Transdermal diffusion stability and clinical efficacy of cosmetic formulations containing *Rosa rubiginosa* rosehip seed oil

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Dissertation submitted in fulfilment of the requirements for the degree *Master of Science* of Pharmaceutics at the Potchefstroom Campus of the North-West University

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October 2016

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Acknowledgements

"Someday, everything will make perfect sense. So for now, laugh at the confusion, smile through the tears and keep reminding yourself that everything happens for a reason." Anonymous

By the Grace of God…I thank you Lord for this opportunity, the life lessons taught and the courage given to remember that my life and days are in Your hands.

I would also like to thank the following people, because without their contribution, this dissertation would not have been possible:

- My husband, Sarel van der Walt, you will never realise how much I appreciate everything you have done. Thank you so much for your love, motivation and unconditional support. Thank you for being there for me (and the kids). My love, my world is truly a better place, because of you.
- Our amazing children, Pieter and Carmi. Thank you for keeping my feet firmly on the ground, for your happy hearts, for being two of my greatest blessings and for making every day, a wonderful day. I love you.
- My parents, for empowering and setting me on this path in the first place. For working hard and sacrificing so much to give me the opportunities that I have received. I can never be more grateful than now and I cannot express how thankful I am for everything you have done for me.
- My in-laws, thank you mom and dad for always helping where you can. For your loving support, encouragement and for being the amazing grand-parents you are.
- Prof Hannes van der Walt, thank you for giving me the opportunity when I truly needed it and for helping me to find my way.
- Prof Banie Boneschans, thank you for your support during challenging times.
- Prof Jeanetta du Plessis, thank you for steering me into the right direction at times when I was not sure which way to go. I appreciate your advice and without your input, this dissertation would not have been the same.
- Prof Jan du Preez, thank you very much for all the time, effort and sometimes tears that prof had to endure. Thank you that prof patiently helped and supported me where possible.
- My friend and colleague, Jani van der Westhuizen, for your support, encouragement and for making all the long days and late nights a whole lot easier. May you and Ivan

have a blessed life together, see and experience the world and may the Lord be with you every day.

- Alicia Brummer, a special thanks to you. Thank you for your hard work and the effort you have put in to help when I really needed it. Your enthusiasm to learn is contagious and it was a great pleasure working with you.
- My fellow students and colleagues, Johan, Candice, Cornel, Trizél, Madél and Lizelle, thank you for being able to share this experience with you guys, may your future be bright, beautiful and blessed beyond believe.
- Sterna van Zyl, thank you for your assistance and help, not only with the clinical studies, but also your kind words of encouragement when times were tough.
- Minja Gerber, thank you for your technical assistance with this dissertation.
- Erika Fourie, thank you the statistical analysis of the clinical studies.
- Julia Handford, thank you very much for your effort and assistance with this dissertation. Thank you for the professional manner in which you have carried out the language editing, you have helped me so much.
- Hanlie Steyn, thank you for your kind words, your help with the translation of the abstract and just being the wonderful person you are.

Abstract

This study aimed at investigating cosmeceutical product development from concept to clinical efficacy testing, using *Rosa rubiginosa* rosehip seed oil, sourced from Southern African soil.

Rosehip seed oil contains various bio-actives, such as vitamin C, tocopherols, phytosterols, bio-flavonoids, triglycerides, fatty acids and tretinoin. Unfortunately, tretinoin assays performed during this study, revealed that no detectable amounts of tretinoin were found in the commercially acquired *R. rubiginosa* seed oil. Research proceeded to assess tretinoin's stability when dissolved in a 100% rosehip seed oil carrier and in newly formulated final products, containing 20% of the tretinoin spiked rosehip oil.

Two investigative cosmetic products (ICPs), namely two oil in water (o/w) emulsions (or emulgels) were formulated during this study and prepared for stability testing purposes. Accelerated stability test procedures were performed, as suggested by the International Conference on Harmonisation (ICH) Guidelines Q1A(R2) and significant physical and/or chemical formulation changes were observed during the long-term (25 ± 2°C/60 ± 5% RH), intermediate (30 ± 2°C/60 ± 5% RH) and accelerated (40 ± 2°C/75 ± 5% RH) storage of the test samples. The stability assessments revealed unacceptably significant changes, higher than 5%, with regards to the following parameters being investigated: the active pharmaceutical ingredient (API) and excipient assays, viscosity and conductivity measurements.

