was purchased from Merck Chemicals (Wadeville, Gauteng) South-Africa Ltd. All the other chemicals and reagents that were used were of analytical grade.

2.2 Methods

2.2.1 Formulations prepared

Four acne preparations were formulated, combining salicylic acid and niacinamide. A gel was prepared containing liquid paraffin (20%), span 60 (0.5%), tween 80 (4.5%), salicylic acid (2%), niacinamide (3%), xanthan gum (2%), methyl paraben (0.4%) and N_2O water (up to 100%). Every ingredient was individually weighed. Niacinamide and water were heated together at 70°C until all of the niacinamide was dissolved. The rest of the ingredients were then heated to 70°C until the salicylic acid was dissolved. The 2 solutions were combined and homogenised with a Heidolph® Diax 600 (Germany) homogeniser at 13500 rpm to 40°C. Thereafter, the mixture was stirred on a Wisestir® Daihan Scientific hs 300 (Daihan Scientific cc, Korea) to 25°C and placed into white plastic containers (50 mm in diameter and 22 mm deep). The Pheroid™ gel contained the same ingredients as the gel formulation; however, butylated hydroxyanisole (BHA) (0.02%), butylated hydroxytoluene (BHT) (0.2%) and Pheroid™, prepared by the North-West University, were added. The cream was prepared containing salicylic acid (2%), niacinamide (4%), methyl paraben (0.2%), liquid paraffin (6%), emulsifying wax (9%), soft paraffin (17%) and N_2O water (up to 100%). The Pheroid™ cream was also formulated with the same ingredients, however, BHA (0.02%), BHT (0.2%) and Pheroid™ manufactured by the North-West University, were added. All of the formulations were prepared by the same method. As in a study conducted by Kuehl et al. (2009) on the preformulation, and *in vivo* efficacy of topically applied apomine, the formulations were smooth, white and homogenous with no phase separation.

2.2.2 Stability testing

The purpose of this study was to evaluate physical and chemical parameters for stability determination in cosmetic formulations (Guaratini et al., 2006), using a gel, Pheroid™ gel, cream

and Pheroid™ cream containing salicylic acid and niacinamide as ingredients. The conditions, under which the formulations were stored, were determined according to the climate and humidity of South-Africa (Medicines Control Council, 2009). All the preparations were stored at 25°C/60% RH (relative humidity), 30°C/60% RH and 40°C/75% RH in Labcon® humidity chambers (Marais Burg, South Africa). Every stability test was conducted on a monthly basis for 6 months, starting at month 0.

2.2.2.1 HPLC method for stability testing (Assay)

The high performance liquid chromatographic (HPLC) system consisted of a Agilent 1100 series auto sampler, Agilent 1100 series variable detector (VWD) and an Agilent 1100 series isocratic pump (Agilent Technologies, Polo Alto, CA). A Phenomenex (Luna C-18, 5 µ, 250 mm x 4.60 mm) column (Phenomenex, Torrance, CA) was used. The flow rate was set at 1.0 ml/min with detection at 220 nm. A mobile phase of 1% orthophosphoric acid/water was prepared. The gradient elution was employed starting at 25% methanol for the first min, followed by a linear immersion to 100% methanol after 8 min. The composition was kept at 100% methanol until 25 min elapsed; thereafter the system was re-equilibrated at starting conditions for 5 min. A volume of 20 µl was injected for all of the samples. The running time was 30 min for each sample. Retention times for niacinamide, methyl paraben, salicylic acid, BHA, BHT and vitamin E were 2.9, 9.2, 10.2, 11.1, 12.9 and 23.2 min, respectively. The solvent used was 100% methanol.

2.2.2.1.1 Sample preparation

A 5 ml syringe was used and filled with the specific formulation. A 15 cm silicone tube was placed over the nozzle in front of the syringe. The syringe with tubing was placed into a tarred 50 ml volumetric flask. Approximately 2 g of each formulation was weighed individually. Methanol was added to fill the volume to 50 ml. The sample was sonicated on a Transsonic 540 (Germany) for 10 min. Another 5 ml syringe was then filled with the solution in the volumetric flask. A pre-filter of 0.45 µm was placed over the nozzle of the syringe and a 23 G sterile needle was added onto the pre-filter. The solution was transferred into vials and analysed on the HPLC in triplicate by the described HPLC assay.

2.2.2.1.2 Standard solution

Approximately 40 mg salicylic acid, 60 mg niacinamide, 8 mg methyl paraben, 0.4 mg BHA, 4 mg BHT and 4 mg vitamin E were weighed. All of the ingredients were dissolved in 50 ml methanol and sonicated for approximately 5 min. The solution was then transferred into vials and analysed on the HPLC system in triplicate.

2.2.2.2 pH determination

The purpose of determining the pH was to establish whether the different formulations were within the pH range of the skin, which is between 4 to 6 (Ademola, 1997); whether the formulations would cause skin irritation as well as to determine whether the formulations were stable during the 6 month period. A Mettler Toledo pH meter was used to measure the pH of the different formulations. A glass Mettler Toledo InLab® 410 (China) was used. Before using the pH meter, it was calibrated each time with a buffer (Mettler Toledo GmbH, Analytical CH-8603 pH-buffer solution) of pH 4.01, 7.00 and 10.01.

2.2.2.3 Rheological measurements

Brookfield Model DV II (USA) with t-bars was used for the rheological measurements. The temperature of the water bath was set at 25°C. The viscosity of the cream and Pheroid™ cream was measured with the S-96 spindle at a speed of 0.3 rpm. The viscosity of the gel and Pheroid™ gel was measured with the S-96 spindle, however at a speed of 1.5 rpm. Viscosity readings for each individual formulation were taken at 10 sec intervals and 32 readings were obtained. Viscosity was measured in centipoise (cP) with the help of the Wingather 32 software program, in order to determine the flow characteristics of each topical formulation (Ademola, 1997).

2.2.2.4 Visual assessment

The different products were visually assessed each month for a period of 3 months. Photos were taken of each individual preparation with a Sony cyber-shot 7.2 mega pixel camera (Japan) and the products were compared to colour charts (Dulux, South Africa) to determine if there was any colour changes.

2.2.2.5 Confocal laser scanning microscopy (CLSM)

CLSM has become an invaluable tool for a wide range of investigations in the medical and biological science for imaging thin optical sections in living and fixed specimens ranging in

thickness up to 100 micrometers (Claxton et al., 2006). A CLSM Nikon D-eclipse C1 si with violet diode laser 400 – 405 nm, a He/Ne laser at 543 nm and an Argon ion laser with 457 – 517 nm and CLSM Nikon PCM2000 with a digital camera DMX1200 with a He/Ne laser at 543 nm and an Argon ion laser with 457 – 514 nm (Nikon cc, Japan) were used to perform the experiments. The CLSM was used to determine whether a sample was homogenous, whether there were any crystals inside the formulations, and to establish the size of the particles in each formulation.

2.2.2.5.1 Sample preparation

0.1 g of each of the 12 formulations (4 formulations each at 3 different temperatures/RH) was accurately weighed into individual 1.5 ml Eppendorf vials and 1 ml distilled water was added to each vial. The vials were mixed with a Stuart Scientific Vortex mixer (South Africa) for approximately 10 sec. Nile red (1 µl) was added to all samples. The vials were then mixed again with the Vortex mixer for approximately 10 sec. The samples were allowed to stand in a darkroom for at least 15 min so that the nile red could charge. Each sample (20 µl) was placed onto individual microscope slides (76 x 26 x 1 mm), avoiding air bubbles. The samples were covered with a cover glass (24 x 60 mm) and numbered. A small amount of Nikon cc for microscopy type A nd = 1.515 (23°C) immersion oil (Nikon cc, Japan) was placed onto the slides before placing them onto the microscope.

2.2.2.6 Mass variation

A Shimadzu AUW 120 calibrated balance (Japan) was used to weigh each container, with the cap and formulation inside, every month in order to determine whether there was weight loss or gain.

2.2.3 Physicochemical properties

2.2.3.1 HPLC method for physicochemical properties and diffusion studies

The HPLC system used was the same as for the assay. A Phenomenex (Luna C-18, 5 µ, 150 mm x 4.60 mm) column was used. The flow rate was set at 1.0 ml/min with UV detection at 262 nm for 4 min and then changed to 302 nm. A mobile phase was prepared of 1-octanesulphonic acid/water which was buffered with 0.1M ammonium hydroxide, 0.1M orthophosphoric acid and 0.1M sodium hydroxide to a pH of 3.5 was prepared. The gradient elution was employed starting at 80% methanol after 3.5 min, followed by 30% methanol until 4 min elapsed; thereafter the

system was re-equilibrated at starting conditions for 4 min. The running time was 8 min for each sample and the retention times for niacinamide and salicylic acid were 2.9 and 5.0 min, respectively. Samples were diluted with 100% methanol.

2.2.3.1.1 Standard preparation

Approximately 5 mg of niacinamide and salicylic acid were accurately weighed and dissolved in 50 ml of phosphate buffer solution (PBS) with a pH 7.4. The standard was transferred into an auto sampler vial and analysed on the HPLC system.

2.2.3.2 Solubility determination

The aqueous solubility of salicylic acid and niacinamide was obtained by preparing a saturated solution in PBS at pH 7.4 according to the standards of the British Pharmacopoeia (British Pharmacopoeia, 2007). The solution was stirred in a Labotec® water bath (South Africa) at 32°C, using magnetic bars for 24 h. An excess amount of salicylic acid and niacinamide was present at all times to provide a saturated solution. After 24 h the solution was filtered and analysed on the HPLC. The experiment was conducted in triplicate (Gerber et al., 2006:33).

2.2.3.3 Experimental octanol-buffer partition coefficient (log D)

250 ml of *n*-octanol and PBS buffer (pH 7.4) solution was poured into one container and vigorously stirred for 24 h. After 24 h, the 2 phases were separated. They were placed into 2 different containers and marked correctly. 1.230 mg of salicylic acid (0.410 mg/ml) and 1.485 mg of niacinamide (0.495 mg/ml) were accurately weighed and placed in 3 test tubes. The test tubes were filled with 3 ml pre-saturated PBS and placed into the Labotec® water bath (South Africa) at 32°C and agitated for 10 min. 3 ml *n*-octanol was placed into the different test tubes and the mixture was again agitated in the water bath (32°C) for 45 min. The mixture was centrifuged on an Eppendorf centrifuge 5804R (Merck, South Africa) at 4000 rpm (32°C) for another 30 min. The aqueous phase was analysed on the HPLC in triplicate. According to the outcome of the aqueous phase, the *n*-octanol phase was calculated and the log D values were determined. (Leo et al., 1971).

2.2.3.4 Permeation experiments

2.2.3.4.1 Preparation of donor solution

The donor solution consisted either of a gel, Pheroid™ gel, cream or Pheroid™ cream that contained salicylic acid as well as niacinamide (see section 2.2.1). The products were prepared 24 h commencing the study.

2.2.3.4.2 Skin preparation

Human skin from abdominal surgery of female Caucasians was used to perform the diffusion experiments (Henning et al., 2008). The study was approved by the Research Ethics Committee of the North-West University with the reference number 04D08. Before any skin could be obtained, a consent form was signed by each patient permitting their skin could to be used for research purposes. The patients remained anonymous. A scalpel was used to separate the skin from the fat layer. The skin (stratum corneum, epidermis and dermis) was punched into circles with a diameter of approximately 15 mm. Only skin without stretch marks, bruises or spots was used. The circles of skin were placed onto Whatman® filter paper. These skin samples were wrapped in aluminium foil and stored in the freezer at -20°C until utilised. The frozen skin samples were taken out of a freezer about 1 h before diffusion study commenced in order to thaw the skin and to check for any defect on the skin, before mounting them onto the Franz cells (Gerber et al., 2006).

2.2.3.4.3 Method of skin permeation

Vertical Franz diffusion cells with a 2.0 ml receptor compartment and a 1.0751 cm^2 diffusion area were used in the permeation studies. The epidermal skin layer was carefully mounted on the lower half of the Franz cell with the stratum corneum facing upwards. The donor compartment was placed on top of the receptor (lower half) and vacuum greased slightly with Dow Corning (USA) high vacuum grease. A clamp was used to fasten the donor and receptor compartments together. Subsequently the receptor compartments were filled with 2.0 ml PBS (pH 7.4) and special care was taken to prevent bubbles from forming in the receptor compartment. Thereafter the Franz cells were filled with donor solution (1 ml), consisting of the gel, Pheroid™ gel, cream or Pheroid™ cream. The donor compartment was covered with Parafilm® to prevent evaporation. Only the receptor compartments containing magnets, in order to stir the solution, were placed into the water bath. The entire receptor volumes were withdrawn and replaced by 37°C fresh PBS

(pH7.4) after 0.5, 1, 2, 4, 6, 8, 10 and 12 h for the gel, Pheroid™ gel, Pheroid™ cream and cream. The experiments were performed over a period of 12 h. Each study contained 10 Franz cells, in order for at least 8 of the 10 Franz cells to be used for results. Subsequently after the receptor phase was withdrawn it was directly analysed on the HPLC (Gerber et al., 2006). Tape stripping was performed after 12 h to determine the concentration of the active ingredients that diffused into the skin.

2.2.3.4.4 Tape stripping

When the donor and receptor compartments were removed at 12 h, the skin was carefully mounted on a piece of Parafilm® which was stapled to a wooden board. The exposed diffusion area (≈ 1.075 cm^2) was clearly marked on the skin, by the imprints of the border from the diffusion cells (≈ 11.7 mm diameter). It was then dabbed dry with tissue paper. 3M Scotch® Magic™ tape was cut into pieces, so that they covered the diffusional area but did not overlap the area outside of the border. The first tape strip was discarded, as it was considered part of the cleaning procedure and could therefore be contaminated with active ingredients from the formulations (Pellet et al., 1997). The following 15 tape strips were placed into a polytop with 5 ml PBS (pH 7.4). If the skin had a glistening appearance, the stratum corneum was successfully removed and the dermal layer remained. The excess skin was then trimmed away from the border of the diffusional area (Pellet et al., 1997). The diffusional area was cut into small pieces and placed into a polytop filled with 2 ml of PBS (pH7.4). Thereafter the polytops were placed into a fridge (4°C) and left to stand overnight. Analysis with the HPLC system commenced the following day.

2.2.3.4.5 Transdermal and statistical data analysis

The concentrations of salicylic acid and niacinamide present in the formulation were determined by means of HPLC. For both the niacinamide and salicylic acid the average cumulative amount per area was plotted against time. The average flux was obtained from the slope of the linear portion of the curve (Fox, 2008). The Pheroid™ gel, Pheroid™ cream, gel and cream plot showed a steady state flux between 6 – 12h. Statistical analysis of data obtained from niacinamide and salicylic acid, involved the examination of the median flux values (Fox, 2008). If there is a large

variation in the experimental flux values, the median (centre) flux is a more precise method for determining flux (Gerber et al., 2008).

3. Results and Discussion

3.1 Stability Testing

3.1.1 Assay results

All the ingredients in the Pheroid™ cream and Pheroid™ gel reduced dramatically from month 0 to 6 at the different storage conditions. It could however be conducted that niacinamide, methyl paraben and salicylic acid were the most stable of all the ingredients. The anti-oxidants namely BHA, BHT and vitamin E were oxygen labile thus, causing oxidation. Oxidation occurs due to the presence of free radicals. This significantly caused the degradation of the active ingredients in the Pheroid™ formulations (Ramchandani & Toddywala, 1997). The gel formulation was more stable over the 6 month period than the Pheroid™ cream and Pheroid™ gel. The gel reduced by a half of its original recovery value, however, niacinamide was the most stable component of the gel formulation. The cream was the most stable formulation of all four of the formulations. Recovery of the cream only reduced by one third of its original recovery value. Components of the cream proved to be stable; however, niacinamide was again, the most stable of all the ingredients. The concentrations of the ingredients after 6 months were not within the acceptable limits (90 – 110%). From these results it was clear that all four formulations were unstable under the specified conditions. A reason for this may be that the active ingredients were not evenly distributed within the formulations, even though confocal laser scanning microscopy predicted uniform distribution of the ingredients. The method for formulating the formulations must be evaluated in order to improve the spreadability of the active ingredients within the different formulations. Different columns were used each month on the HPLC system and could thus, also be an important factor contributing to the dramatic changes in the concentration of each ingredient. Each column has a different plate count and can be more/less sensitive to a specific ingredient. Inconsistency in performing experiments: using exactly the same method each month, at the same time of day, in the same weather conditions; could also be responsible for the changes in concentration.

3.1.2 pH results

The pH range of the dermis tissues is 4 to 7.4 (Hadgraft & Valenta, 2000). The pH of all four of the formulations at different storage conditions were measured between the pH ranges of 3.54 to 4.60. Therefore, the pH of the formulations was of an acidic nature. This was due to the fact that all the formulations contained salicylic acid. A pH of below 4 may cause skin irritation. The pH of a preparation can also influence the solubility of the drug in the formulation (Ademola, 1997).

The only significant change in pH values was for the cream, with a significant decrease in the pH from month 0 (4.71) to month 6 (3.54) at 30°C/60% RH (%RSD = 9.02) and from month 0 (4.71) to month 6 (4.11) at 40°C/75% RH (%RSD = 6.05). This may be due to experimental error and not indicative of a significant pH change, since the data at 25°C/60% RH does not confirm this trend.

3.1.3 The rheological measurements

Measurements of rheological behaviour are important not only to evaluate physical stability (Spiclin et al., 2003; Tadros et al., 2004), but are also parameters indicating system quality, usefulness and purpose. Studies on these properties have become a crucial tool for analysis of cosmetic preparations, due to the possibility of producing correct profiles of physical and structural stability (Soriano et al., 2001).

There were no significant changes in the viscosity of the formulations. The formulations were within the experimental error of 20%. This implies that the formulations were stable and that there was no possible decrease in the molecular weight of the ingredients that could lead to thinning and ultimate failure of the product (Ademola, 1997).

3.1.4 Visual assessment

According to the results for visual assessment of the cream and the gel, both preparations remained white, homogenous and without odour over the 6 month period under all storage conditions. This indicated that the ingredients in the cream and gel were stable and none of them oxidised and caused discoloration. The cream and gel will be compliant with patients based on the visual assessment. However, the Pheroid™ gel and Pheroid™ cream depicted significant colour changes due to ingredients that oxidised during the 6 month period. The Pheroid™ gel went from a white and homogenous gel to a mustard yellow gel, with a fish-like smell. The

Pheroid™ cream went from a white, homogenous cream to a golden, mustard yellow cream also with a fish-like smell. The Pheroid™ cream started to separate into the oil and water phase.

3.1.5 Confocal laser scanning microscopy (CLSM)

Results obtained over the 6 month period indicated that the particle size of each formulation were almost the same size for all formulations. It furthermore showed that all of the formulations were homogenous without any crystals.

3.1.6 Mass variation

Results obtained over the 6 month period for mass variation depicted that the Pheroid™ gel (25.73 – 17.91 g) at a storage condition of 25 °C/60% RH, was the only formulation with a significant weight loss change. However, no significant changes for the rest of the formulations at different storage conditions were observed. Weight loss for the Pheroid™ gel could have been due to the evaporation of volatile constituents caused by a flaw in the container, such as a crack for example (Cannell, 1985).

3.2 Physicochemical properties

3.2.1 Aqueous solubility and experimental log D values of niacinamide and salicylic acid

The aqueous solubility of a drug to optimally permeate the skin should be more than 1 mg/ml (Barry, 2002). For a molecule to permeate well through the skin it must have good solubility in both the water and oil phase; and it should exhibit an octanol-water partition coefficient (log D) of approximately 1 to 3 (Hadgraft, 2004). The solubility of niacinamide and salicylic acid were determined to be 212.95 mg/ml and 4.07 mg/ml, respectively in PBS (pH 7.4) at a temperature of 32°C. The log D values of niacinamide and salicylic acid were -0.32 and 0.33, respectively. According to the solubility of niacinamide and salicylic acid it was expected that both of the active ingredients would permeate through the skin, niacinamide better than the salicylic acid. The results of the log D values for the active ingredients indicated that there would not be optimal permeation, because the values were not within the optimum values of 1 to 3. Salicylic acid would, however, penetrate better than niacinamide.

3.3 Diffusion study

3.3.1 *In vitro* permeation studies

Findings obtained in this study indicated that both niacinamide and salicylic acid, presented flux values from 6 to 12 h. According to the results given in figure 1 a) for niacinamide, the cream depicted a more significant average flux value (2.6985 $\mu g/cm^2.h$) of all the formulations. When compared to the Pheroid™ cream (1.0095 $\mu g/cm^2.h$), the cream depicted approximately double the flux value. Comparing the gel (2.0400 $\mu g/cm^2.h$) to the Pheroid™ gel (1.3157 $\mu g/cm^2.h$) it also showed approximately double the flux value. The reason that the cream depicted the most significant flux value for niacinamide, could be due to the fact that niacinamide is highly soluble in water; it has a small molecular weight and diffuse out of the oily vehicle into the hydrophilic porous shunt pathway (Williams, 2003). The reason for the lower flux value of the gel can also be explained by the hydrophilic character of niacinamide. Niacinamide preferred the water base (gel) and diffused more slowly into the hydrophilic porous shunt (Williams, 2003). As for the Pheroid™ cream and Pheroid™ gel, niacinamide was trapped inside the microsponges of the Pheroid™, thus, caused a delay in the release of the active ingredient into the skin.

Figure 1 a) illustrates a comparison between the average and median flux values of niacinamide. The median flux values are slightly higher for all the formulations when compared to the average flux. The reason being that the median flux is not influenced by outlier values. In this case where biological material was brought into the equation; it was of great value.

Figure 1: Box-plots of the flux values for **a)** niacinamide and **b)** salicylic acid to illustrate the median flux and red lines to illustrate the average flux after application of Pheroid™ gel, Pheroid™ cream, gel and cream

As seen in figure 1 b) for salicylic acid, the gel depicted the highest average flux value (31.952 $\mu g/cm^2.h$). The gel also depicted a higher flux value than the Pheroid™ gel (20.853 $\mu g/cm^2.h$), the cream (11.335 $\mu g/cm^2.h$), and the Pheroid™ cream (10.282 $\mu g/cm^2.h$). This was due to the fact that the salicylic acid has a small molecular weight, is more lipophilic and is more diffusive into the stratum corneum and sweat glands (Williams, 2003). The gel and Pheroid™ gel were porous of nature and it allowed relatively free diffusion of the active ingredient (Barry, 2002). The Pheroid™ cream depicted a lower flux value than the cream and gel due to the active ingredients trapped

within the Pheroid™ system. This entrapment caused the active ingredient to diffuse slower into the skin.

According to the pH-partitioning hypothesis, only the unionised forms of drugs are able pass through lipoidal membranes (Smith & Irwin, 2000). The gel and Pheroid™ gel presented a pH range of 3.98 to 4.14. In this pH range salicylic acid was in a unionised state and therefore caused salicylic acid to penetrate through the skin. However, when salicylic acid diffused into the skin, the pH changed to 7.14, thus causing salicylic acid to be in an ionised state (Singh, 1990). The active ingredient was, thus, trapped within the skin and could not penetrate further. The cream and Pheroid™ cream depicted a higher pH range of 4.60 to 4.71. In this pH range the salicylic acid was in a more ionised state (90%), thus causing the salicylic acid not to permeate effectively through the skin.

Salicylic acid overall depicted higher flux values than niacinamide due to salicylic acid's log D value being closer to the ideal properties. Ogiso and Shintani (1990) suggested that C_{12} groups have an affinity for the skin. This indicates a possible reason that salicylic acid penetrates through the skin more effectively than niacinamide.

Figure 1 b) illustrates a comparison between the average (mean) and median flux values of salicylic acid. The median flux value of the Pheroid™ gel was slightly higher than the average flux. For the Pheroid™ cream, gel and cream the average flux values were a bit higher than the median flux values.

3.3.2 Tape stripping

Figure 2 a) illustrates the comparison between the epidermis and dermis for niacinamide. The gel depicted the highest concentration (2.060 µg/ml) within the epidermis, followed by the cream (2.001µg/ml), the Pheroid™ gel (1.587 µg/ml) and lastly, the Pheroid™ cream (1.097 µg/ml). Concentrations within the dermis were significantly higher than concentrations within the epidermis. The gel depicted the highest concentration (44.749 µg/ml) in the dermis, whereas the Pheroid™ gel was half the concentration (22.764 µg/ml) of the gel. The Pheroid™ cream concentration (18.061 µg/ml) was approximately 2.5 times smaller than the gel concentration and the cream was approximately a third of the gel's concentration (13.363 µg/ml). The dermis

contained significantly higher concentrations of niacinamide than the epidermis. The cream formulation depicted a concentration of 2.001 μg/ml in the epidermis, 13.363 μg/ml in the dermis and 2.5 $\mu g/cm^2.h$ average flux. When compared to the cream, the Pheroid™ cream formulation depicted a concentration of 1.0097 μg/ml in the epidermis, 18.061 μg/ml in the dermis and 1.00 $\mu g/cm^2.h$ average flux. As for the gel formulation, it showed a concentration of 2.060 μg/ml in the epidermis, 44.749 μg/ml in the dermis and 2.00 $\mu g/cm^2.h$ average flux. When compared to the gel formulation, the Pheroid™ gel formulation depicted a concentration of 1.587 μg/ml in the epidermis, 22.764 μg/ml in the dermis and 1.35 $\mu g/cm^2.h$ average flux.

Figure 2: Cumulative concentration (μg/ml) of the epidermis and dermis for **a)** niacinamide and **b)** salicylic acid in the four different formulations

As seen in figure 2 b) the comparison between the epidermis and dermis for salicylic acid depicted that concentrations within the dermis were significantly higher than concentrations in the epidermis. The gel displayed the highest concentration (2.113 μg/ml) within the epidermis, followed by the Pheroid™ gel (1.144 μg/ml), the cream (0.758 μg/ml) and lastly, the Pheroid™ cream (0.411 μg/ml). Pheroid™ gel depicted the highest concentration (95.360 μg/ml) within the dermis. The concentration of the Pheroid™ gel was approximately double the concentration of the gel (49.519 μg/ml), Pheroid™ cream (48.424 μg/ml) and cream (44.729 μg/ml). The cream formulation depicted a concentration of 0.758 μg/ml in the epidermis, 44.729 μg/ml in the dermis and 12.0 $\mu g/cm^2.h$ average flux. When compared to the cream formulation, the Pheroid™ cream depicted a concentration of 0.411 μg/ml in the epidermis, 48.424 μg/ml in the dermis and 10.00 $\mu g/cm^2.h$ average flux. As for the gel formulation, it showed a concentration of 2.113 μg/ml in the epidermis, 49.519 μg/ml in the dermis and 32.0 $\mu g/cm^2.h$ average flux. When compared to the gel, the Pheroid™ gel depicted a concentration of 1.144 μg/ml in the epidermis, 95.360 μg/ml in the dermis and 22.0 $\mu g/cm^2.h$ average flux.

3.3.4 Transdermal and statistical data analysis

Statistical significance is obtained on a 5% level when the p-value is smaller than 0.05 (i.e., $p<0.05$). The Kruskal-Wallis ANOVA test was performed to compare the median flux values and tape stripping values in the epidermis and dermis for salicylic acid from the diffusion studies. There was a significant difference among the formulations with salicylic acid in the diffusion study between the flux values ($p<0.0001$), the epidermis ($p=0.0016$) and the dermis ($p=0.0001$). Pair-wise comparisons between the different formulations for the median flux values were done and it demonstrated that the gel differed significantly from the Pheroid™ cream ($p<0.001$) and from the cream ($p=0.001$). There was also a comparison between the different formulations for the epidermis values here the Pheroid™ cream differed significantly from the cream ($p=0.0016$).

According to Spearman rank correlations there was a statistically significant correlation ($p<.05$) for niacinamide between the flux and dermis. The Kruskal-Wallis ANOVA test was performed to compare the median flux values and tape stripping values in the epidermis and dermis for niacinamide from the diffusion studies. There was a significant difference among the formulations with niacinamide in the diffusion study between the flux values ($p=0.0017$) and the dermis values ($p=0.0001$). Pair-wise comparisons between the different formulations for the median flux values were done and the cream differed significantly from the Pheroid™ cream ($p<0.0017$). There was also a comparison between the different formulations for the epidermis values which revealed that the Pheroid™ cream differed significantly from the cream on a 10% level of significance ($p=0.0731$). There was also a comparison between the different formulations for the dermis values which demonstrated that the cream differed significantly from the gel ($p=0.0001$) (Steyn et al, 1998).

4. Conclusion

The aim of the study was to formulate four different acne preparations (Pheroid^{TM} cream, Pheroid^{TM} gel, cream and gel); combining niacinamide and salicylic acid; evaluating some stability parameters for the different formulations; and comparing the diffusion ability of these formulations. Niacinamide and salicylic acid were chosen in combination for this study, due to the beneficial effects that they have on acne.

According to the conclusive evidence, the cream and gel were the most stable of the four formulations during the determination of the stability parameters. None of the formulations, however, complied with the prerequisites of the Medicines Control Council (MCC). In the diffusion study the cream (in the case of niacinamide) and the gel (in the case of salicylic acid) depicted the highest average and median flux values. The Pheroid^{TM} cream and Pheroid^{TM} gel indicated penetration enhancement of niacinamide into the target site (dermis), however the gel indicated the highest concentration within the dermis. Thus, niacinamide and salicylic acid could act against acne; due to the fact that they were delivered within the target sites. The Pheroid^{TM} gel indicated a significant enhancement of salicylic acid concentrations within the dermis. Overall, the gel and cream displayed the best results within both the studies. Further investigation on the formulation of the preparations should be conducted to improve the stability parameters as well as the penetration enhancement of the active ingredients within the target site.

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FIGURE LEGENDS:

Figure 1: Box-plots of the flux values for **a)** niacinamide and **b)** salicylic acid to illustrate the median flux and red lines to illustrate the average flux after application of Pheroid^{TM} gel, Pheroid^{TM} cream, gel and cream

Figure 2: Cumulative concentration (µg/ml) of the epidermis and dermis for **a)** niacinamide and **b)** salicylic acid in the four different formulations

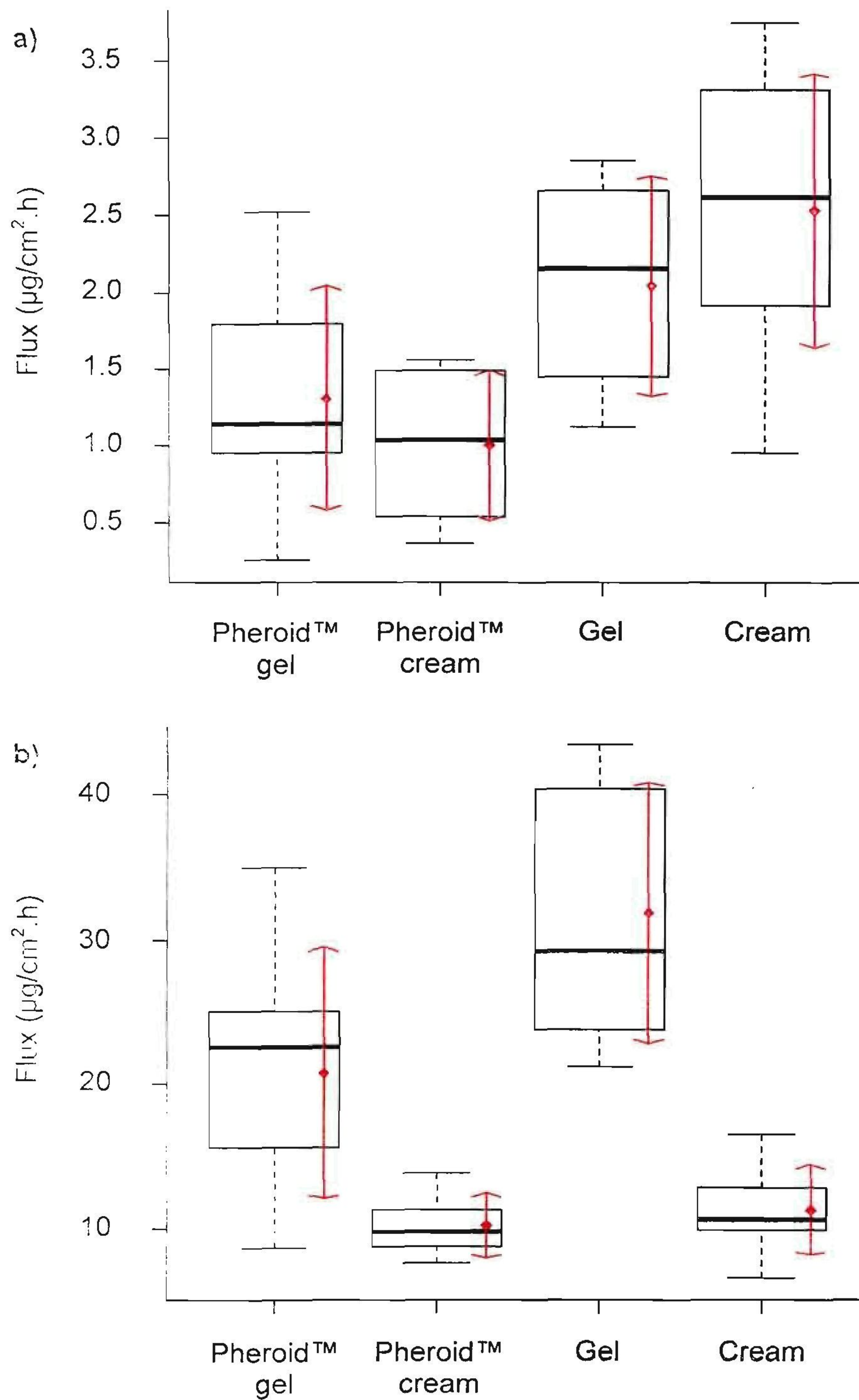

Figure 1: Box-plots of the flux values for **a)** niacinamide and **b)** salicylic acid to illustrate the median flux and red lines to illustrate the average flux after application of Pheroid™ gel, Pheroid™ cream, gel and cream

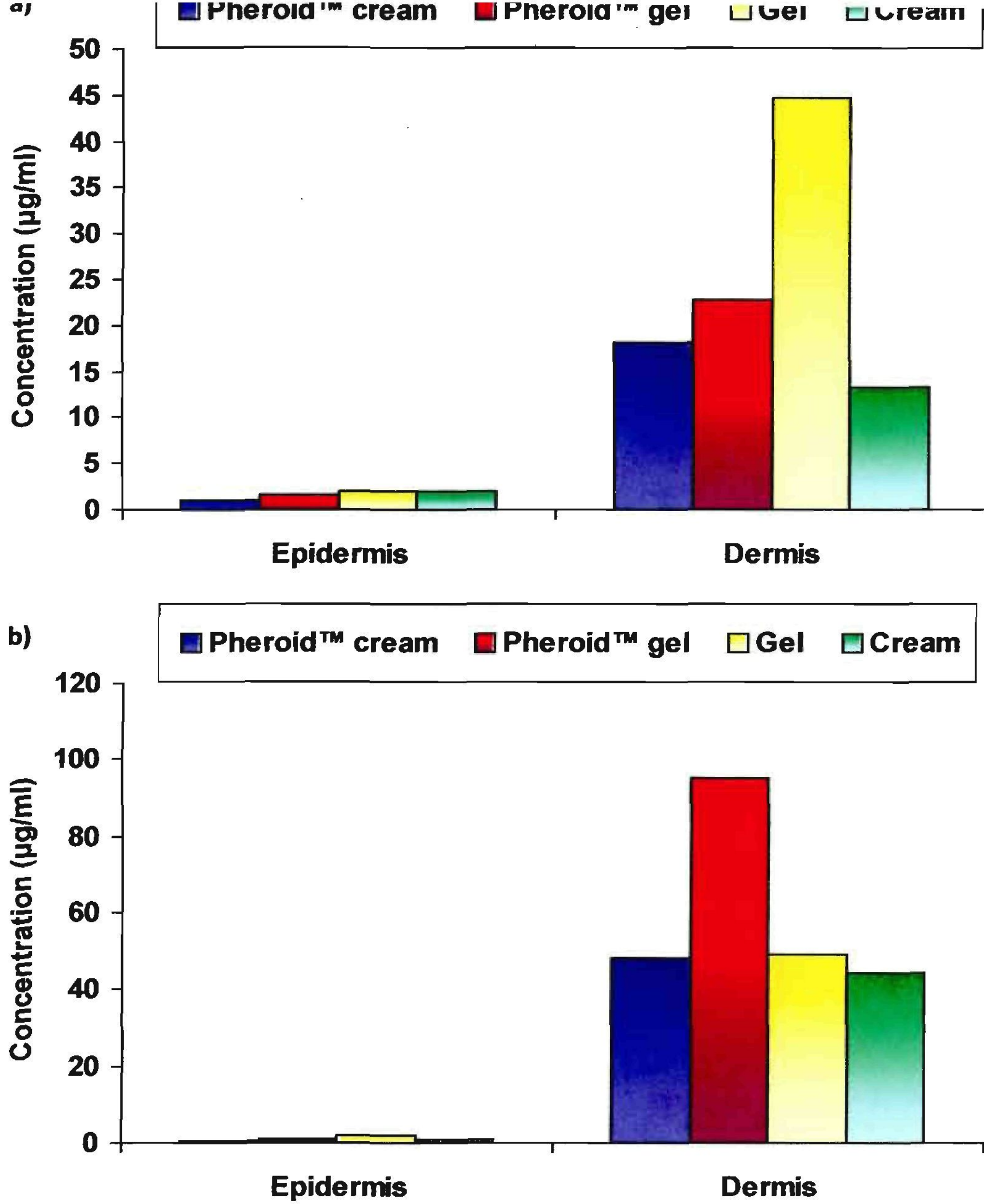

Figure 2: Cumulative concentration (µg/ml) of the epidermis and dermis for **a)** niacinamide and **b)** salicylic acid in the four different formulations

CHAPTER 4: FINAL CONCLUSION AND FUTURE PROSPECTS

There is no proven dietary factor that causes or induces acne. Personal hygiene is of little relevance and excessive cleaning of the skin may aggravate the symptoms. The development of acne is mainly influenced by genetic factors. Sebum is a greasy material produced by pilosebaceous glands, which are mainly distributed over the face, neck and chest. Its production is largely under the control of circulating androgens, which peak in both sexes in early infancy, and again, at puberty (Shaw & Kennedy, 2007:385).

Androgen levels are usually normal, but androgen metabolism at the receptor sites in the skin varies from one individual to another. In acne, an increased secretion of sebum is accompanied by thickening of the epidermis at the outlet to the pilosebaceous follicle. This creates an obstruction to flow, and a comedo develops. Colonisation of the follicle with *Propioni-bacterium acnes* and the host inflammatory response to this play a pivotal role in the development of the typical inflammatory papulopustular lesion (Shaw & Kennedy, 2007:385).

The literature regarding acne is abundant, with more than 8816 papers listed on Medline alone and more than 840 000 acne-related sites on the internet. However, there is no single universally successful treatment (De Souza *et al.*, 2005:40). Partially successful approaches to the treatment of acne contemplate the topical application of a mild exfoliator such as salicylic acid together with an anti-inflammatory agent (Muizzuddin *et al.*, 2008:183) such as niacinamide.

The cosmeceutical actives niacinamide and salicylic acid were chosen in combination, due to the beneficial effects that they have on acne. Niacinamide has an anti-inflammatory action on acne; that reduces redness, dryness and irritation caused by *Propioni-bacterium acnes* that live in the clogged pores of pimples (Acnelabtreatment, 2008:1). Salicylic acid on the other hand is a keratolytic and keratoplastic agent (SAMF, 2005:177).

This study involved the formulation of four different acne products (Pheroid™cream, Pheroid™gel, cream and gel), combining niacinamide and salicylic acid. The evaluation of stability parameters for the different formulations indicated that none of the formulations were stable under the different storage conditions determined by the Medicine Control Council. Nevertheless, the cream and gel were the most stable of the four formulations. Visual assessment of the Pheroid™ formulations with the CLMS was conducted and inconclusive evidence to whether the actives were entrapped within the Pheroids™, was obtained.

The aqueous solubility of a drug to be able to optimally permeate the skin should be more than 1 mg/ml (Barry, 2002:513). For a molecule to permeate well through the skin it must have good solubility in both the water and oil phase, and thus exhibit a log P of approximately 1 to 3 (Hadgraft, 2004:292).

The solubility of niacinamide and salicylic acid in PBS (pH 7.4 at 32°C) were 212.95 mg/ml and 4.07 mg/ml, respectively. The log D values of niacinamide and salicylic acid were determined to be -0.32 and 0.33, respectively. According to the solubility of niacinamide and salicylic acid it was expected that both of the active ingredients would permeate through the skin. However, it is expected that niacinamide will depict enhanced permeation with respect to salicylic acid. The results of the log D value for both of the active ingredients indicated that neither the ingredients would depict optimum penetration because neither values were within the optimum limits. Salicylic acid would be able to show more significant penetration than niacinamide.

Franz cell diffusion studies indicated that the cream (in the case of niacinamide) and gel (in the case of salicylic acid) depicted the highest average and median flux from hours 6 to 12. Results of the tape stripping studies showed that with the gel formulation, concentrations of 2.355 µg/ml and 42.880 µg/ml niacinamide were obtained in the epidermis and dermis respectively. After the Pheroid™ gel was applied, tape stripping depicted only 1.464 µg/ml niacinamide in the epidermis with respect to 25.102 µg/ml niacinamide in the dermis. The cream formulation, on the other hand, showed niacinamide concentrations of 2.017 µg/ml in the epidermis and 12.590 µg/ml in the dermis, whereas with the Pheroid™ cream formulation, concentrations of 1.265 µg/ml and 18.965 µg/ml were obtained in the epidermis and dermis respectively.

Tape stripping results depicted that with the gel formulation, concentrations of 2.420 µg/ml and 47.431 µg/ml salicylic acid were obtained in the epidermis and dermis respectively, whereas the Pheroid™ gel formulation showed salicylic acid, concentrations of 1.023 µg/ml in the epidermis and 97.262 µg/ml in the dermis. The cream formulation, however, depicted salicylic acid concentrations of 0.758 µg/ml in the epidermis and 44.729 µg/ml in the dermis. Lastly, after the Pheroid™ cream was applied, salicylic acid concentrations of 0.382 µg/ml and 48.610 µg/ml in the epidermis and dermis respectively, were measured.

From this study the following aspects were identified which might necessitate further investigation:

- Formulations must be optimised to obtain an acceptable shelf life as well as optimal delivery of the actives.
- Preservatives must be added to the formulation and preservative efficacy studies should be performed in order to determine if the formulation is efficiently preserved.

- *In vivo* studies and clinical efficacy studies should be performed as soon as an acceptable formulation has been designed.

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APPENDIX A: INTERNATIONAL JOURNAL OF PHARMACEUTICS GUIDE FOR AUTHORS

Scope of the journal

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

Areas of particular interest include: pharmaceutical nanotechnology; physical pharmacy; polymer chemistry and physical chemistry as applied to pharmaceutics; excipient function and characterisation; biopharmaceutics; absorption mechanisms; membrane function and transport; novel routes and modes of delivery; responsive delivery systems, feedback and control mechanisms including biosensors; applications of cell and molecular biology to drug delivery; prodrug design; bioadhesion (carrier-ligand interactions); and biotechnology (protein and peptide formulation and delivery).

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Prof A.T. Florence, The School of Pharmacy, University of London, 29-39 Brunswick Square, London WCIN 1AX, U.K., Fax: +44 20 7837 5092; E-mail: ijp@pharmacy.ac.uk

The Americas, Australia and New Zealand

Prof J.H. Rytting, Pharmaceutical Chemistry Dept, University of Kansas, 2095 Constant Avenue, Lawrence, KS 66047, U.S.A., Fax: +1 785 864 5736; E-mail: ijp@ku.edu

Japan and Far East

Prof. T. Sonobe, Department of Pharmaceutical Engineering, School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Shizuoka-shi 422-8526, Japan. Fax: +81-54-264-5614; E-mail: sonobe@gakushikai.jp

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APPENDIX B: FORMULATION OF ACNE PREPARATIONS CONTAINING NIACINAMIDE AND SALICYLIC ACID

B.1 PURPOSE OF THE FORMULATIONS

The objective of the formulations was to formulate the best possible products that could be used in the stability tests and diffusion study.

In these study 4 acne preparations, combining niacinamide and salicylic acid were formulated. A gel, a Pheroid™ gel, a cream and a Pheroid™ cream were developed, after a few attempts. Both the gel and Pheroid™ gel contained 2% salicylic acid and 3% niacinamide, whereas the cream and Pheroid™ cream contained 2% salicylic acid and 4% niacinamide.

In the treatment of acne, the vehicle (that is the gel, Pheroid™ gel, cream or Pheroid™ cream in this case) is just as important as the active ingredients (Russell, 2000:359). The vehicle plays a key role in the appearance, feel and successful application of a topical drug (Buhse *et al.*, 2005:102). It is also very important to assess the patient's skin type first. Gels are normally used by patients with an oily skin type, because the gel has a drying effect on the skin. Creams are appropriate for patients with sensitive or dry skin types which require a non-drying and non-irritating effect (Russell, 2000:359).

B.2 FORMULATION OF A GEL

A gel can be defined as a semisolid dosage form that contains a gelling agent to provide stiffness to a solution or colloidal dispersion for external application to the skin. A gel may contain suspended particles (Buhse *et al.*, 2005:110). The appearance of a gel is usually clear or semi-transparent in a single phase. In a two phase system it can be opaque. The gel should be thick, non-greasy and provide a cooling sensation when applied to the skin (Buhse *et al.*, 2005:110).

Tables B.1 to **B.4** illustrate the composition and percentage per ingredient in the gel formulations.

Table B.1: Formulation of Gel formulation 1.

Composition	% m/m
Propan-2-ol	35%
Propylene glycol	20%
Salicylic Acid	2%
Niacinamide	3%
Hydroxypropyl methyl cellulose (HPMC)	2%
Glycerine	10%
$N_2O.H_2O$	28%
Total	**100%**

Method

1. Propan-2-ol and the propylene glycol were combined.... (A).
2. Salicylic acid was placed into a mortar and pestle and grinded into a fine powder.
3. The fine powder was dissolved into the mixture in (A).
4. Niacinamide was dissolved into the water.
5. HPMC was wetted with the glycerine.
6. All of the ingredients were then mixed together for approximately 5 minutes until a clear gel was formed.

Outcome

A clear gel with a homogonous texture and a sharp odour was formulated. Unfortunately, this formula could not be used, due to the alcohol that would interact with the Pheroid^{TM} ingredients. Subsequently a second formulation was studied.

Table B.2: Formulation of Gel formula 2.

Composition	% m/m
Sodium *di*-hydrogen orthophosphate dihydrate	0,1%
Propylene glycol	62%
Ethanol	16%
Salicylic Acid	2%
Niacinamide	3%
HPMC	2%
$N_2O.H_2O$	14.9%
Total	**100%**

Method

1. Sodium *di*-hydrogen orthophosphate dihydrate was dissolved into 2 g of the $N_2O.H_2O$ ....(A).
2. Propylene glycol and ethanol were combined.... (B).
3. Salicylic acid was grinded with a mortar and pestle into a fine powder.
4. The fine powder of salicylic acid was dissolved into the mixture in (B).... (C).
5. Mixture A and C was then combined.
6. HPMC was slowly poured into the above mixture and stirred well.
7. Niacinamide was dissolved into the rest of the water.
8. All of the ingredients were well mixed, for at least 10 minutes.

Outcome

A white gel was obtained, however, there was a significant amount of air bubbles trapped in the gel. Therefore a third formulation was investigated.

Table B.3: Formulation of Gel formula 3.

Composition	% m/m
Sodium *di*-hydrogen orthophosphate dihydrate	0,1%
Propylene glycol	62%
Ethanol	16%
Salicylic Acid	2%
Niacinamide	3%
HPMC	2%
Glycerine	10%
$N_2O.H_2O$	4.9%
Total	**100%**

Method

1. The sodium *di*-hydrogen orthophosphate dihydrate was dissolved into 2 g of the $N_2O.H_2O$ ....(A).
2. Propylene glycol and ethanol were combined.... (B).
3. Salicylic acid was grinded with a mortar and pestle into a fine powder.
4. The fine powder of salicylic acid was dissolved into the mixture in (B).... (C).
5. Mixture A and C was combined.... (D).
6. HPMC and glycerine was combined.... (E).
7. Mixture E was slowly poured into mixture D and stirred well.
8. The niacinamide was dissolved into the rest of the water.
9. All of the ingredients were then mixed for at least 10 minutes.

Outcome

No gel was formed, only a white suspension. Thus, a fourth formulation was investigated.

Table B.4: Formulation of Gel formula 4.

Composition	**% m/m**
Liquid Paraffin	20%
Span 60	0,5%
Tween 80	4,5%
Salicylic Acid	2%
Niacinamide	3%
Xanthan Gum	2%
Methyl Paraben	0,4%
$N_2O.H_2O$	67.6%
Total	**100%**

Method

1. Niacinamide and 50g of the water were accurately weighed and heated together to 80˚C.... (A).
2. The rest of the water was reheated at a temperature of 80˚C.... (B).
3. Xanthan gum was accurately weighed and slowly added to B while homogenising at 13 500 rpm.... (C).
4. Mixture A and C was then added together and mixed well.... (D).
5. Salicylic acid and liquid paraffin were accurately weighed. The salicylic acid was grinded into a fine powder with a mortar and pestle and added to the liquid paraffin until it was dissolved.... (E).
6. Span 60, Tween 80, and Methyl paraben were accurately weighed and added to E, then heated to 70˚C....(F).
7. Mixture F and E were then combined while homogenising at 13 500 rpm until ≤ 40˚C.... (G).
8. Mixture G was stirred till room temperature on the Wisestir® at 200 rpm.

Outcome

A white homogenous gel was formed.

B.3 FORMULATION OF A PHEROID™ GEL

The Pheroid™ gel contained the same ingredients as gel 4 described in section **B.2**, however with Pheroids™ that were manufactured by the North-West University as well as butylated hydroxyanisol (BHA) 0.02% and butylated hydroxytoluene (BHT) 0.2%. The Pheroids™ consisted of micro-sponges, which are ideal for combination therapies as one drug can be entrapped in the interior volume and the other in the sponge spaces. The geographical separation of active compounds into different interior spaces minimizes interactions between compounds or drug interactions (Grobler, 2004:14).

Outcome

A white homogenous gel, with a fishy odour was formed.

B.4 FORMULATION OF A CREAM

Creams are semisolid emulsions for external application. Oil-in-water emulsions are most useful as water washable bases, whereas water-in-oil emulsions are emollient and cleansing (Barry, 2002:530). A cream can be defined as an emulsion semisolid dosage form that contains > 20% water and volatiles and/or < 50% of hydrocarbons, waxes or polyethylene glycols as the vehicle for external application to the skin (Buhse *et* al., 2005:110). The appearance of a cream can be opaque, viscous, non-greasy to mild greasy and it tends to mostly evaporate or be absorbed when rubbed onto the skin (Buhse *et al.*, 2005:110).

Tables B.5 to **B.7** illustrate the composition and percentage per ingredient in the cream formulations.

Table B.5: Formulation of Cream formula 1.

Composition	% m/m
Salicylic Acid	2%
Niacinamide	4%
Methyl paraben	0,5%
Cetyl Alcohol	17%
Span 60	0,5%
Liquid Paraffin	17%
Tween 80	4,5%
$N_2O.H_2O$	43,5%
Total	**100%**

Method

1. Cetyl alcohol and liquid paraffin were accurately weighed and heated together to approximately 80˚C.... (A).
2. Salicylic acid was accurately weighed and grinded into a fine powder with a mortar and pestle, then added to mixture A and stirred until it dissolved.... (B).
3. Span 60, tween 80 and methyl paraben were accurately weighed, added to B, and heated till 80˚C.... (C).
4. Niacinamide and water were accurately weighed and heated together to 80˚C.... (D).
5. Mixture C and D were combined whilst homogenising at 13 500 rpm until ≤ 40˚C.... (E).
6. Then mixture E was stirred with the Wisestir® at 200 rpm until room temperature (25˚C) was reached.

Outcome

An extremely thick, white cream, with no odour was formulated. A new formula was then attempted.

Table B.6: Formulation of Cream formula 2.

Composition	% m/m
Salicylic Acid	2%
Niacinamide	4%
Methyl paraben	0,2%
Cetyl Alcohol	15%
Propylene glycol	8%
Liquid Paraffin	12%
$N_2O.H_2O$	53,1%
Cremopher EL	2,7%
Total	**100%**

Method

1. Cetyl alcohol and liquid paraffin were accurately weighed and heated together to approximately 80˚C.... (A).

2. Salicylic acid was accurately weighed, grinded into a fine powder with a mortar and pestle, then added to mixture A and stirred until it was dissolved.... (B).