Membrane release studies, using hydrophilic polyvinylidene fluoride (PVDF) synthetic membrane filters and employing the Franz cell diffusion method, were performed. As a result, the release of the API from the test formulation was confirmed. The average cumulative concentration of 28.060 µg/cm^2, also expressed as an average percentage of 4.97% of tretinoin, was released from the formulations through the membranes after 6 h. The average flux that was obtained by the slope of the straight line between 2 h and 6 h for tretinoin was 9.0586 µg/cm^2.h.

In vitro skin diffusion studies, utilising the Franz cell diffusion method and excised adomenoplastic human skin, were performed. The concentration of the tretinoin that had permeated the dermatomed skin and reached the receptor compartment was measured as an average concentration of 0.362 µg/ml. An average percentage of 0.071% of tretinoin of the applied dose had hence diffused from the formulations and through the skin after 12 h. It

was also revealed that 0.049 µg/ml (0.0095%) of isotretinoin had been retained in the receptor fluid after 12 h. The skin fractionation procedure, utilising the tape stripping method, revealed that the average concentration of tretinoin that had been retained in the stratum corneum-epidermis was 0.020 µg/ml, whereas a slightly higher concentration of 0.027 µg/ml was located within the epidermis-dermis.

Finally*, in vivo* clinical studies were performed on human volunteers, utilising various non-invasive, bio-engineering instruments to evaluate the clinical efficacy. Following the results obtained during the *in vivo* clinical studies, it was concluded that beneficial clinical efficacy results had been demonstrated during this study. Hence, *R. rubiginosa* rosehip seed oil could be considered a valuable cosmetic component for the improvement of skin hydration, wrinkle appearance and skin firmness.

Key words: *Rosa rubiginosa,* skin, tretinoin, emulsion, stability, *in vitro, in vivo*

Uittreksel

Hierdie studie was gerig op die ondersoek van kosmetiese produkontwikkeling vanaf konsep tot kliniese doeltreffendheidstoetsing, deur gebruik te maak van *Rosa rubiginosa* roospitolie, verkry vanuit suidelike Afrika bodem.

Roospitolie bevat verskeie bioaktiewe stowwe, soos vitamien C, tokoferol, fitosterol, bioflavonoïede, trigliseriede, vetsure en tretinoïen. Ongelukkig het die tretinoïen-ontleding wat tydens hierdie studie uitgevoer is getoon dat daar geen meetbare hoeveelhede tretinoïen in die kommersieel verkrygde *R. rubiginosa* saadolie gevind is nie. Navorsing is voortgesit om tretinoïen se stabiliteit te assesseer wanneer dit opgelos word in 'n 100% roospitolie draer asook in nuut-geformuleerde formulerings wat 20% van die tretinoïen bevattende roospitolie bevat.

Twee kosmetiese toetsprodukte, naamlik twee olie in water (o/w) emulsies (of emulgels) is voorberei vir stabiliteitstoetsing. Versnelde stabiliteitstoetsprosedures is uitgevoer, soos voorgestel deur die Internasionale Konferensie oor Harmonisering Riglyne Q1A(R2) en betekenisvolle fisiese en/of fisiese en/of chemiese formulerings veranderinge is ondersoek tydens die langtermyn (25 ± 2°C/60 ± 5% RH), intermediêre (30 ± 2°C/60 ± 5% RH) en versnelde (40 ± 2°C/75 ± 5% RH) berging van die toetsmonsters. Die resultaat van die stabiliteitsbepaling het statisties betekenisvolle verandering oor tys uitgewys met betrekking tot die volgende parameters wat ondersoek was: die aktiewe farmaseutiese bestanddeel en hulpstofgehaltebepalings, asook viskositeit en geleidingsvermoë metings.

Membraanvrystellingstudies, wat gebruik maak van hidrofiliese polivinilideen fluoried (PVDF) sintetiese membraanfilters en die Franz seldiffusie metode is uitgevoer. Die vrystelling van die aktiewe farmaseutiese bestanddeel uit die toets formulering is bevestig deur die resultate. Die gemiddelde kumulatiewe konsentrasie van 28.060 µg/cm^2, ook uitgedruk as 'n gemiddelde persentasie 4.97% tretinoïen, is na 6 ure vrygestel uit die formulerings deur die membrane. Die gemiddelde vloei wat verkry is uit die helling van die reguitlyn tussen 2 en 6 ure vir tretinoïen was 9.0586 µg/cm^2.h.