3. Cremopher EL, propylene glycol and methyl paraben were accurately weighed, added to B, and heated to 80˚C....(C).

4. Niacinamide and water were accurately weighed and heated together to 80˚C.... (D).

5. Mixture C and D were added together whilst homogenising at 13 500 rpm until ≤ 40 ˚C.... (E).

6. Mixture E was then stirred with the Wisestir® at 200 rpm until room temperature (25˚C) was reached.

Outcome

A white, very thin cream was formulated with no odour

Table B.7: Formulation of Cream formula 3.

Composition	% m/m
Salicylic Acid	2%
Niacinamide	4%
Methyl paraben	0,4%
Liquid Paraffin	6,0%
Emulsifying wax	9,0%
Soft Paraffin	15%
$N_2O.H_2O$	63.6%
Total	**100%**

Method

1. Emulsifying wax, soft paraffin, methyl paraben and liquid paraffin were accurately weighed and heated together to 70˚C....(A).

2. Salicylic acid was accurately weighed, grinded into a fine powder with a mortar and pestle, then added to mixture A and stirred until it was dissolved.... (B).

3. Niacinamide and the water were accurately weighed and heated together to 80˚C.... (C).

4. Mixture B and C was added together whilst homogenising at 13 500rpm until ≤ 40˚C.... (D).

5. Then mixture D was stirred with the Wisestir® at 200 rpm until room temperature (25°C) was reached.

Outcome

A white homogenous cream, with a soft texture and odourless smell was formed.

B.5 FORMULATION OF A PHEROID™ CREAM

The Pheroid™ cream contained the same ingredients as cream 3 described in section **B.4**, however with Pheroids™ that were manufactured by the North-West University as well as BHA 0.02% and BHT 0.2%.

Outcome

A white homogenous cream, with a soft texture and fishy smell was formed.

B.6 Conclusion

From all of the formulations that were prepared the best four was chosen to take part in die stability testing as well as the diffusion studies. The gel formula 4, Pheroid™ gel formula 1, cream formula 3 and Pheroid™ cream formula 1 were chosen, due to the fact that they gave the best results of all the formulations that were prepared.

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APPENDIX C: VALIDATION OF THE HPLC EXPERIMENTAL METHOD FOR NIACINAMIDE AND SALICYLIC ACID

C.1 PURPOSE OF VALIDATION

The purpose of the validation was to establish a procedure for the validation of an analytical method, which would provide the necessary proof that the method used was sensitive and reliable in the determination of the concentration of salicylic acid and niacinamide that permeated the skin.

C.2 CHROMATOGRAPHIC CONDITIONS

The chromatographic conditions are given in **Table C.1**:

Table C.1: Chromatographic conditions for the detection of salicylic acid and niacinamide.

Analytical instrument	Agilent 1100 series auto sampler, Agilent 1100 series variable detector (VWD) and a Agilent1100 series isocratic pump.
Column	A Phenomenex (Luna C-18, 5 µ, 150 mm x 4.60 mm) column.
Mobile Phase	Sodiumheptanesulphonate/water, pH stabilised with a few drops of 0.1M ammonium hydroxide, then the pH was adjusted to 3.5 with 0.1M orthophosphoric acid as well as 0.1M sodium hydroxide.
Gradient	80% methanol to 3.5 minutes, then 30% methanol to 4 minutes, thereafter the system was re-equilibrated at the starting conditions for 4 minutes.
Flow Rate	1.0 ml/min
Injection volume	50 µl
Detection	UV at 262 nm to 4 minutes then 302 nm
Retention times	Niacinamide – 2.9 minutes Salicylic acid – 5.3 minutes
Stop time	8 minutes
Solvent	100% Methanol

C.3 STANDARD PREPARATION

Approximately 10 mg of salicylic acid and niacinamide were individually and accurately weighed. It was then dissolved in 100 ml of methanol. A further 5 ml was diluted to 50 ml. The last step was performed at least 3 times. The standard solution was transferred into an auto sampler vial and analysed on the High performance liquid chromatography (HPLC) system.

C.4 VALIDATION PARAMETERS

C.4.1 LINEARITY AND RANGE

The linearity of an analytical method is the ability (within a specified range) to obtain test results that are directly proportional to the concentration (amount) of analyte present in the sample. A set of standards to cover the entire concentration range of salicylic acid and niacinamide was prepared. The linear range of both salicylic acid and niacinamide was determined by performing linear regression analysis. Peak ratios versus the concentration (μg/ml) of the standards prepared were plotted onto a graph to determine the relationship.

The acceptance criteria for linear regression analysis should yield a regression coefficient (R^2) of ≥ 0.99. The range is determined as the lowest and highest concentrations between which the response remains linear.

The regression coefficient (R^2) that was obtained for both niacinamide and salicylic acid indicated an excellent linearity with a value of 1. This indicated that the HPLC system was stable. **Figure C.1** and **figure C.2** illustrate the different linear regression curves for both niacinamide and salicylic acid.

Table C.1 and **table C.2** provide the peak area ratio of the standards (μg/ml) of niacinamide and salicylic acid individually.

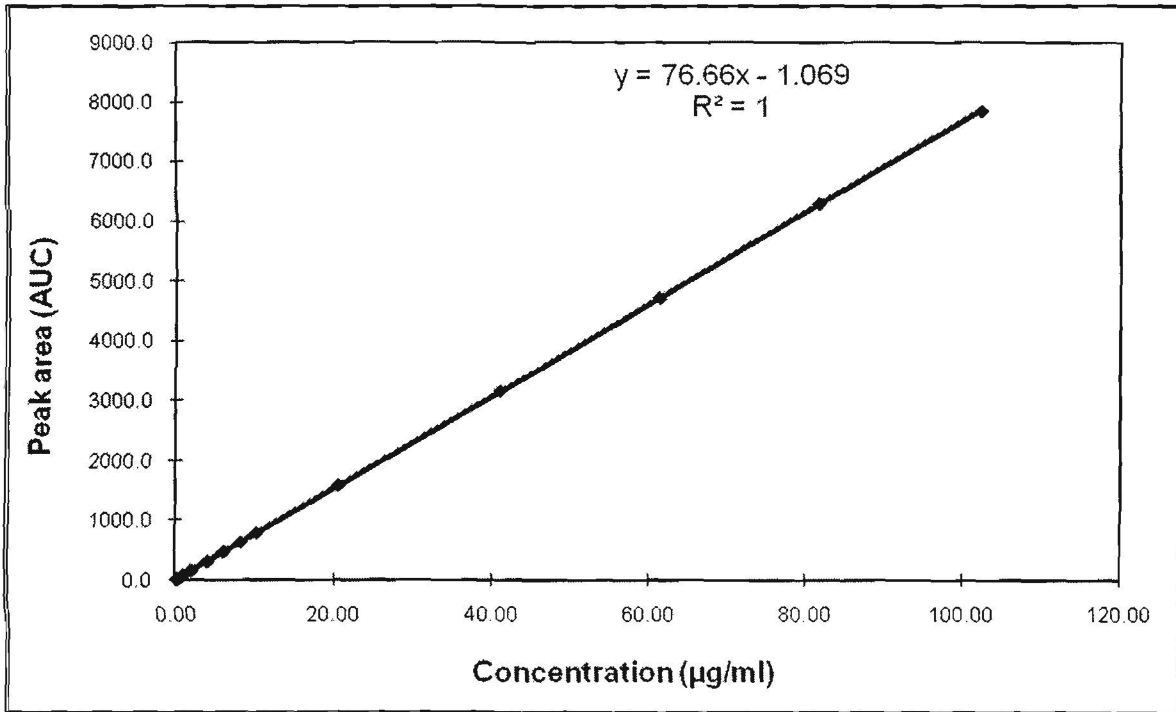


Figure C.1: Linear regression curve of niacinamide.

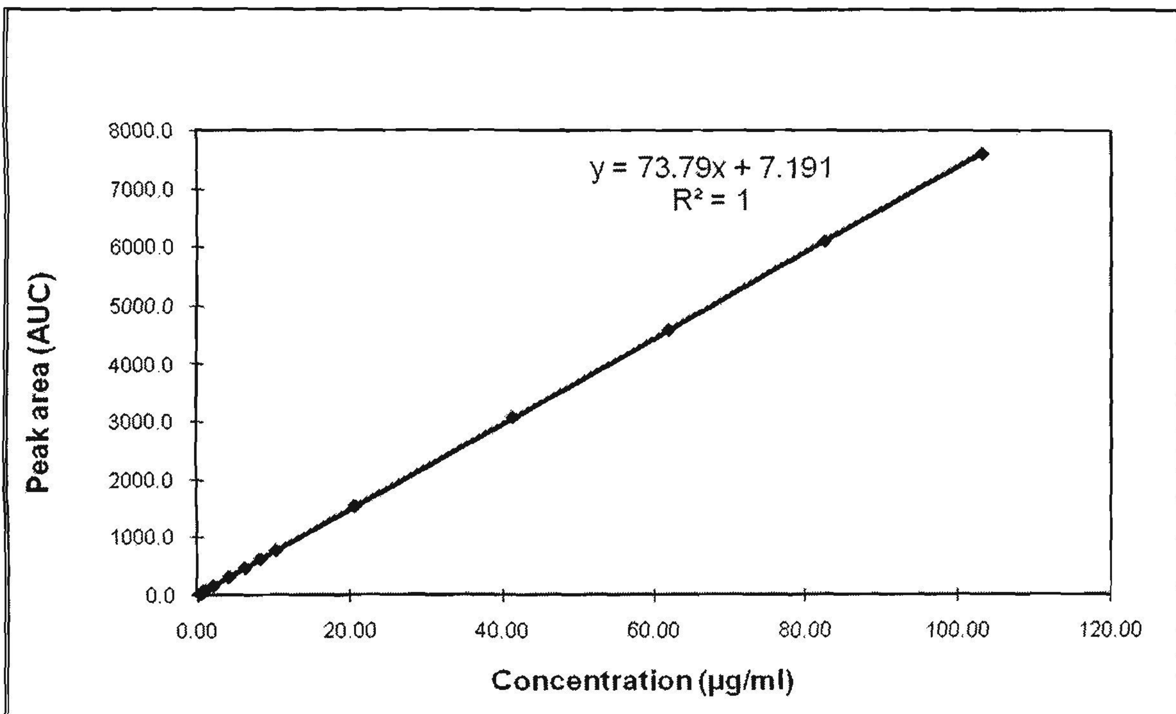


Figure C.2: Linear regression curve of salicylic acid.

Table C.1: Peak ratio values of niacinamide.

Standard (μg/ml)	**Peak area ratio**
0.20	13.2
0.41	28.2
0.61	44.0
0.82	60.4
1.02	75.1
2.05	152.1
4.09	307.0
6.14	466.3
8.18	623.9
10.23	785.5
20.46	1576.2
40.92	3158.3
61.38	4703.4
81.84	6277.1
102.30	7828.4
R^2	0.99
Intercept	-1.07
Slope	76.7

Table C.2: Peak ratio values of salicylic acid.

Standard (μg/ml)	Peak area ratio
0.21	15.7
0.41	30.9
0.62	49.3
0.82	66.7
1.03	81.3
2.06	158.3
4.12	310.1
6.19	460.9
8.25	618.3
10.31	765.7
20.62	1541.1
41.24	3069.8
61.86	4580.1
82.48	6093.1
103.10	7600.2
R^2	0.99
Intercept	7.19
Slope	73.79

C.4.2 ACCURACY AND PRECISION

Accuracy of an analytical method expresses the closeness of the value found to the true value. It is assessed over a specified range and 3 replicates at 3 concentrations must be analysed. Accuracy is expressed as the percentage (%) recovery. The acceptance criteria for accuracy is the following: the recovery must be between 98 - 102%.

Precision of an analytical method expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. Precision is sub-divided into repeatability (within-day variation), intermediate precision (between-day variation) and reproducibility (inter laboratory variation).

C.4.2.1 ACCURACY AND INTRA-DAY PRECISION

HPLC analysis was performed on 3 different samples of standard solution that was 3.003 µg/ml, 30.03 µg/ml and 300.3 µg/ml for niacinamide and 3 µg/ml, 30 µg/ml and 300 µg/ml for salicylic acid in triplicate on the same day.

According to **table C.3** and **table C.4** the accuracy of niacinamide and salicylic acid were both at 102% and was thus within the acceptance criteria.

Table C.3: The accuracy of niacinamide.

Conc.* Spiked (µg/ml)	AUC** 1	AUC** 2	Mean	Recovery (µg/ml)	%
3.003	297.46	279.28	288.37	3.000	99.904
3.003	297.82	299.30	298.56	3.106	103.435
3.003	291.63	294.30	292.97	3.048	101.496
30.03	2593.58	2597.78	2595.68	31.240	104.029
30.03	2601.49	2607.99	2604.74	31.349	104.392
30.03	2598.79	2606.44	2602.62	31.323	104.307
300.3	24580.00	24425.50	24502.75	298.475	99.392
300.3	24547.60	24542.60	24545.10	298.991	99.564
300.3	24376.70	24487.20	24431.95	297.613	99.105
				Mean	101.74
				SD* **	2.17
				% RSD******	2.13

*Conc. refers to concentration (µg/ml)

**AUC refers to area under the curve

***SD refers to standard deviation

****% RSD refers to relative standard deviation

Table C.4: The accuracy of salicylic acid.

Conc.* Spiked (μg/ml)	AUC** 1	AUC** 2	Mean	Recovery (μg/ml)	%
3	254.18	236.02	245.10	2.975	99.171
3	254.71	248.80	251.76	3.056	101.865
3	255.15	251.39	253.27	3.074	102.478
30	2275.11	2272.63	2273.87	31.357	104.522
30	2270.19	2269.13	2269.66	31.299	104.328
30	2266.24	2261.27	2263.76	31.217	104.057
300	18877.10	18686.40	18781.75	312.095	104.032
300	18160.50	17903.00	18031.75	299.633	99.878
300	18014.80	17888.40	17951.60	298.301	99.434
				Mean	102.20
				SD* **	2.09
				% RSD******	2.04

*Conc. refers to concentration (μg/ml)

**AUC refers to area under the curve

***SD refers to standard deviation

****% RSD refers to relative standard deviation

C.4.2.2 INTER-DAY PRECISION

A 100% standard solution was prepared, described in **section C.2**, on 3 different days and analysed on the HPLC in duplicate. The acceptance criteria for inter-day precision are: the % RSD (Relative standard deviation) must be ≤ 5%. According to **table C.5** and **table C.6** the % RSD for niacinamide and salicylic acid, individually, were below 5%.

Table C.5: Inter-day precision of niacinamide.

Concentration (μg/ml)	Day 1	Day 2	Day 3	Between days
	99.1	104.0	102.3	
	98.9	104.3	98.9	
Mean	99.0	104.2	100.6	101.2
SD*	0.1	0.2	2.4	1.3
% RSD**	0.00	0.00	0.02	0.01

*SD refers to standard deviation

**% RSD refers to relative standard deviation

Table C.6: Inter-day precision of salicylic acid.

Concentration (µg/ml)	Day 1	Day 2	Day 3	Between days
	99.9	104.5	99.9	
	99.4	104.3	100.1	
Mean	99.6	104.4	100.0	101.3
SD*	0.4	0.1	0.00	0.1
% RSD**	0.00	0.00	0.00	0.00

*SD refers to standard deviation

**% RSD refers to relative standard deviation

C.4.3 RUGGEDNESS

C.4.3.1 STABILITY OF SAMPLE SOLUTION

A standard sample was prepared as described in section **C.2**. The sample was injected on the HPLC system and analysed at hourly intervals for up to 24 hours in order to determine the stability of the sample. The pump was programmed to reduce the flow rate to 0.1 ml/min after elution of the peak and it was to reset the flow rate to 1 ml/min, 5 minutes before the next sample was injected.

Table C.7 and **table C.8** provide the results for niacinamide and salicylic acid stability determination, individually.

Table C.7: The stability parameters of niacinamide.

Time (hours)	AUC*	%
0	3612.64	100.0
1	3640.86	100.8
2	3653.77	101.1
3	3666.79	101.5
4	3680.59	101.9
5	3658.21	101.3
6	3726.63	103.2
7	3693.32	102.2
8	3670.66	101.6
9	3655.65	101.2
10	3703.48	102.5
11	3692.28	102.2
12	3671.02	101.6
13	3657.01	101.2
14	3668.32	101.5
15	3654.54	101.2
16	3725.10	103.1
17	3675.23	101.7
18	3659.77	101.3
19	3669.57	101.6
20	3619.83	100.2
21	3619.30	100.2
22	3616.40	100.1
23	3602.84	99.7
24	3630.36	100.5
Mean	3661.0	101.6
SD**	32.14	0.78
% RSD***	0.88	0.76

*AUC refers to area under the curve

**SD refers to standard deviation

**% RSD refers to relative standard deviation

Table C.8: The stability parameters of salicylic acid.

Time (hours)	AUC*	%
0	2917.018	100.0
1	2911.444	99.8
2	2901.937	99.5
3	2912.467	99.8
4	2927.585	100.4
5	2911.388	99.8
6	2932.797	100.5
7	2933.941	100.6
8	2940.709	100.8
9	2992.272	102.6
10	2936.544	100.7
11	2942.972	100.9
12	2932.271	100.5
13	2934.652	100.6
14	2926.826	100.3
15	2928.396	100.4
16	2932.844	100.5
17	2936.023	100.7
18	2882.611	98.8
19	2962.659	101.6
20	2949.823	101.1
21	2959.664	101.5
22	2952.036	101.2
23	2980.552	102.2
24	2966.434	101.7
Mean	2936.2	100.5
SD**	23.88	0.75
% RSD***	0.81	0.74

*AUC refers to area under the curve

**SD refers to standard deviation

**% RSD refers to relative standard deviation

C.4.4.2 SYSTEM REPEATABILITY

HPLC analysis was performed on 6 replicates from the same standard solution (100%) with known concentrations of niacinamide and salicylic acid separately. **Table C.9** and **table C.10** illustrate the results for niacinamide and salicylic acid. Both niacinamide and salicylic acid had a % RSD of less than 2% and this complies with the acceptance criteria for system repeatability.

Table C.9: System repeatability parameters for niacinamide

	AUC*	Retention times (minutes)
	3216	3.062
	3223	3.071
	3222	3.060
	3193	3.063
	3235	3.056
	3236	3.064
Mean	3221	3.063
SD**	14.48	0.005
% RSD***	0.45	0.148

*AUC refers to area under curve

**SD refers to standard deviation

***% RSD refers to relative standard deviation

Table C.10: System repeatability parameters for salicylic acid.

	AUC*	Retention times (minutes)
	61.51	5.243
	60.65	5.233
	61.68	5.240
	60.98	5.239
	61.93	5.235
	62.32	5.235
Mean	62.00	5.238
SD**	0.56	0.003
% RSD***	0.91	0.066

*AUC refers to area under

*SD refers to standard deviation

**% RSD refers to relative standard deviation

C.4.5 SPECIFICITY

Standard samples were prepared in water, 0.1M hydrochloric acid, 0.1M sodium hydroxide and 0.1M perchloric acid. It was placed in an oven at 60°C. The samples were neutralised and analysed on the HPLC system. There were additional peaks added on the chromatogram, but none of them interfered with the peak areas of salicylic acid and niacinamide.

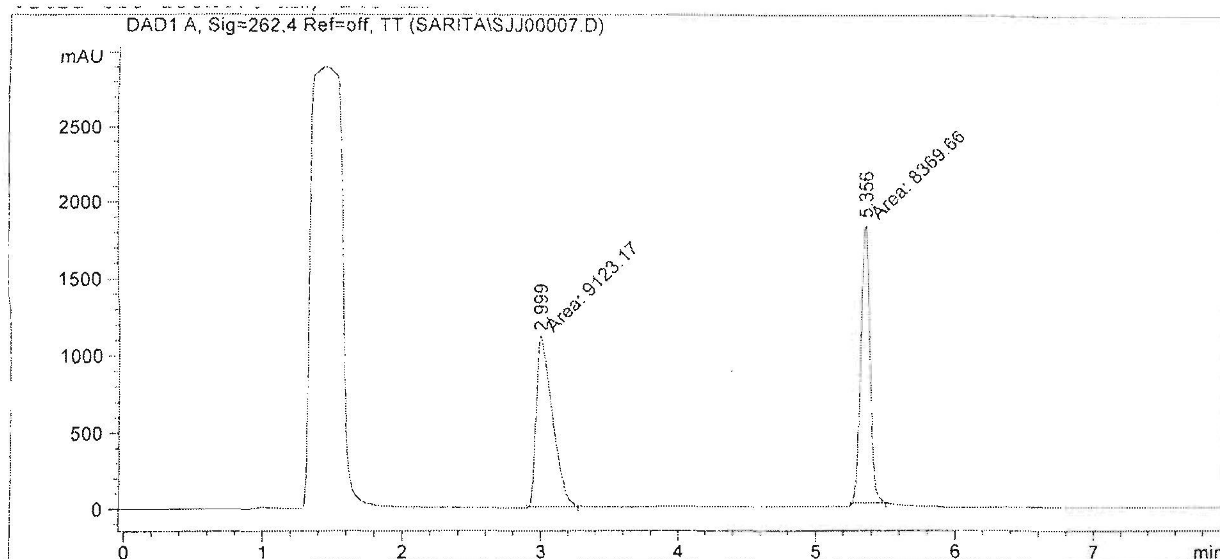


Figure C.3: Standard sample stressed in 10% perchloric acid (H_2O_2) and placed in 60°C oven.

C.5 CONCLUSION

The development of the HPLC system method has proven to be reliable and sensitive for the determination of niacinamide and salicylic acid's concentrations.

APPENDIX D: VALIDATION OF THE HPLC EXPERIMENTAL METHOD FOR A PHEROID™ GEL CONTAINING NIACINAMIDE AND SALICYLIC ACID

D.1 PURPOSE OF THE VALIDATION

The purpose of validation was to establish a procedure for the validation of an analytical method which would provide the necessary proof that the method used was sensitive and reliable. Intended for the determination of the concentration of the ingredients in the Pheroid™ gel such as salicylic acid, niacinamide, methyl paraben, Butylated hydroxyanisole (BHA), Butylated hydroxytoluene (BHT) and vitamin E that permeated the skin and for stability determination.

D.2 CHROMATOGRAPHIC CONDITIONS

The chromatographic conditions are given in **Table D.1**:

Table D.1: Chromatographic conditions for the detection of salicylic acid and niacinamide.

Analytical instrument	Agilent 1100 series auto sampler, Agilent 1100 series variable detector (VWD) and a Agilent1100 series isocratic pump.
Column	A Phenomenex (Luna C-18, 5 μ, 250 mm x 4.60 mm) column.
Mobile Phase	1% orthophosphoric acid/water with a pH of 3.5.
Gradient	25% methanol to 1minute, then 100% methanol to 25.10 minutes and thereafter the system was re-equilibrated at the starting conditions for 5 minutes.
Flow Rate	1.0ml/min
Injection volume	20 μl
Detection	UV at 220 nm
Retention times	Niacinamide – 3.2 minutes Methyl paraben – 9.2 minutes Salicylic acid – 10.1 minutes BHA – 11.1 minutes BHT – 13.0 minutes and Vitamin E – 23.1 minutes
Stop time	30 minutes
Solvent	100% Methanol

D.3 STANDARD PREPARATION (100%)

Approximately 40 mg salicylic acid, 60 mg niacinamide, 4 mg vitamin E, 8 mg methyl paraben, 0.4 mg BHA and 4 mg BHT were accurately weighed and dissolved in 50 ml of methanol. The solution was then sonicated for at least 10 minutes and then transferred into an auto sampler vial for analysis.

D.4 SAMPLE PREPARATION (100%)

Approximately 2 g of Pheroid™ gel was accurately weighed and dissolved in 100 ml of methanol. The solution was then sonicated for at least 10 minutes and transferred into an auto sampler vial for analysed.

D.5 VALIDATION PARAMETERS

D.5.1 LINEARITY AND RANGE

See **Appendix C.4.1** for a detailed description of Linearity and Range.

A range of 70%, 80%, 100%, 120% and 130% of standard preparations were prepared. Inject 20 μl of each preparation in duplicate on the HPLC system.

The regression coefficient (R^2) that was obtained for all of the ingredients mentioned in **section D.2** indicated an excellent linearity with a value of 0.99. This indicated that the HPLC system was stable. **Figures D.1** to **D.6** illustrate the different linear regression curves for niacinamide, salicylic acid, methyl paraben, vitamin E, BHA and BHT.

Tables D.1 to **D.6** provide the peak area ratio of the standards (μg/ml) of all of the above mentioned ingredients individually.

Table D.1: Peak ratio values of niacinamide in the Pheroid^{TM} gel.

Standard (µg/ml)	Peak area ratio
336	51762.7
384	59216.3
480	72208.9
577	84383.3
624	88150.4
R^2	0.995
Intercept	127.6
Slope	9864

Table D.2: Peak ratio values of methyl paraben in the Pheroid^{TM} gel.

Standard (µg/ml)	Peak area ratio
45.2	3133.0
51.7	3537.8
64.6	4325.3
77.5	5132.9
84	5492.3
R^2	0.999
Intercept	61.11
Slope	375.9

Table D.3: Peak ratio values of salicylic acid in the Pheroid™ gel.

Standard (µg/ml)	Peak area ratio
227	22937.7
259.4	26338.25
324.3	32167.55
389.2	39386.65
421.6	42032.6
R^2	0.998
Intercept	98.86
Slope	511.0

Table D.4: Peak ratio values of BHA in the Pheroid™ gel.

Standard (µg/ml)	Peak area ratio
3.4	502.2
3.9	562.0
4.9	686.0
5.9	881.7
6.4	952.8
R^2	0.991
Intercept	153.1
Slope	-33.62

Table D.5: Peak ratio values of BHT in the Pheroid™ gel.

Standard (µg/ml)	Peak area ratio
22.4	2152.6
25.6	2474.1
32	3061.6
38.4	3693.9
41.6	3983.4
R^2	0.999
Intercept	95.33
Slope	22.37

Table D.6: Peak ratio values of vitamin E .in the Pheroid™ gel.

Standard (µg/ml)	Peak area ratio
22	2390.6
25.1	2746.0
31.4	3384.9
37.7	4080.4
40.8	4436.2
R^2	0.999
Intercept	107.9
Slope	19.33

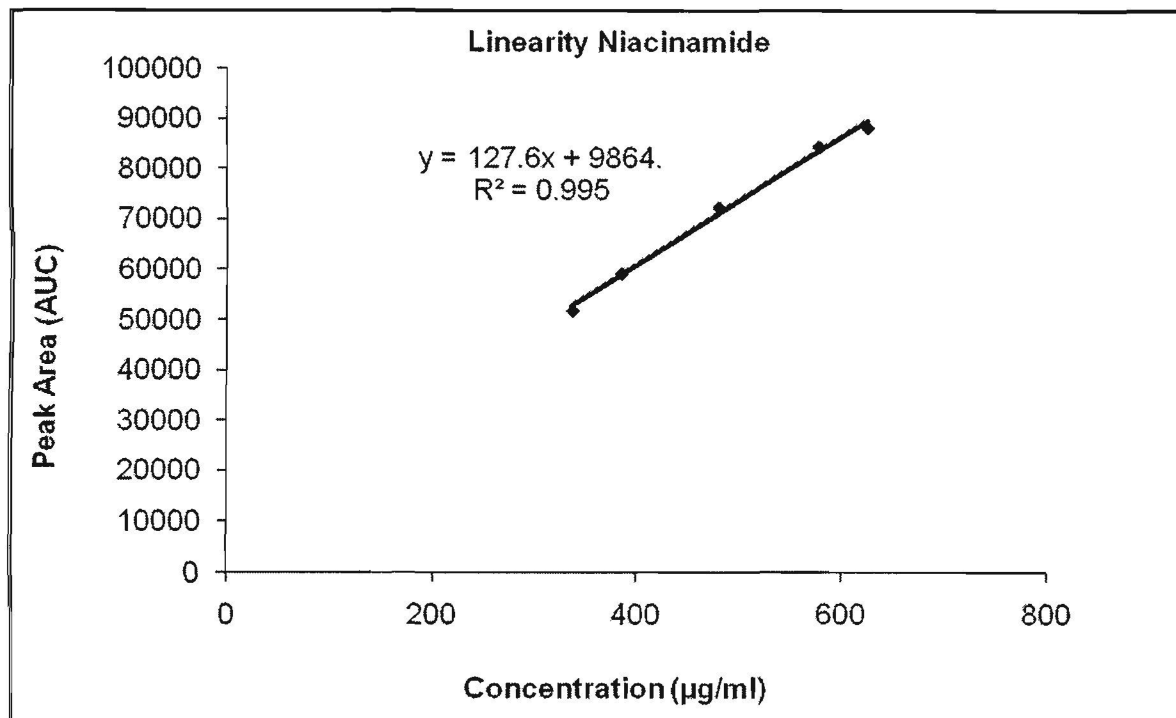


Figure D.1: Linear regression curve of niacinamide in the Pheroid™ gel.

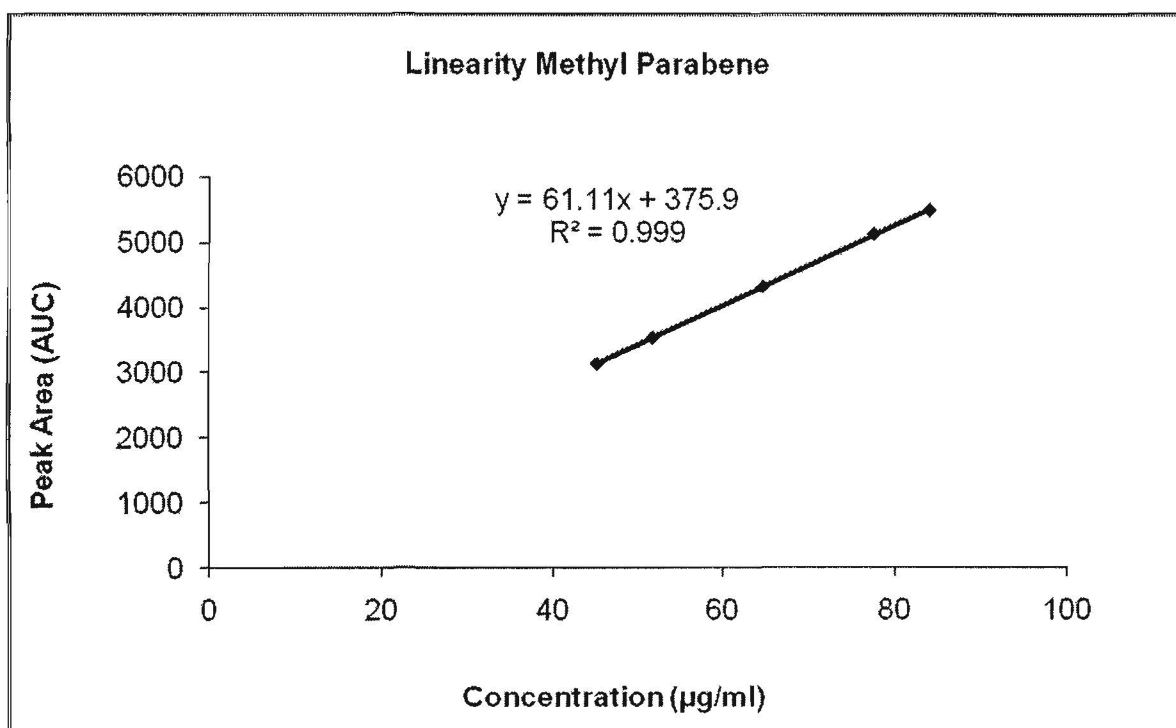


Figure D.2: Linear regression curve of methyl paraben in the Pheroid™ gel.

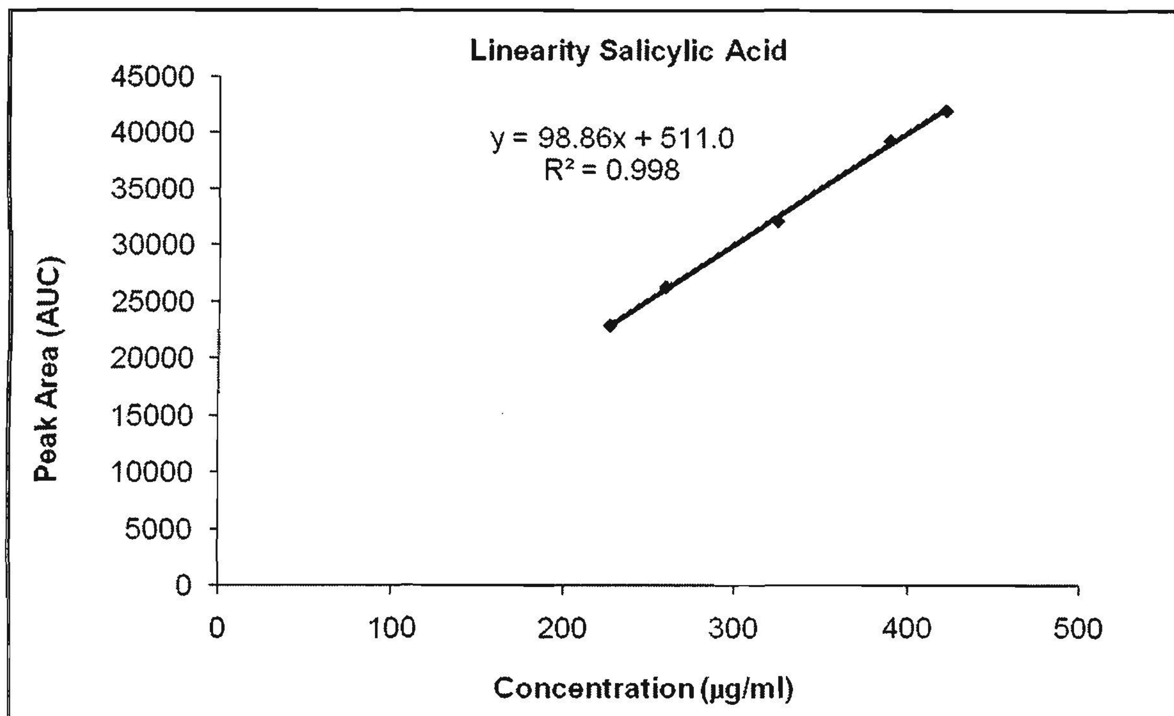


Figure D.3: Linear regression curve of salicylic acid in the Pheroid™ gel.

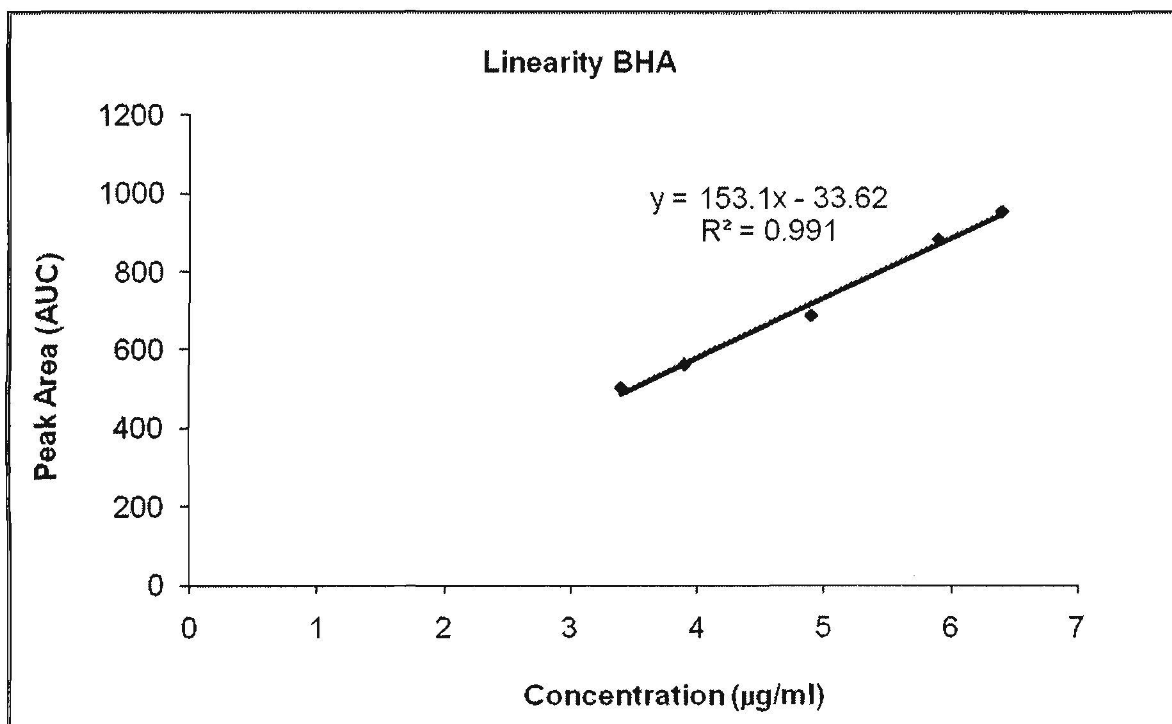


Figure D.4: Linear regression curve of BHA in the Pheroid™ gel.

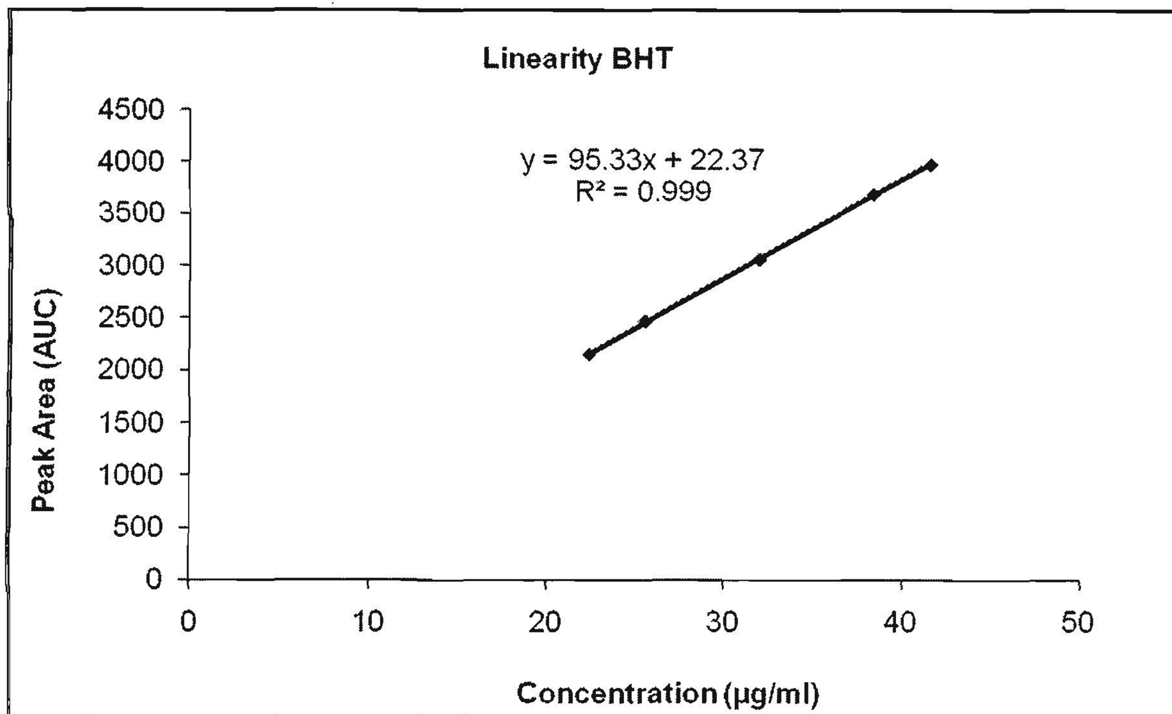


Figure D.5: Linear regression curve of BHT in the Pheroid™ gel.

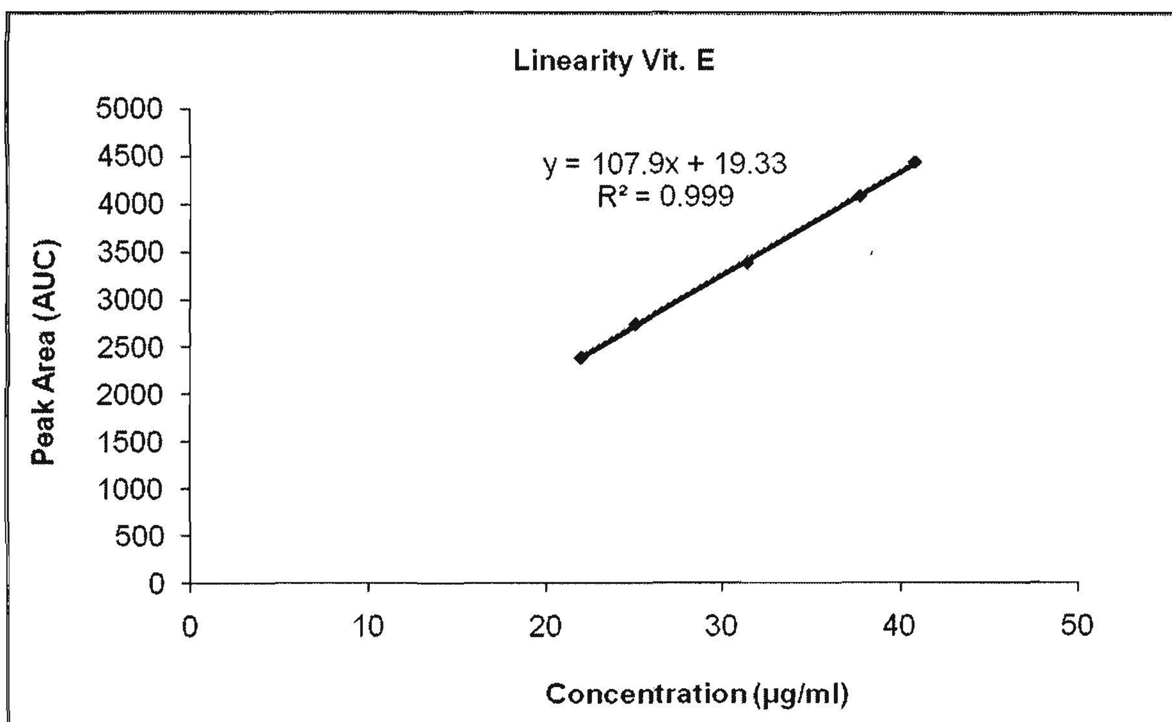


Figure D.6: Linear regression curve of vitamin E in the Pheroid™ gel.

D.5.2 ACCURACY

See **Appendix C.4.2** for a detailed description of accuracy.

A placebo of all matrix components except those to be tested, was prepared. An accurate amount of 80%, 100% and 120% placebo were weighed into 100ml volumetric flasks. Standard solutions (as prescribed in section **D.3**) of 80%, 100% and 120% were prepared. The placebos were spiked with the standard preparations, example the 80% placebo with 80% standard solution. All of the mixtures were filled to 100 ml with methanol and sonicated for 10 minutes. Samples where then transferred into auto sampler vials and analyse.

The recovery for all of the ingredients was between 98 – 102% of the limits. **Tables D.7** to **D.12** depicts the results of the ingredient accuracy.

Table D.7: Accuracy of niacinamide.

Conc.* spiked (µg/ml)	AUC** 1	AUC** 2	Mean	Recovery (µg/ml)	%
396.50	60423.60	60628.90	60526.25	413.52	104.29
396.50	61289.30	60576.30	60932.80	416.30	104.99
396.50	60984.80	61378.00	61181.40	418.00	105.42
498.88	72102.90	72161.00	72131.95	492.82	98.78
498.88	71956.80	71891.50	71924.15	491.40	98.50
498.88	71783.90	71927.40	71855.65	490.93	98.41
579.41	78567.80	78555.60	78561.70	536.74	92.64
579.41	79338.10	79549.50	79443.80	542.77	93.68
579.41	79557.70	79893.30	79725.50	544.70	94.01
				Mean	98.97
				SD* **	4.71
				% RSD****	4.76

*Conc. refers to concentration (µg/ml)
**AUC refers to area under the curve
***SD refers to standard deviation
****% RSD refers to relative standard deviation

Table D.8: Accuracy of methyl paraben.

Conc.* spiked (µg/ml)	AUC** 1	AUC** 2	Mean	Recovery (µg/ml)	%
52.9	3570.5	3581.9	3576.2	55.0	104.0
52.9	3593.1	3586.0	3589.6	55.2	104.4
52.9	3561.1	3586.5	3573.8	55.0	104.0
65.3	4276.2	4255.6	4265.9	65.6	100.4
65.3	4294.7	4232.7	4263.7	65.6	100.4
65.3	4208.7	4290.2	4249.5	65.4	100.1
77.3	4746.8	4714.9	4730.9	72.8	94.2
77.3	4749.7	4727.9	4738.8	72.9	94.3
77.3	4722.9	4707.1	4715.0	72.5	93.9
				Mean	99.5
				SD* **	4.1
				% RSD******	4.1

*Conc. refers to concentration (µg/ml)
**AUC refers to area under the curve
***SD refers to standard deviation
****% RSD refers to relative standard deviation

Table D.9: Accuracy of salicylic acid.

Conc.* spiked (µg/ml)	AUC** 1	AUC** 2	Mean	Recovery (µg/ml)	%
264.3	26008.9	26064.1	26036.5	270.8	102.5
264.3	26397.5	26072.5	26235.0	272.9	103.2
264.3	26294.8	26426.7	26360.8	274.2	103.7
326.6	31600.6	31592.8	31596.7	328.7	100.6
326.6	31536.0	31536.7	31536.4	328.1	100.4
326.6	31154.3	31505.9	31330.1	325.9	99.8
386.3	34880.6	34899.6	34890.1	362.9	94.0
386.3	35288.4	35329.4	35308.9	367.3	95.1
386.3	35270.4	35291.3	35280.9	367.0	95.0
				Mean	99.4
				SD***	3.5
				% RSD******	3.6

Table D.10: Accuracy of BHA.

Conc.* spiked (µg/ml)	AUC** 1	AUC** 2	Mean	Recovery (µg/ml)	%
2.6	282.6	302.1	292.4	2.8	106.1
2.6	295.8	290.5	293.2	2.8	106.4
2.6	296.9	296.6	296.8	2.8	107.7
3.3	349.2	358.0	353.6	3.4	103.8
3.3	349.5	348.4	349.0	3.3	102.5
3.3	340.4	383.4	361.9	3.5	106.3
3.9	363.3	357.7	360.5	3.5	89.5
3.9	371.9	398.6	385.3	3.7	95.7
3.9	390.1	398.1	394.1	3.8	97.9
				Mean	101.8
				SD* **	5.8
				% RSD****	5.7

*Conc. refers to concentration (µg/ml)
**AUC refers to area under the curve
***SD refers to standard deviation
****% RSD refers to relative standard deviation

Table D.11: Accuracy of BHT.

Conc.* spiked (µg/ml)	AUC** 1	AUC** 2	Mean	Recovery (µg/ml)	%
26.4	2233.1	2250.7	2241.9	28.0	106.0
26.4	2342.5	2298.3	2320.4	29.0	109.7
26.4	2334.4	2412.6	2373.5	29.7	112.2
32.7	2696.9	2729.8	2713.4	33.9	103.8
32.7	2636.7	2658.7	2647.7	33.1	101.3
32.7	2592.0	2550.5	2571.3	32.1	98.4
42.3	2854.9	2838.9	2846.9	35.6	84.1
42.3	2923.0	2970.6	2946.8	36.8	87.1
42.3	2967.3	3015.2	2991.3	37.4	88.4
				Mean	99.0
				SD* **	9.7
				% RSD****	9.8

Table D.12: Accuracy of vitamin E.

Conc.* spiked (µg/ml)	AUC** 1	AUC** 2	Mean	Recovery (µg/ml)	%
26.4	1217.3	1231.4	1224.3	30.6	115.7
26.4	1287.2	1234.1	1260.7	31.5	119.1
26.4	1265.8	1257.3	1261.5	31.5	119.2
32.7	1368.6	1373.6	1371.1	34.2	104.9
32.7	1185.3	1337.5	1261.4	31.5	96.5
32.7	1145.8	1146.3	1146.1	28.6	87.6
42.3	1394.3	1391.2	1392.8	34.8	82.2
42.3	1538.9	1533.4	1536.2	38.4	90.7
42.3	1542.7	1552.5	1547.6	38.7	91.4
				Mean	100.8
				SD***	13.5
				% RSD****	13.4

*Conc. refers to concentration (µg/ml)
**AUC refers to area under the curve
***SD refers to standard deviation
****% RSD refers to relative standard deviation

D.5.3 PRECISION

See **Appendix C.4.3** for a detailed description of precision.

D.5.3.1 INTRA-DAY PRECISION (REPEATABILITY)

Sample preparations (as seen in section **D.4**) of 80%, 100% and 120% were accurately weighed and dissolved in 100 ml methanol. A single standard solution (as seen in section **D.3**) was also prepared. The samples as well as the standard were transferred into auto sampler vials and analysed. The samples were injected in duplicate on the HPLC system.

See **tables D.13** to **D.18** for the results of the ingredients.

Table D.13: Intra-day precision parameters of niacinamide in the Pheroid^{TM} gel.

Conc.* spiked (µg/ml)	AUC** 1	AUC** 2	Mean	Recovery (µg/ml)	%
387.6	62980.6	60737.2	61858.9	408.1	105.3
387.6	60815.5	61721.4	61268.5	404.2	104.3
387.6	61765.9	61763.5	61764.7	407.5	105.1
483.0	74980.9	74841.9	74911.4	494.2	102.3
483.0	74941.2	74920.5	74930.9	494.4	102.4
483.0	73902.7	73888.1	73895.4	487.5	100.9
577.5	85947.7	86176.5	86062.1	567.8	98.3
577.5	86065.9	86185.9	86125.9	568.2	98.4
577.5	86196.0	86200.1	86198.1	568.7	98.5
				Mean	101.7
				SD* **	2.7
				% RSD****	2.7

*Conc. refers to concentration (µg/ml)
**AUC refers to area under the curve
***SD refers to standard deviation
****% RSD refers to relative standard deviation

Table D.14: Intra-day precision parameters of methyl paraben in the Pheroid™ gel.

Conc.* spiked (µg/ml)	AUC** 1	AUC** 2	Mean	Recovery (µg/ml)	%
51.7	3732.6	3532.8	3632.7	52.9	102.3
51.7	3551.3	3611.3	3581.3	52.1	100.8
51.7	3592.1	3589.1	3590.6	52.3	101.1
64.4	4339.5	4348.5	4344.0	63.2	98.2
64.4	4337.1	4332.2	4334.7	63.1	98.0
64.4	4297.7	4300.9	4299.3	62.6	97.2
77.0	5068.3	5079.4	5073.9	73.8	95.9
77.0	5038.5	5075.8	5057.2	73.6	95.6
77.0	5044.0	5028.7	5036.4	73.3	95.2
				Mean	98.2
				SD* **	2.4
				% RSD******	2.5

*Conc. refers to concentration (µg/ml)
**AUC refers to area under the curve
***SD refers to standard deviation
****% RSD refers to relative standard deviation

Table D.15: Intra-day precision parameters of salicylic acid in the Pheroid™ gel.

Conc.* spiked (µg/ml)	AUC** 1	AUC** 2	Mean	Recovery (µg/ml)	%
258.4	24573.4	25389.8	24981.6	247.7	95.9
258.4	25868.4	26471.7	26170.1	259.5	100.4
258.4	26491.3	26537.5	26514.4	262.9	101.8
322.0	32629.2	32664.7	32647.0	323.7	100.5
322.0	32626.8	32638.8	32632.8	323.6	100.5
322.0	32260.6	32236.6	32248.6	319.8	99.3
385.0	38803.6	38881.5	38842.6	385.2	100.0
385.0	38846.0	38853.5	38849.8	385.2	100.1
385.0	38811.1	38822.7	38816.9	384.9	100.0
				Mean	99.8
				SD* **	1.5
				% RSD******	1.5

Table D.16: Intra-day precision parameters of BHA in the Pheroid^{TM} gel.

Conc.* spiked (µg/ml)	AUC** 1	AUC** 2	Mean	Recovery (µg/ml)	%
2.6	208.4	229.2	218.8	2.0	76.4
2.6	264.7	285.1	274.9	2.5	96.0
2.6	282.9	291.9	287.4	2.6	100.3
3.2	360.6	360.7	360.7	3.3	102.3
3.2	367.4	367.9	367.7	3.3	104.3
3.2	363.7	364.1	363.9	3.3	103.2
3.9	437.5	436.5	437.0	4.0	101.7
3.9	438.5	437.4	438.0	4.0	101.9
3.9	436.0	439.7	437.9	4.0	101.9
				Mean	98.7
				SD* **	8.2
				% RSD****	8.3

*Conc. refers to concentration (µg/ml)
**AUC refers to area under the curve
***SD refers to standard deviation
****% RSD refers to relative standard deviation

Table D.17: Intra-day precision parameters of BHT in the Pheroid^{TM} gel.