In vitro vel diffusie studies wat gebruik maak van die Franz seldiffusie metode en verwyderde abdomenoplastiese menslike vel, is uitgevoer. Die tretinoïen konsentrasie wat die dermatoom vel deurdring het en die reseptorkompartement bereik het, is gemeet as 'n gemiddelde konsentrasie van 0.362 µg/ml. 'n Gemiddelde persentasie van 0.071% tretinoïen van die aangewende dosis het uit die formulerings gediffundeer deur die vel na 12

ure. Daar is ook aangetoon dat 0.049 µg/ml (0.0095%) isotretinoïen teenwoordig was in die reseptor vloeistof (na 12 ure). Die vel fraksionering prosedure, wat gebruik maak van die kleefband stroping metode het getoon dat die gemiddelde konsentrasie tretinoïen wat behoue gebly het in die stratum corneum-epidermis was 0.020 µg/ml, teenoor die effens hoër konsentrasie van 0.027 µg/ml wat in die epidermis-dermis gevind is.

Laastens is *in vivo* kliniese studies uitgevoer op menslike vrywilligers, deur gebruik te maak van verskeie nie-indringende, bio-ingenieur instrumente om sodanig kliniese effektiwiteit te evalueer. Na aanleiding van die resultate verkry uit die *in vivo* kliniese studies, is die gevolgtrekking gemaak dat voordelige kliniese effektiwiteit resultate gedemonstreer is. Gevolglik kan *R. rubiginosa* roospitolie beskou word as 'n waardevolle kosmetiese komponent vir die verbetering van vel hidrasie, voorkoms van plooie en fermheid van die vel.

Kernwoorde: *Rosa rubiginosa,* vel, tretinoïen, emulsie, stabiliteit, *in vitro, in vivo*

Chapter 1

Introduction and Problem statement

1.1 Introduction

"Newcomers to cosmetic manufacturing sometimes think that because they have used a product themselves with no apparent problems, or because the ingredients are “natural,” “organic,” or “botanical,” the product must be safe. This assumption is not correct” (FDA, 2015). Botanicals, extracted from plant sources, have a wide range of applications in the food, pharmaceutical and cosmetic industries. It is not extraordinary to use natural oils, mixtures of oils, plants, or other materials in cosmetic, or personal skin care formulations for protection, healing, or soothing purposes and have this been done for thousands of years (Draelos, 2005:432; Kumar, 2005:1263; Sharafzadeh, 2013:234). What is surprising, though, is the fact that the efficacy, safety and toxicology data of these natural materials that are so commonly used, are most often not scientifically reviewed. Indeed, topical formulation ingredients of natural origin may, similarly to their synthetic therapeutic equivalents, cause allergic, skin irritation, sensitivity, or even toxic reactions (Kumar, 2005:1270; Mitsui, 1997:121; Nohynek *et al.*, 2010:243). Although widely used, only a small percentage of herbal, or natural cosmetic raw materials are therefore found to be well documented (Kumar, 2005:1270; Nohynek *et al.,* 2010:243).

According to the literature, *Rosa rubiginosa* (rosehip) seed oil contains a wide array of bio-actives and unsaturated fatty acids, but was it the all-*trans*-retinoic acid, otherwise known as tretinoin, which caught the attention of our research team. *R. rubiginosa* is the only rose family that is documented to contain this vitamin A derivative. Cosmeceuticals that concentrate on the anti-ageing segment, are for the most part relying on the available information that *R. rubiginosa* seed oil contains this biologically active pharmaceutical ingredient (API) (Concha *et al.*, 2006:771; Ioele *et al.,* 2005:251). A significant challenge is the fact that natural ingredients are not standardised constituents. For instance, the analysis of *R. rubiginosa* seed oils revealed that, apart from genetic diversity and geographical distribution, even the different extraction methods could largely influence the concentration of the tretinoin being present in the oil, ranging between 0.051 and 0.324 mg/L, according to available reports (Concha *et al.*, 2006:772).

Advertisements and label claims for rosehip seed oil are found to be, to a certain extent, quite generic, offering typical skin care and anti-ageing solutions, such as skin moisturisation, anti-wrinkle properties, and the improvement of the elasticity and brightening of the skin. However, the claims that have raised the interest of researchers were those relating to the treatment of

eczema, psoriasis and the fading of scars (Elegance and Beauty Reviews.com, 2015), because, if the oil indeed contained the stated tretinoin concentrations, some of such claims were actually found worth investigating.

1.2 Problem statement and Aim of the study

Components of rosehip seed oil from different rose species have been investigated over recent years, but according to the available literature, the rosehip seed oil, originating from the wild growing *R. rubiginosa* roses, harvested in the mountains of Lesotho, South Africa, have not yet been studied. There may be a percentage of genetic diversity within the *R. rubiginosa* population that originate from different eco-regions, due to the different soil compositions (Aguirre *et al.*, 2009:183). Numerous studies have been conducted to analyse *R. rubiginosa* constituents in whole rosehip, and in their seeds and flesh. The assays being performed were mostly done, using oil that was extracted in a controlled laboratory environment. This study however, aimed at investigating *R. rubiginosa* seed oil that are commercially available and sold by suppliers. This was to ensure that the research outcomes were representative of rosehip seed oil that is typically acquired and used in commercial manufacturing purposes, when subjected to conditions that are normally encountered during the supply chain process.