Conc.* spiked (µg/ml)	AUC** 1	AUC** 2	Mean	Recovery (µg/ml)	%
25.8	3074.8	2974.3	3024.6	22.9	88.6
25.8	2663.3	3004.0	2833.7	21.4	83.1
25.8	2970.8	3011.2	2991.0	22.6	87.7
32.2	3384.2	3322.8	3353.5	25.4	78.8
32.2	3674.0	3663.3	3668.7	27.7	86.2
32.2	3357.3	3676.5	3516.9	26.6	82.6
38.5	4509.9	4079.9	4294.9	32.5	84.4
38.5	4077.3	4106.3	4091.8	30.9	80.4
38.5	4066.1	4103.8	4085.0	30.9	80.2
				Mean	83.5
				SD* **	3.3
				% RSD****	3.9

Table D.18: Intra-day precision parameters of vitamin E in the Pheroid™ gel.

Conc.* spiked (µg/ml)	AUC** 1	AUC** 2	Mean	Recovery (µg/ml)	%
25.8	1278.5	1293.3	1285.9	20.6	79.7
25.8	1313.8	1309.8	1311.8	21.0	81.3
25.8	1274.2	1260.5	1267.4	20.3	78.6
32.2	1663.2	1667.4	1665.3	26.6	82.7
32.2	1602.2	1587.7	1595.0	25.5	79.3
32.2	1599.3	1681.0	1640.2	26.2	81.5
38.5	2024.6	1944.6	1984.6	31.8	82.5
38.5	1915.1	1964.0	1939.6	31.0	80.6
38.5	2026.9	2012.5	2019.7	32.3	83.9
				Mean	81.1
				SD* **	1.7
				% RSD****	2.0

*Conc. refers to concentration (µg/ml)
**AUC refers to area under the curve
***SD refers to standard deviation
****% RSD refers to relative standard deviation

D.5.3.1 INTER-DAY PRECISION

A 100% sample was prepared, as described in **section D.4**, on 3 different days and analysed on the HPLC in triplicate. The results may be observed in **tables D.19** to **D.24** for all the ingredients.

Table D.19: Inter-day precision parameters for niacinamide in the Pheroid™ gel.

Concentration (µg/ml)	Day 1	Day 2	Day 3	Between days
	98.8	102.3	101.7	
	98.5	102.4	101.2	
	98.4	100.9	101.2	
Mean	98.6	101.9	101.3	100.6
SD*	0.2	0.8	0.3	0.3
% RSD**	0.0	0.0	0.0	0.0

*SD refers to standard deviation
**% RSD refers to relative standard deviation

Table D.20: Inter-day precision parameters for methyl paraben in the Pheroid^{TM} gel.

Concentration (µg/ml)	Day 1	Day 2	Day 3	Between days
	100.4	98.2	97.4	
	100.4	98.0	97.2	
	100.1	97.2	97.2	
Mean	100.3	97.8	97.3	98.4
SD*	0.2	0.5	0.1	0.2
% RSD**	0.0	0.0	0.0	0.0

*SD refers to standard deviation
**% RSD refers to relative standard deviation

Table D.21: Inter-day precision parameters for salicylic acid in the Pheroid^{TM} gel.

Concentration (µg/ml)	Day 1	Day 2	Day 3	Between days
	100.6	100.5	100.0	
	100.4	100.5	99.4	
	99.8	99.3	99.3	
Mean	100.3	100.1	99.6	100.0
SD*	0.4	0.7	0.4	0.2
% RSD**	0.0	0.0	0.0	0.0

*SD refers to standard deviation
**% RSD refers to relative standard deviation

Table D.22: Inter-day precision parameters for BHA in the Pheroid^{TM} gel.

Concentration (µg/ml)	Day 1	Day 2	Day 3	Between days
	103.8	102.3	92.8	
	102.5	104.3	103.2	
	106.3	103.2	102.6	
Mean	104.2	103.3	99.6	102.3
SD*	1.9	1.0	5.9	2.6
% RSD**	0.0	0.0	0.1	0.0

*SD refers to standard deviation
**% RSD refers to relative standard deviation

Table D.23: Inter-day precision parameters for BHT in the Pheroid^{TM} gel.

Concentration (µg/ml)	Day 1	Day 2	Day 3	Between days
	103.8	78.8	102.5	
	101.3	86.2	101.6	
	98.4	82.6	100.9	
Mean	101.2	82.5	101.7	95.1
SD*	2.7	3.7	0.8	1.5
% RSD**	0.0	0.0	0.0	0.0

*SD refers to standard deviation
**% RSD refers to relative standard deviation

Table D.24: Inter-day precision parameters for vitamin E in the Pheroid^{TM} gel.

Concentration (µg/ml)	Day 1	Day 2	Day 3	Between days
	104.9	82.7	80.5	
	96.5	79.3	78.7	
	87.6	81.5	82.4	
Mean	96.3	81.2	80.5	86.0
SD*	8.6	1.8	1.9	3.9
% RSD**	0.1	0.0	0.0	0.0

*SD refers to standard deviation
**% RSD refers to relative standard deviation

D.5.4 RUGGEDNESS

D.5.4.1 STABILITY OF SAMPLE SOLUTION

A sample was prepared as described in section **D.4**. The sample was injected on the HPLC system, and reanalysed at hourly intervals for up to 12 hours in order to determine the stability of the specific sample. The pump was programmed to reduce the flow rate to 0.1 ml/min after elution of the peak, and then reset the flow rate to 1 ml/min 5 minutes before the next sample was injected.

Tables D.25 to **D.30** provide the results of all the ingredients for the stability determination.

Table D.25: The stability parameters of niacinamide in the Pheroid^{TM} gel.

Time (hours)	AUC*	%
0	77730.1	100.0
1	77829.3	100.1
2	77784.4	100.1
3	78882.6	101.5
4	77898.9	100.2
5	78566.5	101.1
6	79151.3	101.8
7	78084.2	100.5
8	78857.7	101.5
9	77971.2	100.3
10	79105.8	101.8
11	78171.7	100.6
12	78905.7	101.5
Mean	78380.0	100.8
SD**	520.61	0.67
% RSD***	0.66	0.66

*AUC refers to area under curve
**SD refers to standard deviation
***% RSD refers to relative standard deviation

Table D.26: The stability parameters of methyl paraben in the Pheroid^{TM} gel.

Time (hours)	AUC*	%
0	4532.8	100.0
1	4656.4	102.7
2	4636.9	102.3
3	4656.1	102.7
4	4601.9	101.5
5	4633.4	102.2
6	4663.6	102.9
7	4650.5	102.6
8	4820.3	106.3
9	4714.7	104.0
10	4700.3	103.7
11	4678.0	103.2
12	4708.1	103.9
Mean	4665.6	102.9
SD**	64.13	1.41
% RSD***	1.37	1.37

*AUC refers to area under curve
**SD refers to standard deviation
***% RSD refers to relative standard deviation

Table D.27: The stability parameters of salicylic acid in the Pheroid^{TM} gel.

Time (hours)	AUC*	%
0	34298.8	100.0
1	34273.9	99.9
2	34246.5	99.8
3	34679.3	101.1
4	34301.5	100.0
5	34554.5	100.7
6	34743.9	101.3
7	34326.2	100.1
8	34742.4	101.3
9	34335.8	100.1
10	34764.6	101.4
11	34403.5	100.3
12	34783.4	101.4
Mean	34496.5	100.6
SD**	208.53	0.61
% RSD***	0.60	0.60

*AUC refers to area under curve
**SD refers to standard deviation
***% RSD refers to relative standard deviation

Table D.28: The stability parameters of BHA in the Pheroid™ gel.

Time (hours)	AUC*	%
0	382.5	100.0
1	380.7	99.5
2	380.9	99.6
3	382.7	100.1
4	377.2	98.6
5	381.1	99.6
6	384.0	100.4
7	378.7	99.0
8	380.2	99.4
9	372.4	97.4
10	378.8	99.0
11	375.9	98.3
12	378.0	98.8
Mean	379.5	99.2
SD**	3.01	0.79
% RSD***	0.79	0.79

*AUC refers to area under curve
**SD refers to standard deviation
***% RSD refers to relative standard deviation

Table D.29: The stability parameters of BHT in the Pheroid™ gel.

Time (hours)	AUC*	%
0	3170.0	100.0
1	3119.5	98.4
2	3113.0	98.2
3	3149.2	99.3
4	3084.6	97.3
5	3128.1	98.7
6	3137.5	99.0
7	3126.4	98.6
8	3108.0	98.0
9	3105.5	98.0
10	3153.3	99.5
11	3103.6	97.9
12	3104.3	97.9
Mean	3123.3	98.5
SD**	23.05	0.73
% RSD***	0.74	0.74

*AUC refers to area under curve
**SD refers to standard deviation
***% RSD refers to relative standard deviation

Table D.30: The stability parameters of vitamin E in the Pheroid™ gel.

Time (hours)	AUC*	%
0	1728.1	100.0
1	1728.4	100.0
2	1723	99.7
3	1737.6	100.5
4	1705	98.7
5	1716.7	99.3
6	1729	100.1
7	1705.3	98.7
8	1717.7	99.4
9	1694.7	98.1
10	1710.7	99.0
11	1680	97.2
12	1699.7	98.4
Mean	1713.5	99.2
SD**	15.66	0.91
% RSD***	0.91	0.91

*AUC refers to area under curve
**SD refers to standard deviation
***% RSD refers to relative standard deviation

D.5.4.2 SYSTEM REPEATABILITY

A sample was prepared according to **section D.4** and then injected six times, to determine the repeatability of the peak area as well as the retention time.

The results of the system repeatability are given individually in **tables D.31** to **D.36.** The results proofed that all of the ingredients had a % RSD less than 2% which was within the range of the acceptance criteria.

Table D.31: The system repeatability parameters of niacinamide.

	Peak Area	**Retention times (minutes)**
	70658	3.202
	71935	3.214
	71993	3.216
	71914	3.218
	72033	3.215
	71511	3.218
Mean	71674	3.214
SD*	485.59	0.005
% RSD**	0.68	0.171

*SD refers to standard deviation
**% RSD refers to relative standard deviation

Table D.32: The system repeatability parameters of methyl paraben.

	Peak Area	**Retention times (minutes)**
	4942	9.179
	4944	9.177
	4916	9.178
	4938	9.176
	4925	9.192
	4901	9.197
Mean	4928	9.183
SD*	15.41	0.008
% RSD**	0.31	0.089

*SD refers to standard deviation
**% RSD refers to relative standard deviation

Table D.33: The system repeatability parameters of salicylic acid.

	Peak Area	Retention times (minutes)
	32321	10.121
	32702	10.157
	32676	10.175
	32804	10.195
	32813	10.199
	32636	10.197
Mean	32659	10.174
SD*	164.03	0.028
% RSD**	0.50	0.275

*SD refers to standard deviation
**% RSD refers to relative standard deviation

Table D.34: The system repeatability parameters of BHA.

	Peak Area	Retention times (minutes)
	450	11.137
	438	11.144
	438	11.143
	439	11.144
	440	11.150
	435	11.153
Mean	440	11.145
SD*	4.88	0.005
% RSD**	1.11	0.046

*SD refers to standard deviation
**% RSD refers to relative standard deviation

Table D.35: The system repeatability parameters of BHT.

	Peak Area	Retention times (minutes)
	4670	12.988
	4720	12.972
	4716	12.977
	4718	12.983
	4722	12.991
	4699	13.008
Mean	4708	12.987
SD*	18.38	0.012
% RSD**	0.39	0.089

*SD refers to standard deviation
**% RSD refers to relative standard deviation

Table D.36: The system repeatability parameters of vitamin E.

	Peak Area	Retention times (minutes)
	1280	22.944
	1379	22.864
	1421	22.860
	1375	22.884
	1352	23.050
	1264	23.068
Mean	1345	22.945
SD*	55.63	0.085
% RSD**	4.14	0.372

*SD refers to standard deviation
**% RSD refers to relative standard deviation

D.5.5 SPECIFICITY

Pheroid™ gel samples were prepared in water, 0.1M hydrochloric acid, 0.1M sodium hydroxide and 0.1M perchloric acid. It was then placed in an oven at approximately 60°C. The samples were neutralised and analysed on the HPLC system. There were additional peaks added on the chromatogram, but none of them interfered with the peak areas of niacinamide, methyl paraben, salicylic acid, BHA, BHT and vitamin E.

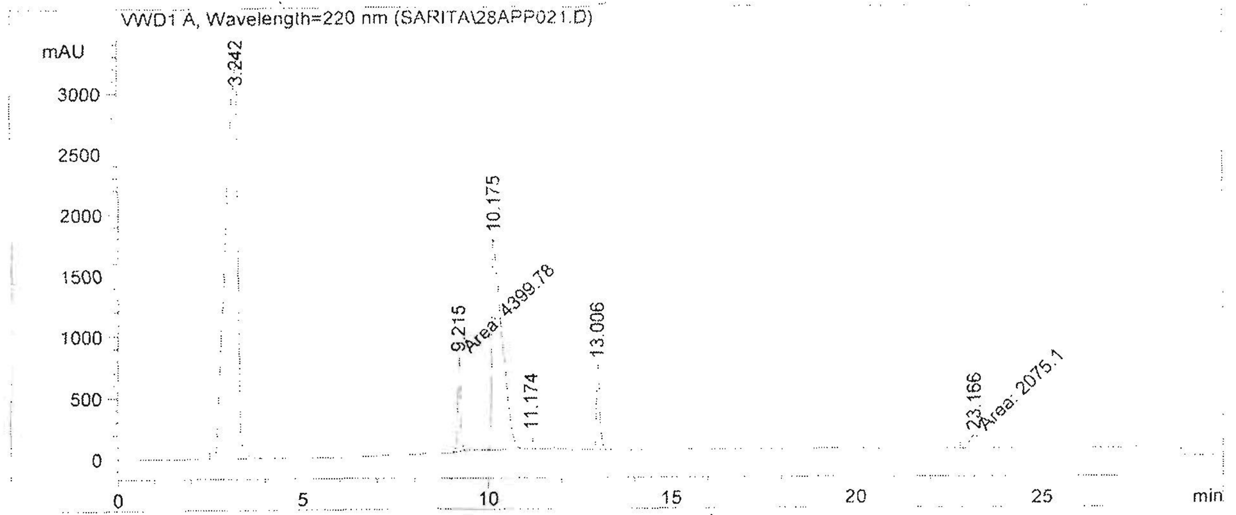


Figure D.7: Sample stressed in water at 60 °C in an oven.

D.6 CONCLUSION

The HPLC system method development for the Pheroid™ gel, has proven to be reliable and sensitive in order to determine the ingredients inside the Pheroid™ gel. This method was necessary in the assay determination for all four of the formulations during stability testing.

APPENDIX E: STABILITY TESTING

E.1 PURPOSE OF THE STABILITY TESTING

The purpose of stability testing for cosmetic formulations was to ensure that a new or modified formulation meets the intended physical, chemical and microbiological quality standards, as well as functionality and aesthetics when stored under appropriate conditions (Marx, 2004:1).

In this study the conditions under which the formulations were stored, were determined according to the climate changes in South Africa (MCC, 2006:1-24). All four of the formulations were stored at 25°C/60% RH, 30°C/60% RH and 40°C/75% RH (thus, 12 formulations in total) for 6 months.

All of the stability tests were conducted on a monthly basis, starting at month 0. The tests that were performed on the 12 formulations are given in **table E.1**:

Table E.1: Stability tests performed on the twelve formulations.

Test	Cream	Pheroid™ Cream	Gel	Pheroid™ Gel
Assay	√	√	√	√
pH	√	√	√	√
Viscosity	√	√	√	√
Visual assessment	√	√	√	√
Confocal	√	√	√	√
Mass variation	√	√	√	√

E.2 STABILITY METHODS

E.2.1 ASSAY DETERMINATION

Chromatographic methods were used to identify and quantify ingredients. The evaluation of a formulation component, over many time intervals, reveals its stability profile under the specified conditions. In this study, the assay was conducted using HPLC analysis (ANVISA, 2004:36). See **appendix D.2** for a detailed description of the chromatographic conditions.

E.2.2 PH DETERMINATION

Three different aspects related to pH values must be compatible: stability of the formulation, ingredients effectiveness, and safety of the product (ANVISA, 2004:13).

A Mettler Toledo pH meter with a glass Mettler Toledo LE 409 electrode was used to determine the pH of each formulation. The pH meter was calibrated each time before use, with three different buffer solutions (pH 4.01, 7.00 and 10.01, respectively).

E.2.3 VISCOSITY

The Brookfield Viscometer is a rotational viscometer. A number of spindles of various geometries, including cylinders, t-bars and cone-plate configurations are available to provide scientific rheologic data for liquids, pastes and other semi-solid materials (Martin, 1993:465).

This viscometer rotates a spindle in the specific formulation and then it measures the rotating force that is necessary to overcome the resistance to create movement. The spindle rotates at a specific speed that is measured in rpm (BELI, 2009:3).

A Brookfield Model DV II was used to conduct the experiments with. The viscosity of the cream and Pheroid™ cream was measured with the S-96 spindle at a speed of 0.3 rpm. The viscosity of the gel and Pheroid™ gel was also measured with the S-96 spindle, however at a speed of 1.5 rpm.

E.2.4 VISUAL ASSESSMENT

The visual appearance of a product is very important. If the quality of the product is poor it may lead to non-compliance with patients (Barry, 2002:531).

For the visual assessment, different shades of colour cards were used to document any change in colour of the products over a period of 6 months. Photos were taken of the 12 individual products every month, for 6 months in order to display whether there were any changes in colour.

E.2.5 CONFOCAL LASER SCANNING MICROSCOPY (CLSM)

Confocal laser scanning microscopy (CLSM) has become an invaluable tool for a wide range of investigations in the medical and biological science for imaging thin optical sections in living and fixed specimens ranging in thickness up to 100 micrometers (Claxton *et al.*, 2006:1).

reduce the background information away from the focal plane, and it has the capability to collect serial optical sections from thick specimens (Claxton *et al.*, 2006:1).

Figure E.1 depicts a schematic diagram of the optimal pathway and principle components in a laser scanning confocal microscope (Claxton *et al.*, 2006:1).

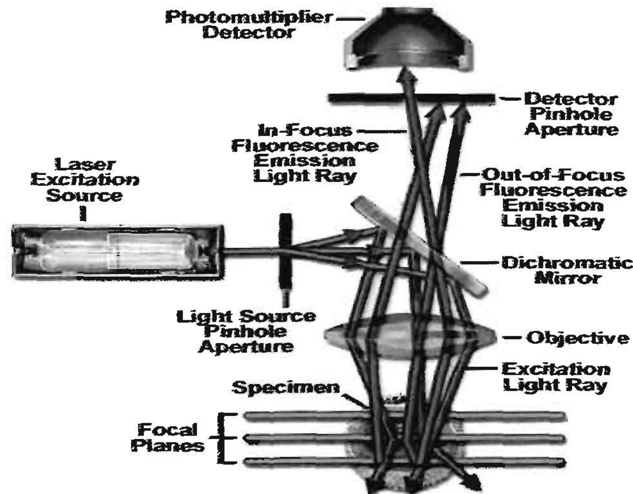


Figure E.1: Schematic diagram of the optical pathway and principal components in a laser scanning confocal microscope (Claxton *et al.*, 2006:3).

Coherent light are emitted by the laser system to pass through a pinhole opening that is situated in a confocal with a scanning point on the specimen and a second pinhole opening positioned in front of the detector. As the laser is reflected by a dichromatic mirror and scanned across a specimen in a defined focal plane, secondary fluorescence emitted from points on the specimen pass back through the dichromatic mirror and is focused as a confocal point at the detector pinhole aperture (Claxton *et al.*, 2006:3).

The significant amount of fluorescence emission that occurs at points above and below the objective focal plane is not in confocal with the pinhole, (termed Out-of Focus Light Rays in **figure E.1**) and forms extended airy disks in the opening plane (Claxton *et al.*, 2006:3).

Most of this extraneous light is not detected by the photomultiplier and does not contribute to the resulting image, because only a small fraction of the out-of-focus fluorescence emission is delivered through the pinhole opening. The dichromatic mirror, barrier filter and excitations filter perform similar functions to identical components in a wide field epi-fluorescence microscope (Claxton *et al.*, 2006:4).

specimen to a new plane that becomes confocal with the pinhole apertures of the light source and detector (Claxton *et al.*, 2006:4).

The phenoxazine dye nile red that was added to all of the samples, is used to localise and quantitate lipids, particularly neutral lipid droplets within the cells. It is selective for neutral lipids such as cholesteryl esters and is suitable for staining lysosomal phospholipid inclusions. Nile red is almost non-fluorescent in water and other polar solvents, but undergoes fluorescence enhancement and large absorption emission blue shifts in non-polar environments (Haugland, 2005:630-631).

A CLSM Nikon D-eclipse C1 si with violet diode laser 400-405 nm, a He/Ne laser at 543 nm and an Argon ion laser with 457-517 nm and CLMS Nikon PCM2000 with a digital camera DMX1200 with a He/Ne laser at 543 nm and an Argon ion laser with 457-514 nm were used to perform the experiments.

E.2.6 MASS VARIATION

Weight loss or gain measurements should always be conducted in order to confirm the adequacy of the container to preserve the product without loss by evaporation of volatile ingredients, or to protect it from atmospheric moisture, oxygen and carbon dioxide (Cannell, 1985:299).

It is also important to perform mass variation to determine whether there is a possible interaction between the product and the container. The interactions can be: the container absorbs some of the ingredients in the formulations, leaching of ingredients of the container by the product, and corrosion (Cannell, 1985:292-293).

A Shimadzu AUW 120 calibrated balance was used to weigh each container, with the cap and formulation inside, every month in order to determine whether there was any weight loss or gain.

E.3 RESULTS AND DISCUSSIONS

E.3.1. ASSAY DETERMINATION

Tables E.2 to **E.5** depict the results of the assay determination for each of the formulations at different storage conditions, from month 0 to 6. The results indicated that there were significant changes in the percentage (%) of label claim for each ingredient in the specific formulation. The concentrations of these ingredients after 6 months were not within the acceptable limits (90 – 110%).

the **Pheroid™ cream**.

25°C/60% RH								
Formulation ingredient	Month					Mean*	SD**	%RSD***
	0	1	2	3	6			
Niacinamide	104.26	95.10	91.24	85.41	68.28	88.86	11.98	13.48
Methyl paraben	122.17	100.80	95.57	72.40	61.58	90.50	21.44	23.69
Salicylic acid	106.14	103.73	101.30	88.62	53.29	90.62	19.62	21.65
BHA	185.82	150.80	130.90	25.75	0.00	98.65	72.67	73.66
BHT	118.63	86.60	78.24	37.39	34.00	70.97	31.82	44.84
Vit E	103.20	47.15	25.95	21.65	8.80	41.35	33.30	80.53
30°C/60% RH								
Formulation ingredients	Month					Mean*	SD**	%RSD***
	0	1	2	3	6			
Niacinamide	123.41	95.10	94.88	88.14	50.76	90.46	23.27	25.73
Methyl paraben	144.63	100.80	91.77	78.94	37.56	90.74	34.57	38.09
Salicylic acid	133.63	101.30	99.52	87.70	32.91	91.01	32.81	36.05
BHA	300.46	183.66	130.90	16.06	8.92	128.00	109.14	85.27
BHT	88.66	86.60	66.93	27.54	17.38	57.42	29.71	51.74
Vit E	103.20	52.61	16.24	10.31	8.54	38.18	36.26	94.96
40°C/75% RH								
Formulation ingredients	Month					Mean*	SD**	%RSD***
	0	1	2	3	6			
Niacinamide	111.28	95.10	94.07	44.01	42.27	77.35	28.59	36.97
Methyl paraben	137.71	100.80	80.17	45.54	37.01	80.25	36.87	45.95
Salicylic acid	113.78	101.30	96.47	40.56	28.13	76.05	34.74	45.68
BHA	181.10	159.88	130.90	12.71	1.55	97.23	75.35	77.50
BHT	86.60	44.70	13.17	5.03	4.84	30.87	31.48	101.99
Vit E	103.20	12.50	2.30	1.73	3.29	24.60	39.49	160.52

*Mean refers to average.

**SD refers to standard deviation.

***%RSD refers to relative standard deviation.

the **Pheroid™ gel**.

25°C/60% RH								
Formulation ingredient	Month					Mean*	SD**	%RSD***
	0	1	2	3	6			
Niacinamide	116.29	90.30	80.52	70.10	65.80	84.60	17.98	21.26
Methyl paraben	162.10	93.40	72.54	69.43	60.50	91.59	36.87	40.25
Salicylic acid	110.84	84.00	78.87	67.50	47.53	77.75	20.75	26.69
BHA	122.82	112.83	103.60	21.53	0.00	72.16	50.95	70.61
BHT	88.20	81.93	67.44	29.96	19.45	57.40	27.73	48.31
Vit E	85.20	31.32	14.50	10.00	6.99	29.60	29.04	98.11
30°C/60% RH								
Formulation ingredients	Month					Mean*	SD**	%RSD***
	0	1	2	3	6			
Niacinamide	99.37	94.99	90.30	74.34	66.77	85.15	12.49	14.67
Methyl paraben	110.61	93.40	79.13	77.69	54.44	83.05	18.60	22.39
Salicylic acid	95.17	93.38	84.00	70.93	42.93	77.28	19.21	24.85
BHA	160.92	127.69	103.60	18.86	13.05	84.82	59.13	69.71
BHT	88.20	88.03	44.85	20.32	18.02	51.88	31.04	59.83
Vit E	85.20	58.18	7.60	2.79	0.00	30.75	34.58	112.46
40°C/75% RH								
Formulation ingredients	Month					Mean*	SD**	%RSD***
	0	1	2	3	6			
Niacinamide	99.85	90.30	83.17	65.10	58.16	79.32	15.54	19.59
Methyl paraben	101.29	93.40	79.02	62.40	54.99	78.22	17.62	22.53
Salicylic acid	92.62	84.00	83.29	55.36	41.34	71.32	19.55	27.41
BHA	156.12	103.60	80.20	15.39	2.70	71.60	56.83	79.37
BHT	88.20	50.59	20.66	7.30	0.00	33.35	32.43	97.23
Vit E	85.20	20.70	3.29	2.50	2.17	22.77	31.99	140.48

*Mean refers to average.

**SD refers to standard deviation.

***%RSD refers to relative standard deviation.

the **Cream**.

25°C/60% RH								
Formulation	Month					Mean*	SD**	%RSD***
ingredient	0	1	2	3	6			
Niacinamide	137.18	95.30	94.60	94.48	91.50	102.61	17.33	16.89
Methyl paraben	145.81	101.56	98.50	92.28	81.02	103.83	22.13	21.32
Salicylic acid	139.54	102.72	97.61	91.80	75.40	101.41	21.16	20.87
30°C/60% RH								
Formulation	Month					Mean*	SD**	%RSD***
ingredients	0	1	2	3	6			
Niacinamide	135.69	98.58	93.90	91.50	86.22	101.18	17.71	17.50
Methyl paraben	140.92	98.50	98.18	91.12	79.14	101.57	20.89	20.57
Salicylic acid	133.10	97.50	91.80	87.60	79.64	97.93	18.53	18.92
40°C/75% RH								
Formulation	Month					Mean*	SD**	%RSD***
ingredients	0	1	2	3	6			
Niacinamide	135.55	95.10	95.05	92.45	91.50	101.93	16.87	16.55
Methyl paraben	147.84	98.50	94.50	81.26	79.64	100.35	24.84	24.76
Salicylic acid	127.58	98.46	95.10	91.80	73.95	97.38	17.31	17.78

*Mean refers to average.

**SD refers to standard deviation.

***%RSD refers to relative standard deviation.

the **Gel**.

25°C/60% RH								
Formulation	Month					Mean*	SD**	%RSD***
ingredient	0	1	2	3	6			
Niacinamide	93.20	92.69	80.59	76.71	56.70	79.98	13.34	16.68
Methyl paraben	107.16	93.10	78.10	66.76	23.27	73.68	28.66	38.90
Salicylic acid	101.82	91.80	84.76	82.44	38.25	79.81	21.85	27.37
30°C/60% RH								
Formulation	Month					Mean*	SD**	%RSD***
ingredients	0	1	2	3	6			
Niacinamide	95.23	93.20	90.02	62.06	61.86	80.47	15.21	18.90
Methyl paraben	106.45	93.10	75.46	68.01	50.58	78.72	19.46	24.72
Salicylic acid	98.17	91.80	78.01	66.00	41.91	75.18	20.02	26.63
40°C/75% RH								
Formulation	Month					Mean*	SD**	%RSD***
ingredients	0	1	2	3	6			
Niacinamide	96.48	93.20	91.87	72.00	60.42	82.79	14.11	17.04
Methyl paraben	105.38	93.10	76.11	75.54	41.91	78.41	21.40	27.29
Salicylic acid	99.28	91.80	76.48	65.36	39.35	74.45	21.15	28.40

*Mean refers to average.

**SD refers to standard deviation.

***%RSD refers to relative standard deviation.

E.3.2 PH DETERMINATION

Table E.6 presents the pH results of the different formulations at the different storage conditions. The only significant change in pH values was for the cream, with a significant decrease in the pH from month 0 (4.71) to month 6 (3.54) at 30°C/60% RH (%RSD = 9.02), as well as a significant decrease in pH from month 0 (4.71) to month 6 (4.11) at 40°C/75% RH (%RSD = 6.05).

25°C/60% RH

Formulation	Month					Mean*	SD**	%RSD***
	0	1	2	3	6			
Pheroid™ cream	4.60	4.28	4.25	4.25	4.25	4.32	0.14	3.17
Pheroid™ gel	3.98	3.80	4.22	4.02	4.05	4.01	0.13	3.34
Gel	4.14	4.17	4.23	3.96	4.01	4.10	0.10	2.53
Cream	4.71	4.40	4.12	4.29	4.18	4.34	0.21	4.81

30°C/60% RH

Formulation	Month					Mean*	SD**	%RSD***
	0	1	2	3	6			
Pheroid™ cream	4.60	4.37	4.06	4.28	4.21	4.30	0.18	4.18
Pheroid™ gel	3.98	4.22	4.28	4.04	3.93	4.09	0.14	3.35
Gel	4.14	3.91	4.02	4.07	3.95	4.02	0.08	2.06
Cream	4.71	4.18	4.30	4.19	3.54	4.18	0.38	9.02

40°C/75% RH

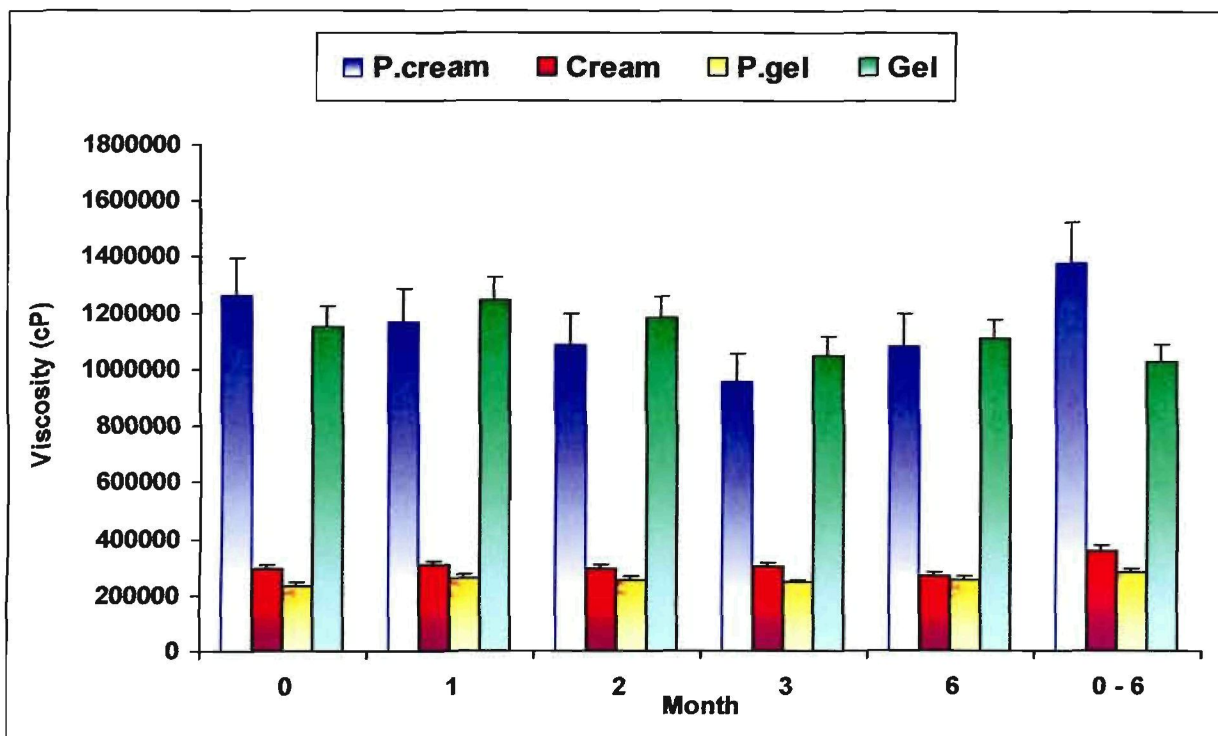
Formulation	Month					Mean*	SD**	%RSD***
	0	1	2	3	6			
Pheroid™ cream	4.60	4.15	4.37	4.24	4.15	4.30	0.17	3.92
Pheroid™ gel	3.98	4.01	4.31	3.85	3.86	4.00	0.17	4.15
Gel	4.14	3.84	3.96	3.97	3.89	3.96	0.10	2.55
Cream	4.71	4.26	4.05	4.02	4.11	4.23	0.26	6.05

*Mean refers to average.

**SD refers to standard deviation.

***%RSD refers to relative standard deviation.

As seen in **figure E.2**, there was no significant change in the viscosity of the formulations. The formulations were within the experimental error of 20%.



P.cream refers to Pheroid™ cream.

P.gel refers to Pheroid™ gel.

Figure E.2: Viscosity of the 4 different formulations from month 0 to month 6 at 25°C/60% RH compared to month 0 to 6 at 25°C/60% RH.

E.3.4 VISUAL ASSESSMENT

E.3.4.1 VISUAL ASSESSMENT WITH IMAGES

As seen in **figures E.3** to **E.6**, the gel and cream depicted no changes regarding the colour. Both remained white, homogenous and presented no smell. As for the Pheroid™ cream and Pheroid™ gel there were a significant change in the colour and both formulations presented a fish like smell.

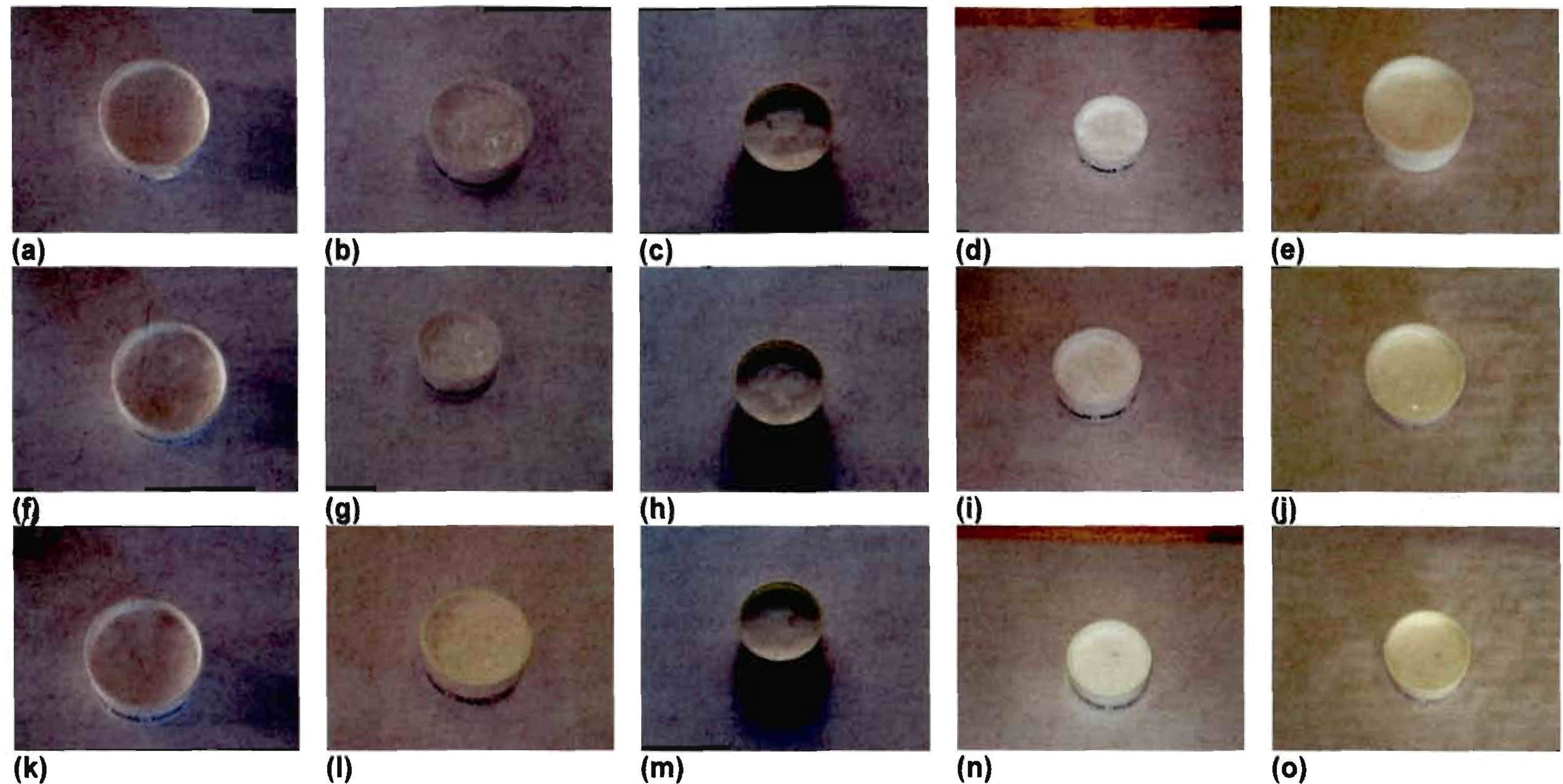


Figure E.3: Visual evaluation by means of photographs of the **gel** taken at each storage condition over 6 months **(a)** month 0 - 25°C/60% RH, **(b)** month 1 - 25°C/60% RH, **(c)** month 2 - 25°C/60% RH, **(d)** month 3 - 25°C/60% RH, **(e)** month 6 - 25°C/60% RH, **(f)** month 0 - 30°C/60% RH, **(g)** month 1 - 30°C/60% RH, **(h)** month 2 - 30°C/60% RH, **(i)** month 3 - 30°C/60% RH, **(j)** month 6 - 30°C/60% RH, **(k)** month 0 - 40°C/75% RH, **(l)** mor 1 - 40°C/75% RH, **(m)** month 2 - 40°C/75% RH, **(n)** month 3 - 40°C/75% RH, **(o)** month 6 - 40°C/75% RH.

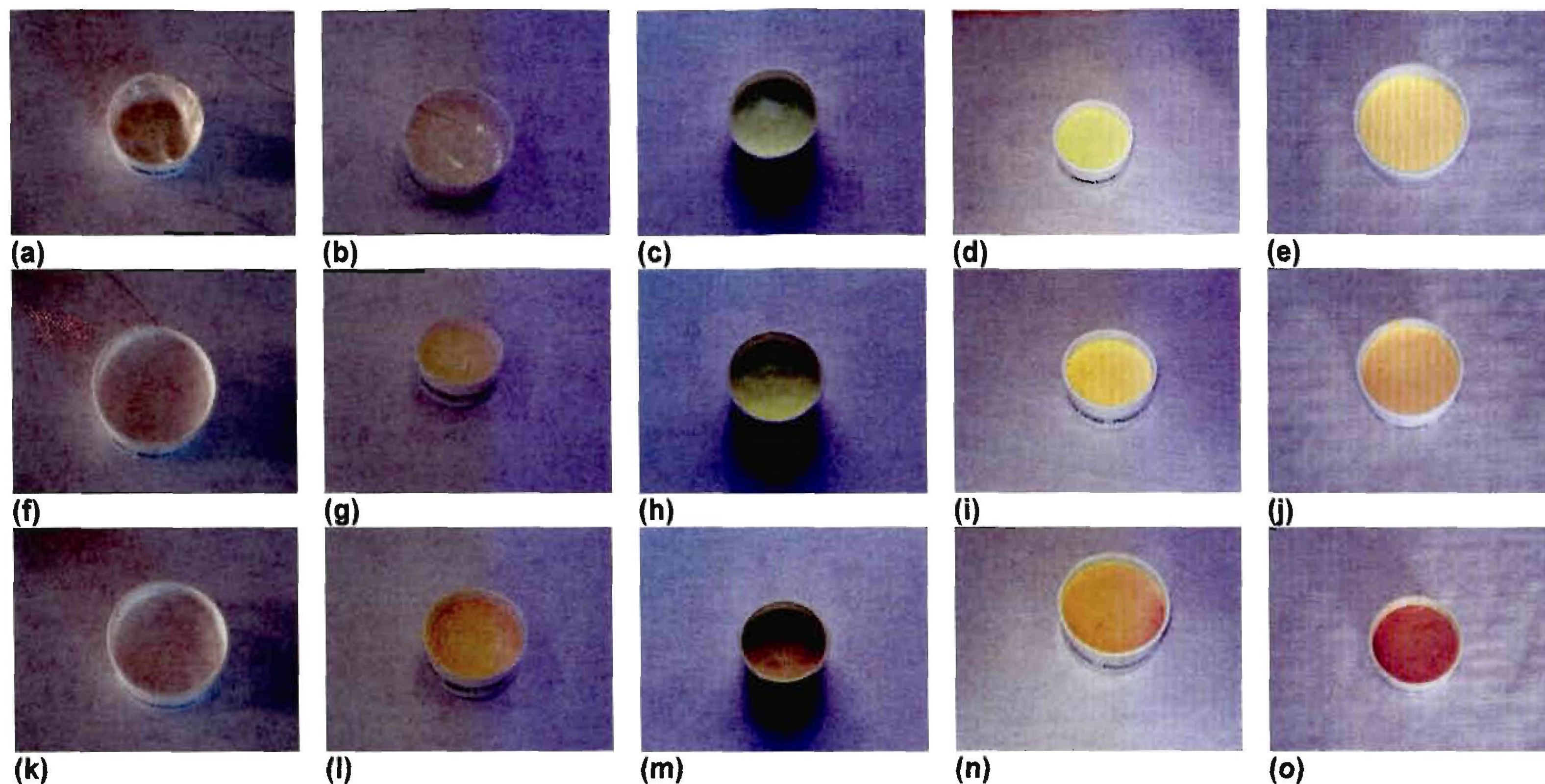


Figure E.4: Visual evaluation by means of photographs of the **Pheroid™ gel** taken at each storage condition over 6 months **(a)** month 0 - 25˚C/60 RH, **(b)** month 1 - 25˚C/60% RH, **(c)** month 2 - 25˚C/60% RH, **(d)** month 3 - 25˚C/60% RH, **(e)** month 6 - 25˚C/60% RH, **(f)** month 0 - 30˚C/60% RH **(g)** month 1 - 30˚C/60% RH, **(h)** month 2 - 30˚C/60% RH, **(i)** month 3 - 30˚C/60% RH, **(j)** month 6 - 30˚C/60% RH, **(k)** month 0 - 40˚C/75% RH, **(l)** month 1 - 40˚C/75% RH, **(m)** month 2 - 40˚C/75% RH, **(n)** month 3 - 40˚C/75% RH, **(o)** month 6 - 40˚C/75% RH.

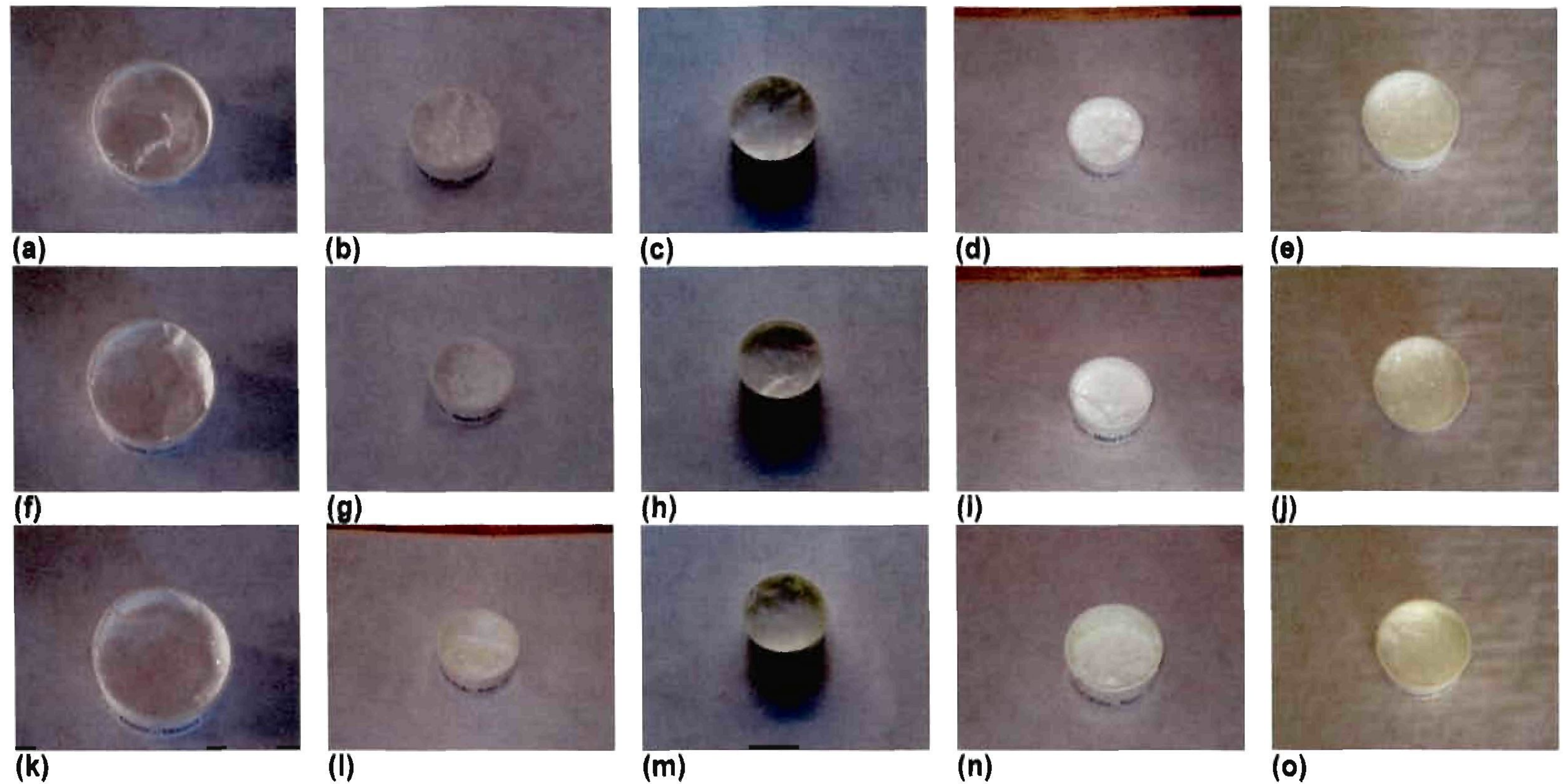


Figure E.5: Visual evaluation by means of photographs of the **cream** taken at each storage condition over 6 months **(a)** month 0 - 25˚C/60% RH, **(** month 1 - 25˚C/60% RH, **(c)** month 2 - 25˚C/60% RH, **(d)** month 3 - 25˚C/60% RH, **(e)** month 6 - 25˚C/60% RH, **(f)** month 0 - 30˚C/60% RH, **(g)** month 1 - 30˚C/60% RH, **(h)** month 2 - 30˚C/60% RH, **(i)** month 3 - 30˚C/60% RH, **(j)** month 6 - 30˚C/60% RH, **(k)** month 0 - 40˚C/75% RH, **(l)** mont 1 - 40˚C/75% RH, **(m)** month 2 - 40˚C/75% RH, **(n)** month 3 - 40˚C/75% RH, **(o)** month 6 - 40˚C/75% RH.

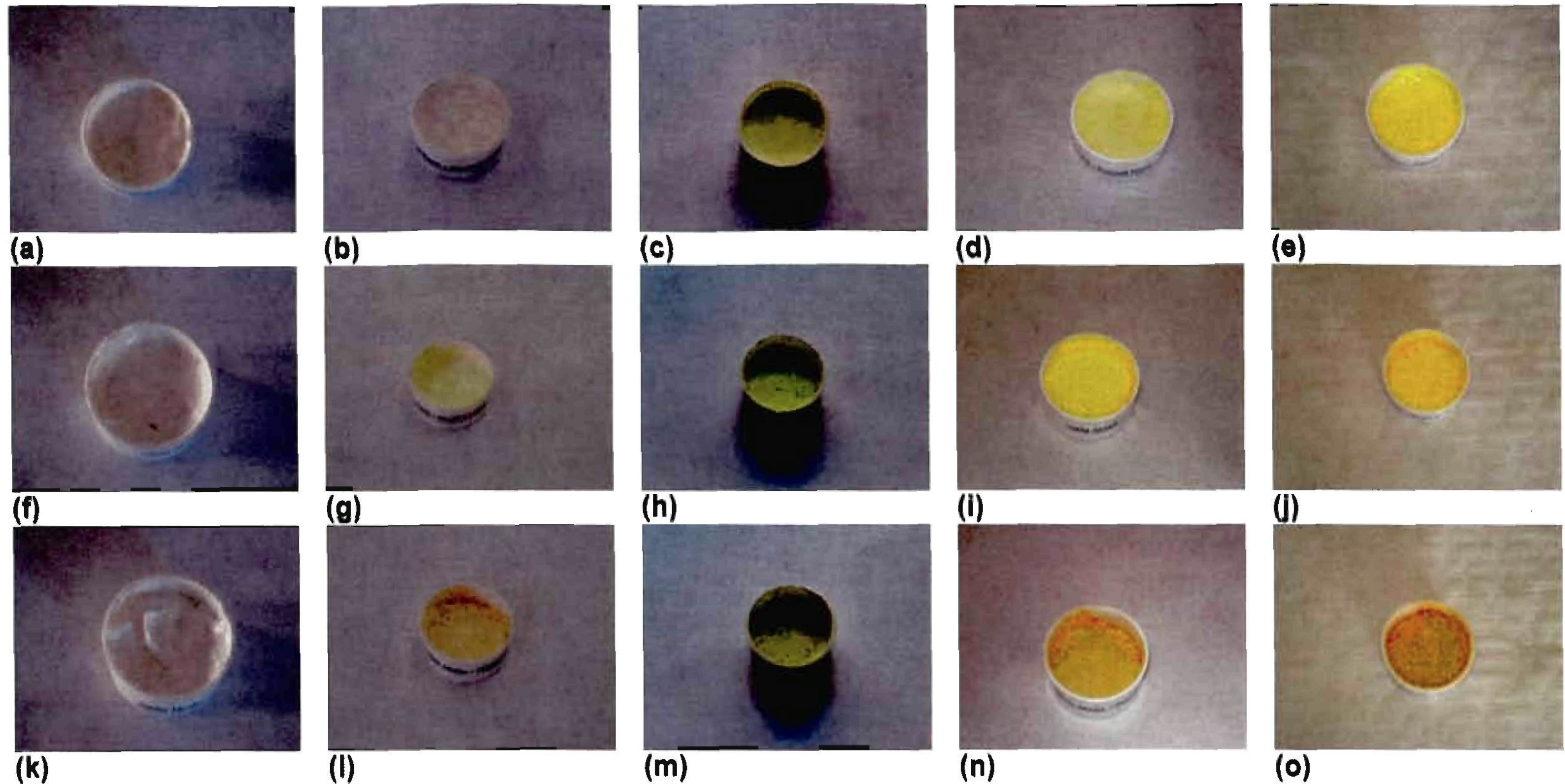


Figure E.6: Visual evaluation by means of photographs of the **Pheroid™ cream** taken at each storage condition over 6 months **(a)** month 0 - 25˚C/60% RH, **(b)** month 1 - 25˚C/60% RH, **(c)** month 2 - 25˚C/60% RH, **(d)** month 3 - 25˚C/60% RH, **(e)** month 6 - 25˚C/60% RH, **(f)** month 0 - 30˚C/60% RH, **(g)** month 1 - 30˚C/60% RH, **(h)** month 2 - 30˚C/60% RH, **(i)** month 3 - 30˚C/60% RH, **(j)** month 6 - 30˚C/60% RH, **(k)** month 0 - 40˚C/75% RH, **(l)** month 1 - 40˚C/75% RH, **(m)** month 2 - 40˚C/75% RH, **(n)** month 3 - 40˚C/75% RH, **(o)** month 6 - 40˚C/75% RH.

E.3.4.2 VISUAL ASSESSMENT WITH COLOUR CHARTS

Each formulation was visually assessed over 6 months and compared to colour charts obtained from Dulux. The colours for each formulation during the 6 month period are presented and in **tables E.7** to **E.9**, the colour charts are presented in **figure E.7**.

Table E.7: Colour chart assessment at 25°C/60% RH.

25°C/60% RH					
Formulations	**Month 0**	**Month 1**	**Month 2**	**Month 3**	**Month 6**
Gel	Chiffon white	Chiffon white	Chiffon white	Chiffon white	Chiffon white
Pheroid™ gel	Chiffon white	Baked alaska	Spring breeze 3	Spring breeze 2	Buttercup fool 1
Cream	Chiffon white	Chiffon white	Chiffon white	Chiffon white	Chiffon white
Pheroid™ cream	Chiffon white	Pale honey	Spring breeze 4	Spring breeze 3	Buttercup fool 2

Table E.8: Colour chart assessment at 30°C/60% RH.

30°C/60% RH					
Formulations	**Month 0**	**Month 1**	**Month 2**	**Month 3**	**Month 6**
Gel	Chiffon white	Chiffon white	Chiffon white	Chiffon white	Chiffon white
Pheroid™ gel	Chiffon white	Banana dream 4	Spring breeze 1	Banana dream 2	Sun dust 1
Cream	Chiffon white	Chiffon white	Chiffon white	Chiffon white	Chiffon white
Pheroid™ cream	Chiffon white	Yellow Mallow	Spring breeze 2	Easter morn 2	Buttercup fool 1

Table E.9: Colour chart assessment at 40°C/75% RH.

40°C/75% RH					
Formulations	**Month 0**	**Month 1**	**Month 2**	**Month 3**	**Month 6**
Gel	Chiffon white	Chiffon white	Chiffon white	Chiffon white	Chiffon white
Pheroid™ gel	Chiffon white	Easter morn 1	Sun dust 1	Sun dust 1	Mustard flower
Cream	Chiffon white	Chiffon white	Chiffon white	Chiffon white	Chiffon white
Pheroid™ cream	Chiffon white	Dessert island 4	Dessert island 3	Sun dust 2	Oriole

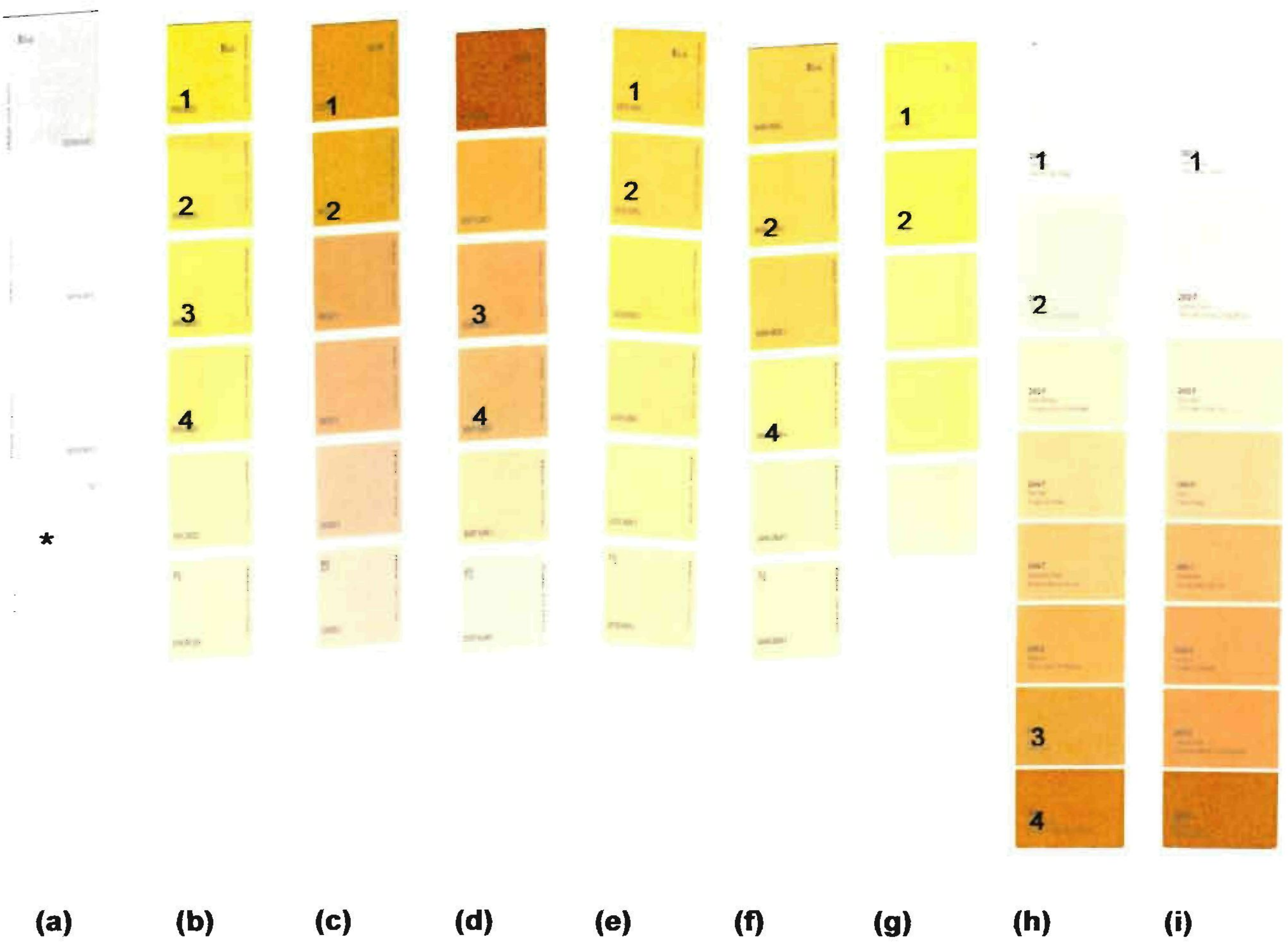


Figure E.7: **(a)** Chiffon white*, **(b)** Spring breeze 1 to 4, **(c)** Sun dust 1 and 2, **(d)** Dessert island 3 and 4, **(e)** Eastern morn 1 and 2, **(f)** Banana dream 2 and 4, **(g)** Buttercup fool 1 and 2, **(h)** 1 – Pale honey, 2 – Yellow mellow, 3- Oriole and 4 – Mustard flower and **(i)** 1 – Baked alaska.

E.3.5 CONFOCAL LASER SCANNING MICROSCOPY (CLSM)

As depicted in **figure E.8** to **E.11**, all of the images showed that the particle size of each formulation was almost the same size. It furthermore indicated that all of the formulations were homogenous, without any crystals. The CLSM micrograph (**figure E.9.n**) displayed perfectly sphere oil drops formed in the Pheroid™ gel under storage conditions: 40°C/75% RH. In **figure E.11 (g)** the waxes inside the Pheroid™ cream are well displayed.

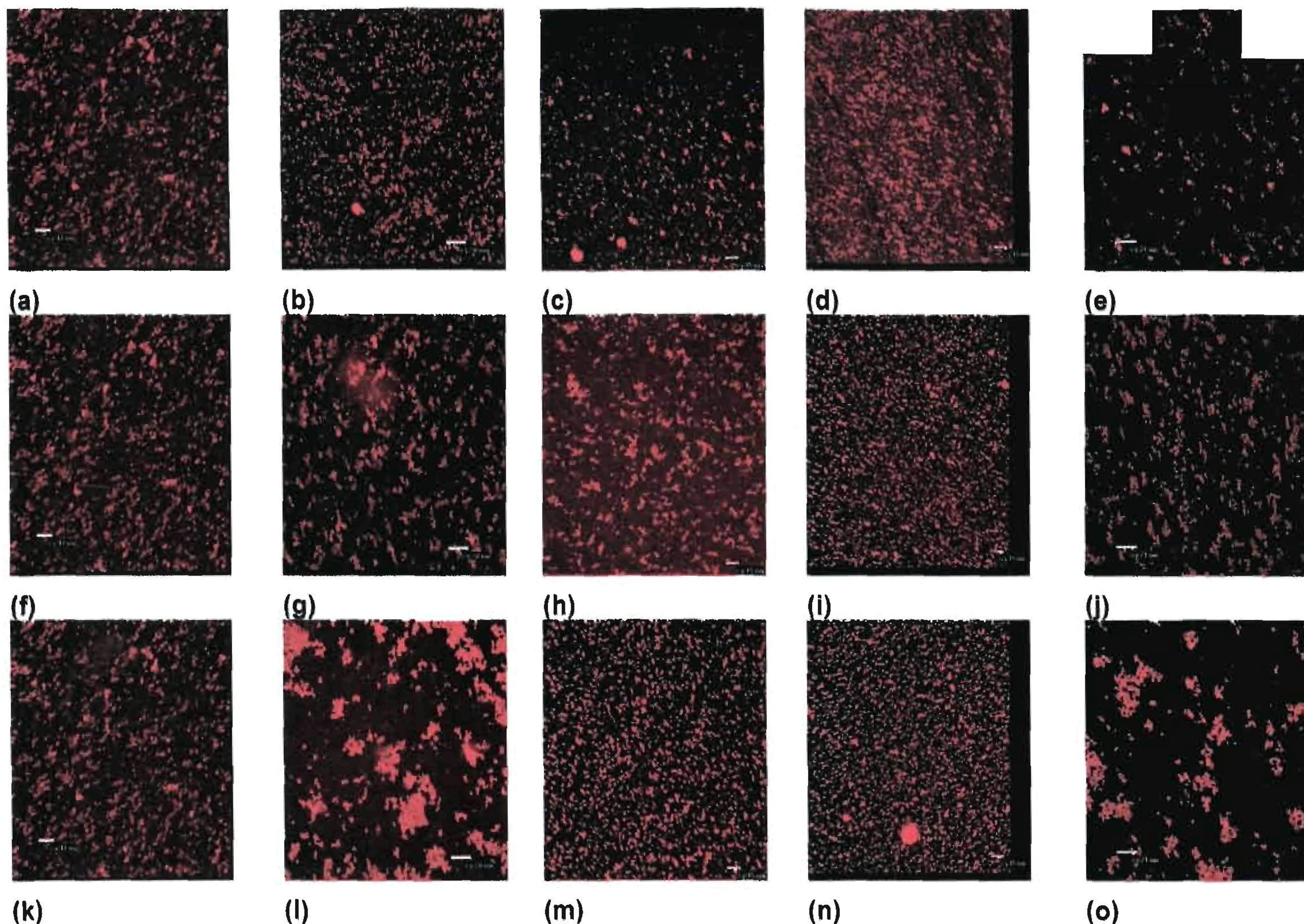


Figure E.8: CLSM micrographs of the **gel** taken at each storage condition over 6 months **(a)** month 0 - 25˚C/60% RH, **(b)** month 1 - 25˚C/60% RH, **(c)** month 2 - 25˚C/60% RH, **(d)** month 3 - 25˚C/60% RH, **(e)** month 6 - 25˚C/60% RH, **(f)** month 0 - 30˚C/60% RH, **(g)** month 1 - 30˚C/60% RH, **(h)** month 2 - 30˚C/60% RH, **(i)** month 3 - 30˚C/60% RH, **(j)** month 6 - 30˚C/60% RH, **(k)** month 0 - 40˚C/75% RH, **(l)** month 1 - 40˚C/75% RH, **(m)** month 2 - 40˚C/75% RH, **(n)** month 3 - 40˚C/75% RH, **(o)** month 6 - 40˚C/75% RH.

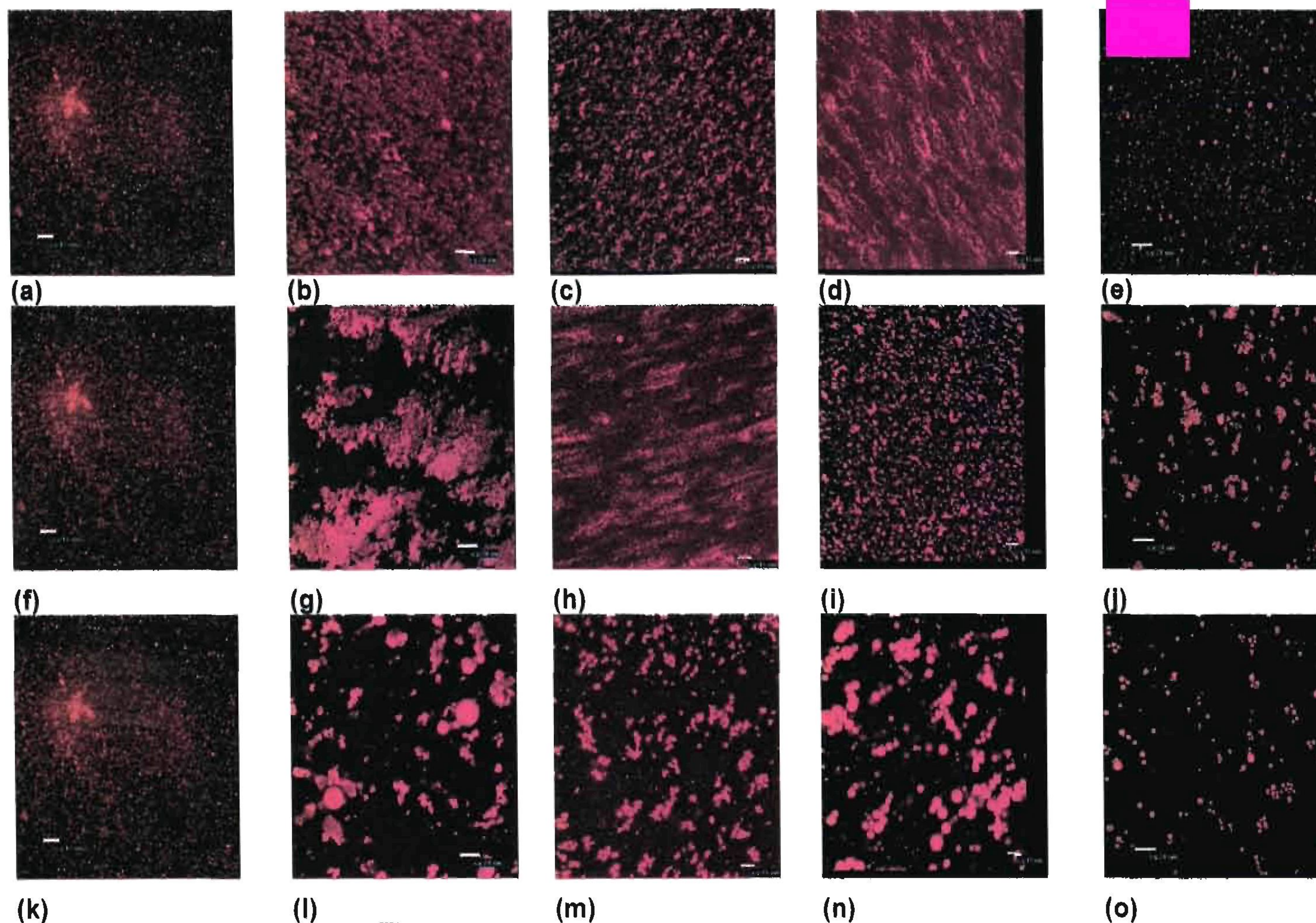


Figure E.9: CLSM micrographs of the **Pheroid™ gel** taken at each storage condition over 6 months **(a)** month 0 - 25˚C/60% RH, **(b)** month 1 - 25˚C/60% RH, **(c)** month 2 - 25˚C/60% RH, **(d)** month 3 - 25˚C/60% RH, **(e)** month 6 - 25˚C/60% RH, **(f)** month 0 - 30˚C/60% RH, **(g)** month 1 - 30˚C/60% RH, **(h)** month 2 - 30˚C/60% RH, **(i)** month 3 - 30˚C/60% RH, **(j)** month 6 - 30˚C/60% RH, **(k)** month 0 - 40˚C/75% RH, **(l)** month 1 - 40˚C/75% RH, **(m)** month 2 - 40˚C/75% RH, **(n)** month 3 - 40˚C/75% RH, **(o)** month 6 - 40˚C/75% RH.

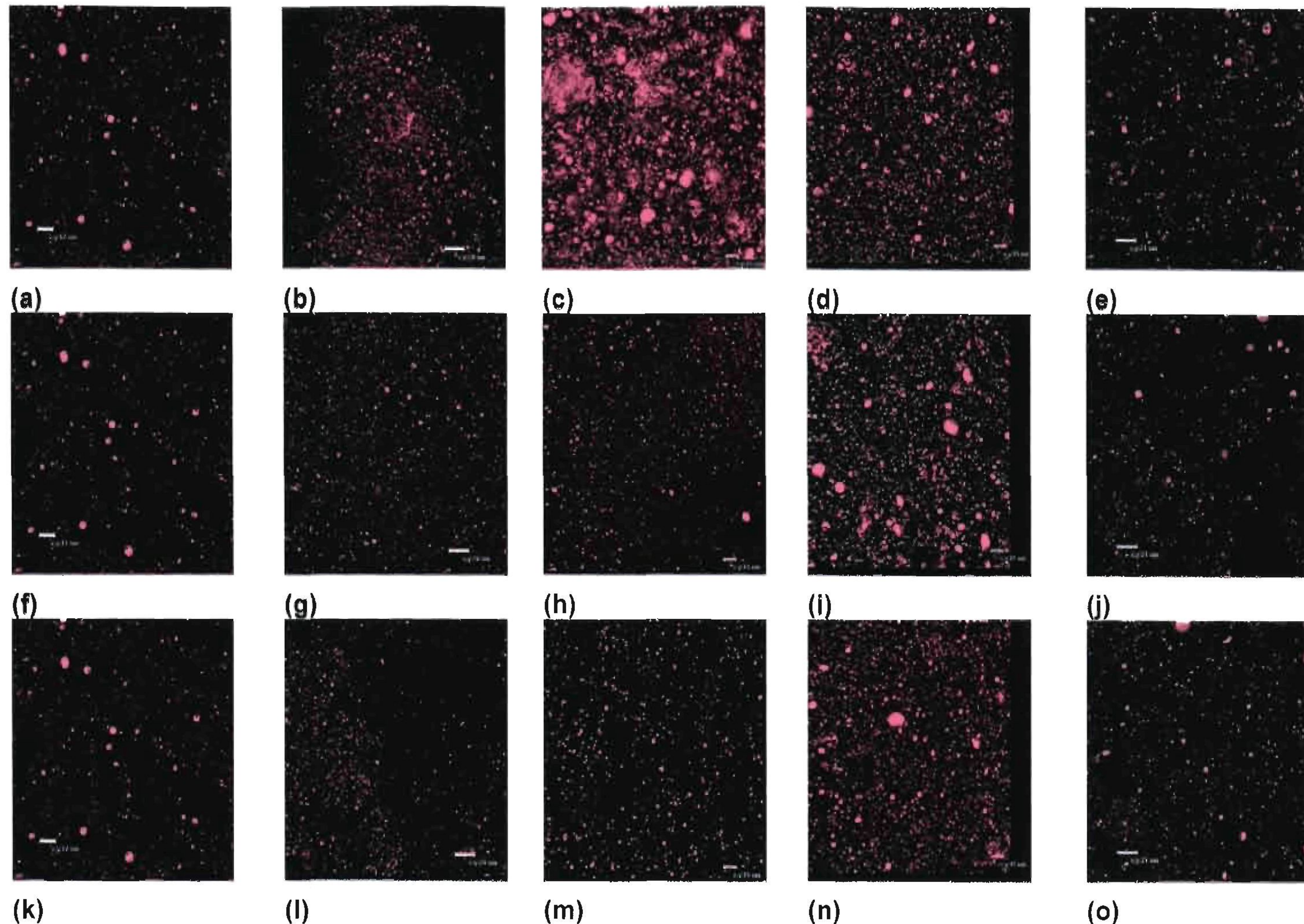


Figure E.10: CLSM micrographs of the **cream** taken at each storage condition over 6 months **(a)** month 0 - 25˚C/60% RH, **(b)** month 1 - 25˚C/60% RH, **(c)** month 2 - 25˚C/60% RH, **(d)** month 3 - 25˚C/60% RH, **(e)** month 6 - 25˚C/60% RH, **(f)** month 0 - 30˚C/60% RH, **(g)** month 1 - 30˚C/60%RH, **(h)** month 2 - 30˚C/60% RH, **(i)** month 3 - 30˚C/60% RH, **(j)** month 6 - 30˚C/60% RH, **(k)** month 0 - 40˚C/75% RH, **(l)** month 1 - 40˚C/75% RH, **(m)** month 2 - 40˚C/75% RH, **(n)** month 3 - 40˚C/75% RH, **(o)** month 6 - 40˚C/75% RH.

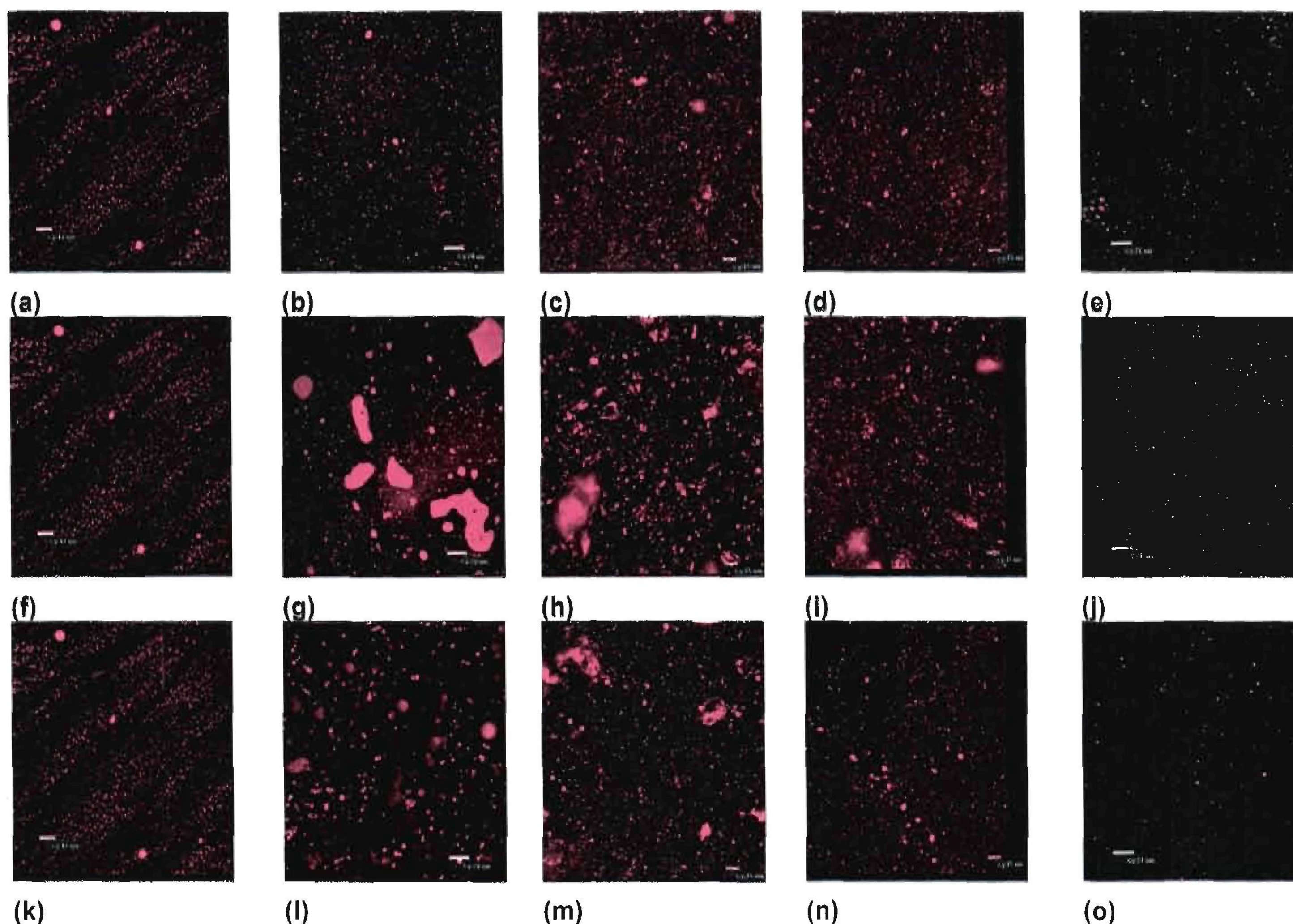


Figure E.11: CLSM micrographs of the **Pheroid™ cream** taken at each storage condition over 6 months **(a)** month 0 - 25˚C ; 60%RH, **(b)** month 1 25˚C/60% RH, **(c)** month 2 - 25˚C/60% RH, **(d)** month 3 - 25˚C/60% RH, **(e)** month 6 - 25˚C/60% RH, **(f)** month 0 - 30˚C/60% RH, **(g)** month 1 - 30˚C/60% RH, **(h)** month 2 - 30˚C/60% RH, **(i)** month 3 - 30˚C/60% RH, **(j)** month 6 - 30˚C/60% RH, **(k)** month 0 - 40˚C/75% RH, **(l)** month 1 - 40˚C/75% RH, **(m)** month 2 - 40˚C/75% RH, **(n)** month 3 - 40˚C/75% RH, **(o)** month 6 - 40˚C/75% RH.

E.3.6 MASS VARIATION

Table E.10 depicts the results of mass variation for each formulation at the different storage conditions. The Pheroid™ gel at a storage condition of 25°C/60% RH, was the only formulation with a significant weight loss change.

Table E.10: Mass variation from month 0 – 6 at the different storage conditions.

Formulation	Month					Mean*	SD**	%RSD***
	0	1	2	3	6			
25°C/60% RH								
Pheroid™ cream	20.20	20.72	20.69	20.67	20.58	20.57	0.19	0.92
Pheroid™ gel	25.73	25.01	24.97	24.92	17.91	23.71	2.92	12.30
Gel	27.34	27.02	26.89	26.88	26.63	26.95	0.23	0.86
Cream	20.71	20.49	20.36	20.35	20.21	20.42	0.17	0.83
30°C/60% RH								
Pheroid™ cream	20.13	20.12	20.07	20.02	19.98	20.07	0.06	0.29
Pheroid™ gel	25.80	25.68	25.58	25.53	25.32	25.58	0.16	0.63
Gel	27.80	27.72	27.46	27.17	27.06	27.44	0.29	1.07
Cream	20.28	20.24	20.21	20.10	20.07	20.18	0.08	0.39
40°C/75% RH								
Pheroid™ cream	20.12	20.09	20.05	20.04	19.58	19.97	0.20	0.99
Pheroid™ gel	24.24	25.62	25.21	25.12	25.06	25.05	0.45	1.80
Gel	26.21	26.89	26.93	26.84	26.70	26.71	0.26	0.98
Cream	20.17	20.50	20.28	19.92	19.85	20.14	0.24	1.17

*Mean refers to average.

**SD refers to standard deviation.

***%RSD refers to relative standard deviation.

E.4 CONCLUSION

None of the formulations were stable over the 6 month period regarding the assay determination. There was no significant change regarding the viscosity determination of the cream, gel, Pheroid™ gel and Pheroid™ cream. Only the cream at a storage condition at 30°C/60% RH, had a significant decrease in the pH values over the 6 month period (RSD = 9.02).

From these results, it was clear that overall, the gel and cream were significantly more stable than the Pheroid™ cream or Pheroid™ gel during the 6 months stability testing. Considering colour and odour changes, the gel and cream formulations were significantly more stable over the 6 month period compared to the Pheroid™ cream and Pheroid™ gel formulations. From the CLSM studies, no change in the particle size or shape could be established for either the cream or gel formulation, over the 6 month period. However, the formulation of oil drops and an oil layer could be seen for the Pheroid™ cream and Pheroid™ gel, over the same time period.

Only the Pheroid™ gel at 25°C/60% RH, depicted a significant weight loss over the 6 month period. The gel, cream and Pheroid™ cream presented no significant change over the same time period.

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APPENDIX F: DIFFUSION STUDY

F.1 NIACINAMIDE AND SALICYLIC ACID

The aim of the study was to compare the Pheroid™ cream and Pheroid™ gel with the cream and the gel formulations, in order to determine whether the Pheroid™ delivery system enhanced the flux of niacinamide and salicylic acid.

F.1.1 NIACINAMIDE PERMEATION PROFILE

In **figures F.1** to **F.4** the average cumulative amount per area was plotted against time, for the Pheroid™ cream, Pheroid™ gel, cream and gel.

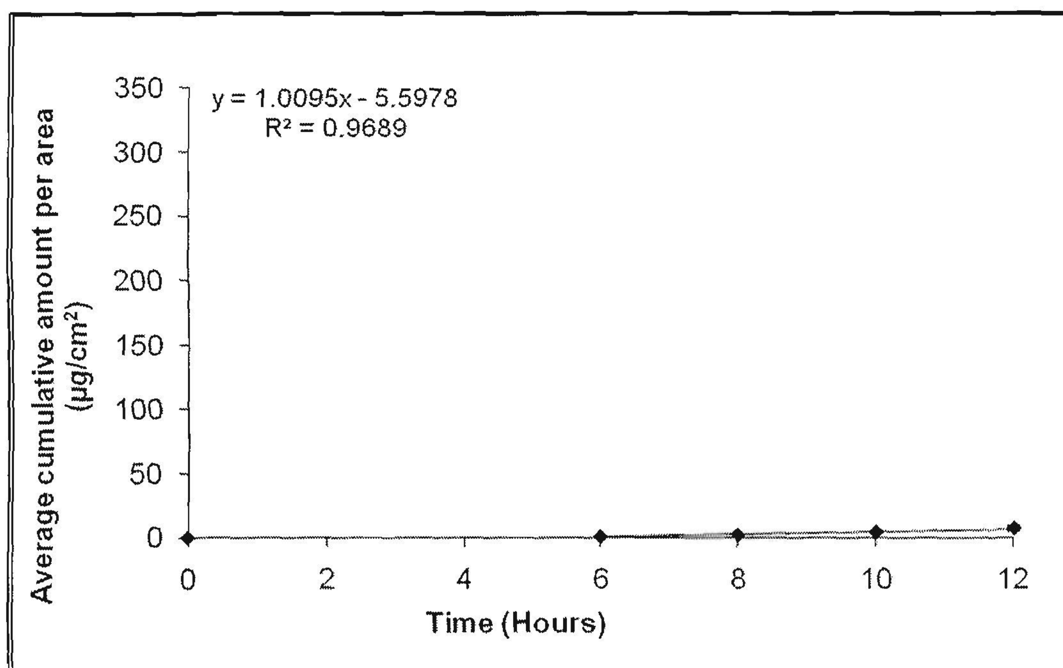

Figure F.1: Cumulative amount per area of niacinamide in the **Pheroid™ cream** plotted against time.

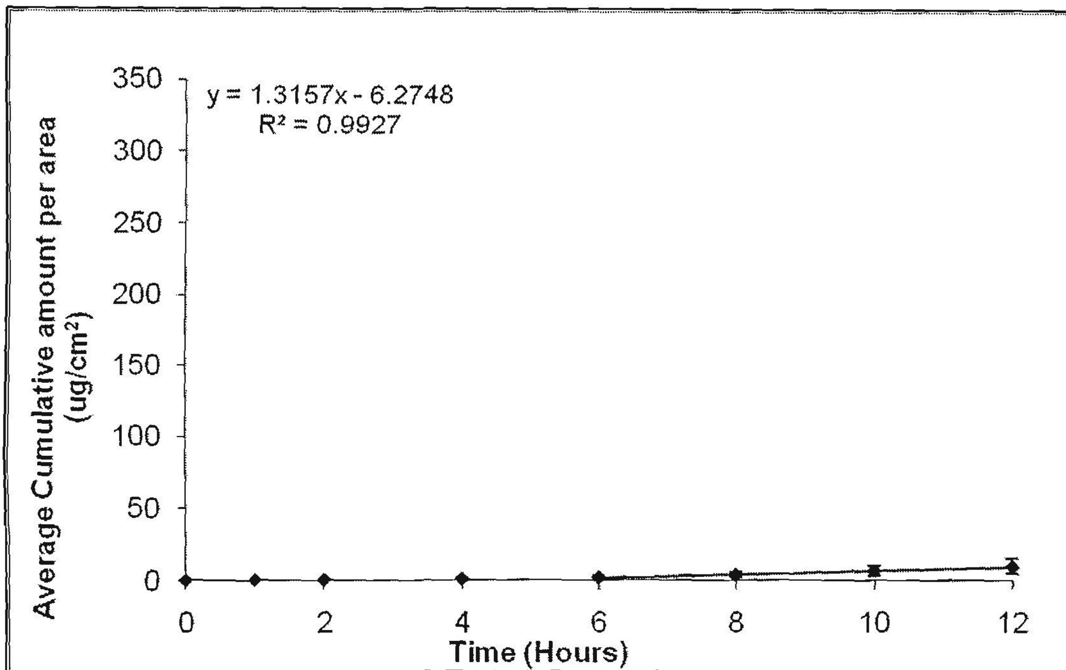

Figure F.2: Cumulative amount per area of niacinamide in the **Pheroid™gel** plotted against time.

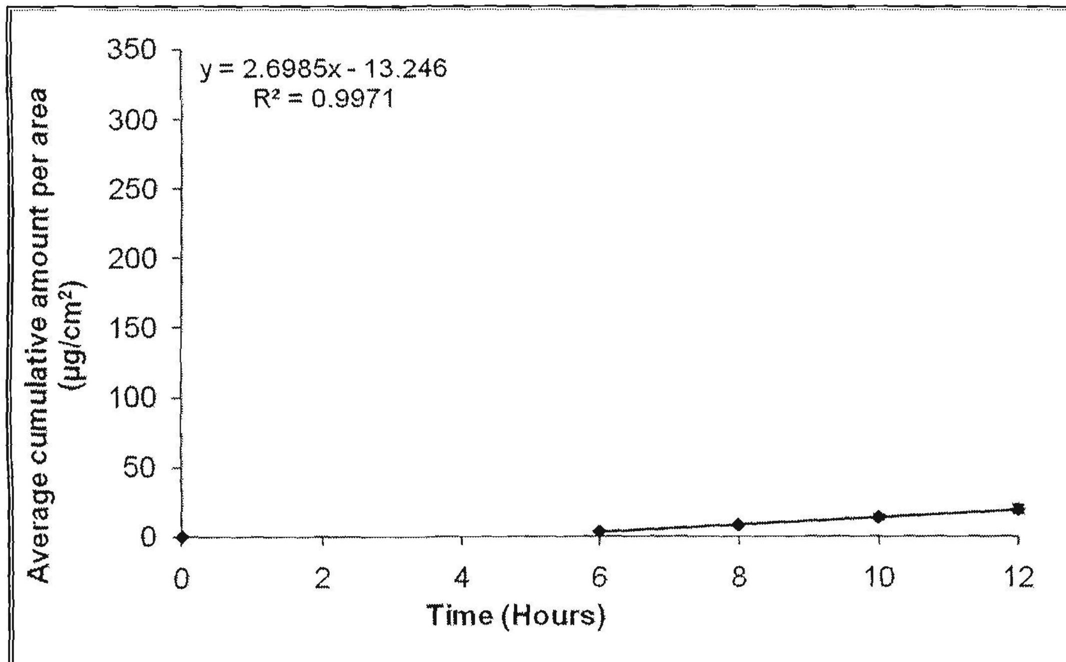

Figure F.3: Cumulative amount per area of niacinamide in the **cream** plotted against time.

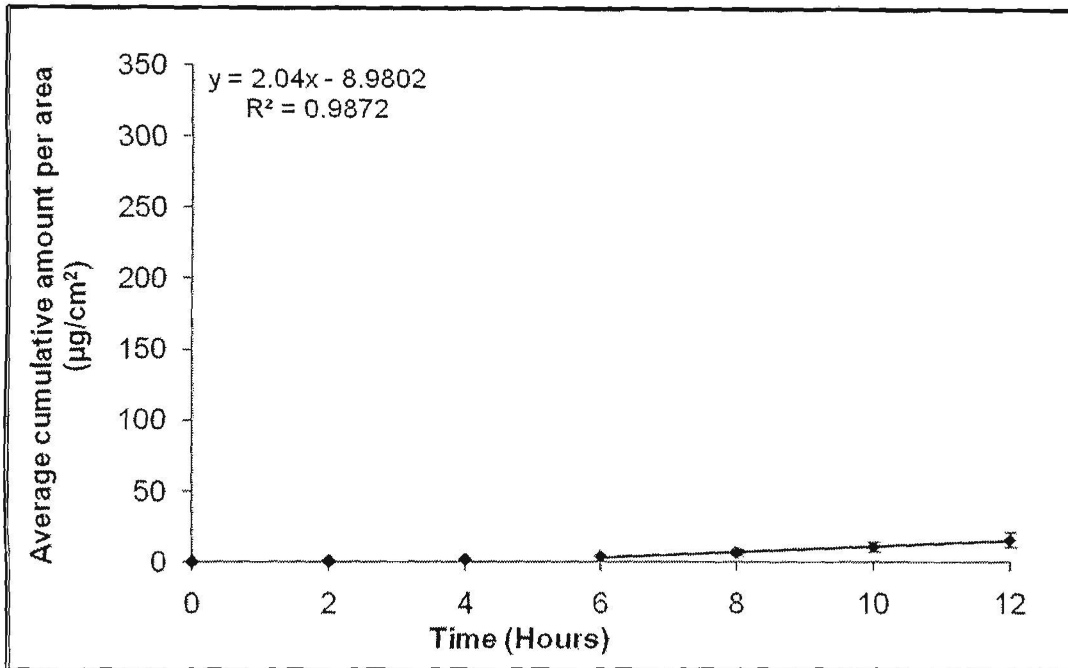

Figure F.4: Cumulative amount per area of niacinamide in the **gel** plotted against time.

F.1.2 SALICYLIC ACID PERMEATION PROFILE

In **figures F.5** to **F.8** the average cumulative amount per area was plotted against time, for the Pheroid™ cream, Pheroid™ gel, cream and gel.

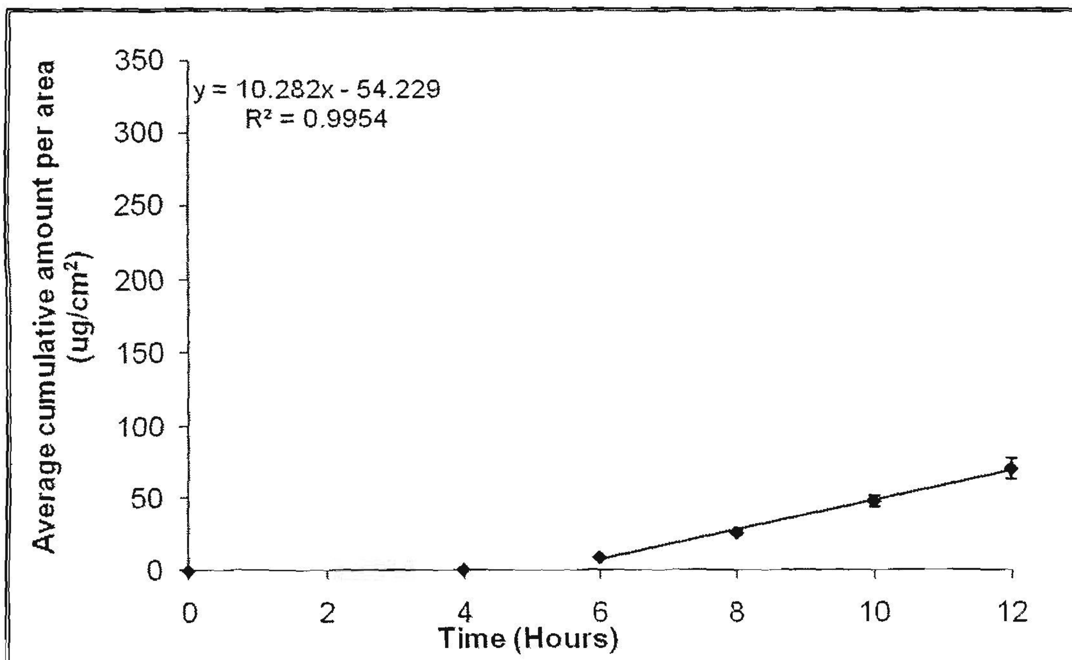

Figure F.5: Cumulative amount per area of salicylic acid in the **Pheroid™ cream** plotted against time.

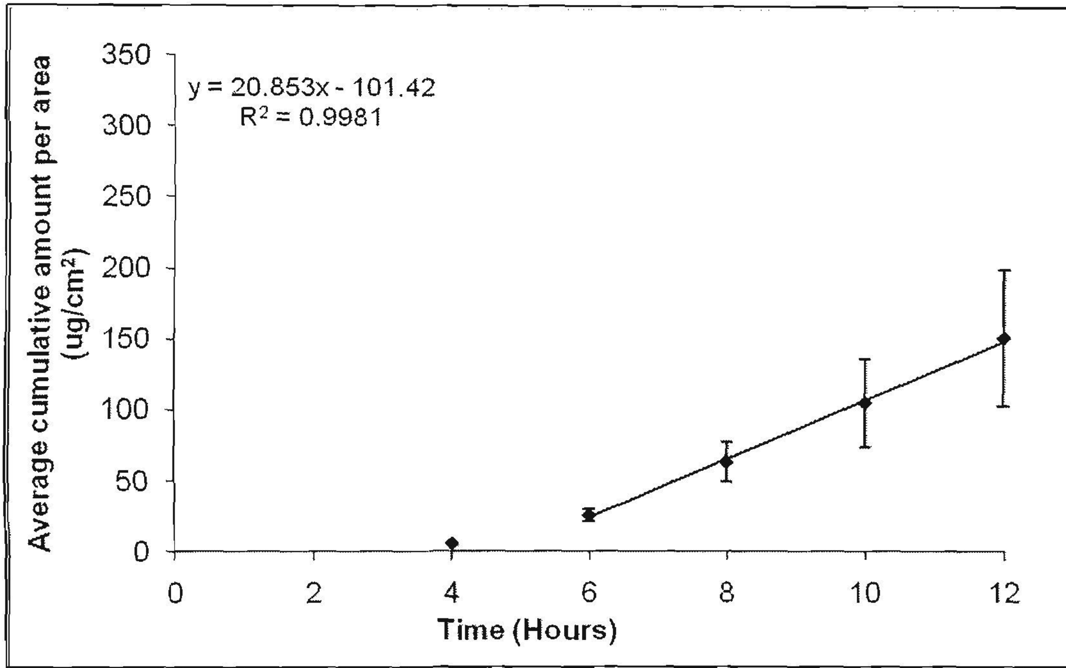

Figure F.6: Cumulative amount per area of salicylic acid in the **Pheroid™ gel** plotted against time.

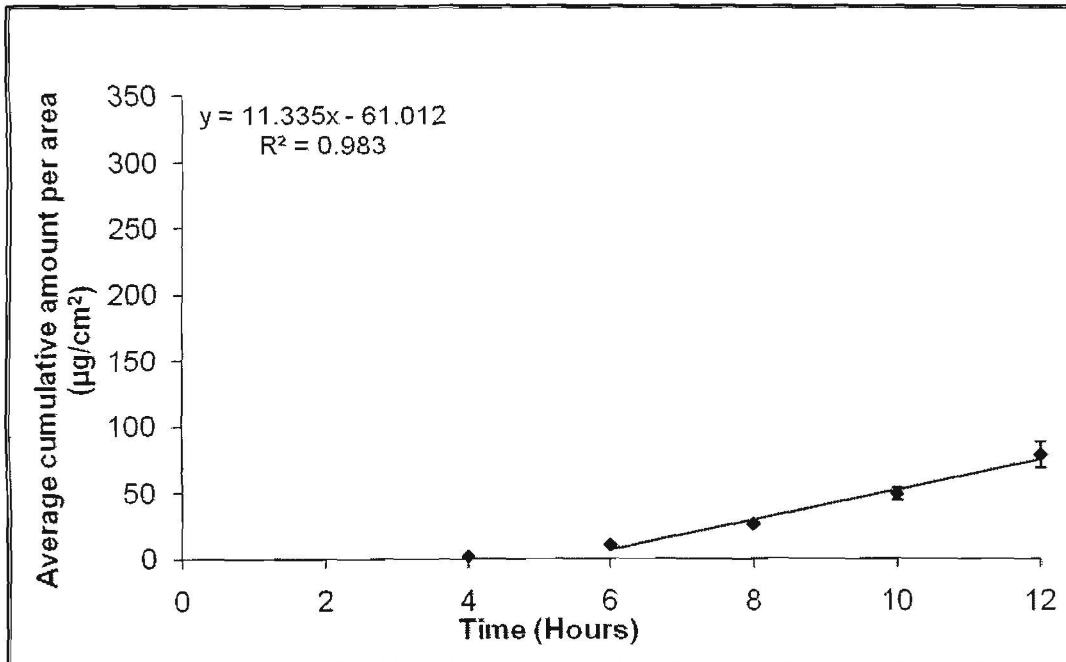

Figure F.7: Cumulative amount per area of salicylic acid in the **cream** plotted against time.

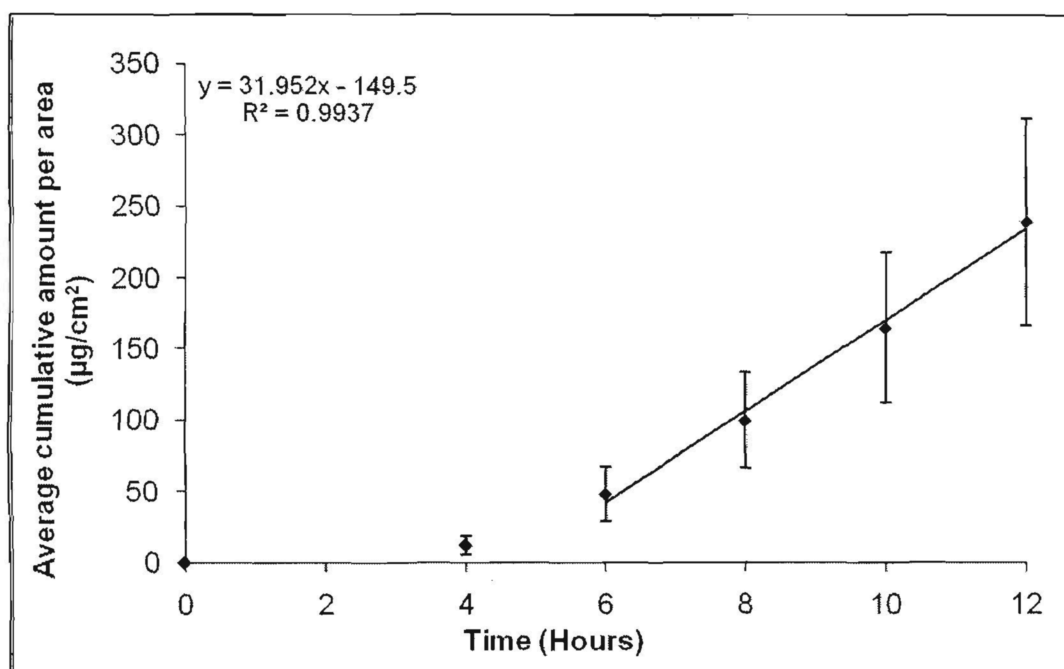

Figure F.8: Cumulative amount per area of salicylic in the **gel** plotted against time.

F.2 CONCLUSION

The flux value of each figure was calculated with the data points of the cumulative amount per area from 6 to 12 hours. **Figures F.1** to **F.4**, depicted the cumulative amount per area plotted against time for niacinamide. The cream depicted the highest flux of 2.70 $\mu g/cm^2.h$, followed by the gel (2.04 $\mu g/cm^2.h$), the Pheroid™ gel (1.32 $\mu g/cm^2.h$) and lastly the Pheroid™ cream (1.01 $\mu g/cm^2.h$).

Figures F.5 to **F.8**, depicted the cumulative amount per area plotted against time for salicylic acid. The gel showed the highest flux of 31.95 $\mu g/cm^2.h$, followed by the Pheroid™ gel (20.85 $\mu g/cm^2.h$), the cream (11.34 $\mu g/cm^2.h$) and lastly the Pheroid™ cream 10.28 $\mu g/cm^2.h$.

This indicated that the cream (in the case of niacinamide) and the gel (in the case of salicylic acid) penetrated through the skin more effectively than the rest of the formulations.

APPENDIX G: PHOTOS OF APPARATUS USED DURING THE DIFFUSION STUDY, SAMPLE ANALYSIS AND STABILITY TESTS

Photo G.1: A Grant water bath.

Photo G.2: A vertical Franz diffusion cell with a receptor and donor compartment.

Photo G.3: Assembled Franz diffusion cells on a Variomag® magnetic stirring plate.

Photo G.4: Syringes used to withdraw samples from the receptor compartments of the Franz cells.

Photo G.5: Parafilm® where each skin from

Photo G.6: 3M Scotch® Magic™ tape that

stripping.

Photo G.7: 15 Tapestrips in a politop with 5 ml PBS.

Photo G.8: Auto sampler vials that was analysed on the HPLC.

Photo G.9: Agilent 1100 HPLC machine.

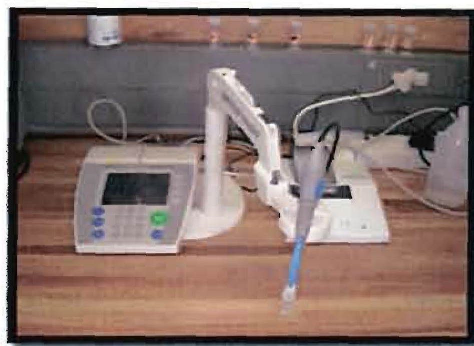

Photo G.10: Mettler Toledo pH meter.

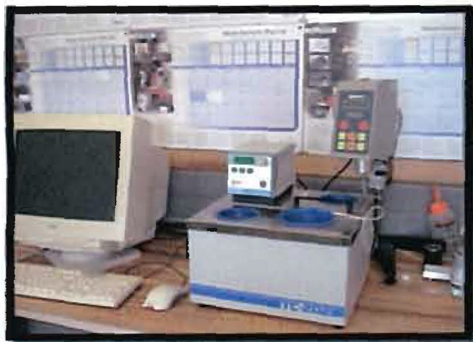

Photo G.11: Brookfield viscosity meter.

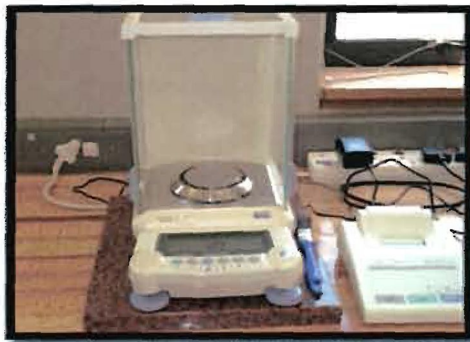

Photo G.12: Shimadzu AUW 120 Balance.

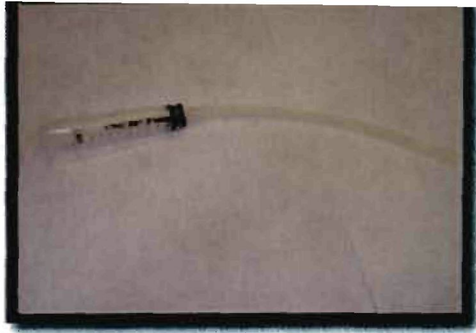

Photo G.13: A 5 ml syringe with a 15 cm silicone tube over the nozzle.

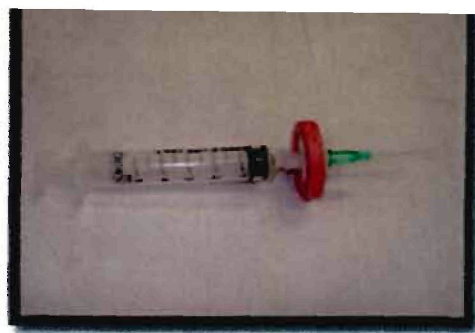

Photo G.14: A 5 ml syringe, with a 0.45 µm pre-filter and a 23G sterile needle.