For the purpose of this study, the aims were broadly to investigate the concentration of tretinoin (all-*trans*-retinoic acid) that is present in commercially acquired *R. rubiginosa* rosehip seed oil, obtained from Southern African soil and to determine the *in vivo* clinical efficacy thereof. During the development and evaluation of new cosmeceuticals, it is important to investigate an API's penetration through the stratum corneum and its target deposition, prior to conducting significant clinical trials, to substantiate clinical efficacy claims (Millikan, 2001:371). This study aimed at contributing towards the available data of natural raw material monographs.

1.3 Study objectives

To achieve these aims, the following objectives were set:

1. Develop and validate a high performance liquid chromatography (HPLC) method to quantitatively determine the concentrations of: a) tretinoin in the *R. rubiginosa* seed oil and b) the API and excipients in an investigational cosmetic product (ICP) to assess their concentration changes, when subjected to accelerated storage conditions (stability studies).

2. Formulate an oil in water (o/w) emulsion, utilising commercially acquired rosehip seed oil.

3. Perform stability tests on the ICP, when stored at long-term (25°C / 60% RH), intermediate (30°C / 60% RH) and accelerated (40°C / 75% RH) storage conditions. The following evaluations were performed at 0, 1, 2, 3 and 6 months: assays of the concentrations of the API and excipients, pH, viscosity, conductivity, particle size, visual appearance and creaming index.

4. Perform membrane release studies, utilising vertical Franz cell methods to determine the API release from the ICP.

5. Perform transdermal diffusion studies by employing *in vitro* vertical Franz cell methods, followed by tape stripping to determine and compare transdermal and topical delivery of the API from the ICP, respectively.

6. *In vivo* clinical efficacy trials, evaluating *R. rubiginosa* seed oil (100% oil) alone and in the formulated ICP (20% rosehip seed oil o/w emulsion). The following clinical efficacy trials were performed:

    - A short-term study (over 4 h): to measure skin hydration and the improvement thereof by the ICPs.

    - A long-term study (over 84 days): to evaluate skin hydration and the anti-ageing effects of the ICPs.

    - An erythema study: to investigate the anti-inflammatory properties of *R. rubiginosa* seed oil.

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Chapter 2

Cosmeceutical product development: from concept to clinical efficacy testing

2.1 Introduction

Since immemorial time, primitive people had relied upon natural extracts and on various mixtures of oils, plants, or other materials for vital protection, healing and/or soothing purposes. Skin care products had been prepared for centuries, long before technical formulation design, clinical efficacy testing, or manufacturing technology became understood. Parts of plants were collected and incorporated into any possible dosage form available. Even today, approximately 80% of those populations in developing countries are reliant upon medicinal herb and plant extracts for the treatment, or prevention of diseases (Draelos, 2009:432; Kumar, 2005:1263, Vermaak *et al.,* 2011:920). In recent years, modern civilisation has developed a renewed interest in the so called, *natural* cosmetic market. Consumers have become keener to purchase eco-friendly products, causing a continuously growing market trend towards *going green*, as observed since the late 1970's (Kumar, 2005:1263, 1270; Sharafzadeh, 2013:234; Vermaak *et al.,* 2011:920).

Research and development scientists in the personal care market sector have transformed formulation strategies through the implementation of a *back to nature* approach, by substituting conventional synthetic ingredients with their *natural* counterparts. The development of these so called *cosmeceutical* formulations, which incorporate such natural bio-active ingredients, has become a successful marketing tool that has resulted in continuous annual growth in the natural skin care market section in recent years (Vermaak *et al.,* 2011:920).

According to the United States Food and Drug Administration (FDA), *cosmeceuticals* refer to cosmetic formulations that deliver a specified pharmacological action, by using bio-active ingredients, which influence the biological functions of the skin to correct non-pathological variations of normal skin (Brandt *et al.,* 2011:141; Nohynek *et al.,* 2010:251). Despite the wide use of the term, cosmeceuticals, it is not a unanimously recognised regulatory term. Current European Union (EU) regulations allow for topical formulations only to be classified as either a *medicine*, or a *cosmetic (*European Commission, 2009:59). Some cosmeceuticals, close to representing therapeutic borderline products, may possibly incur a regulatory re-classification from cosmetic to medicine. It is clearly stated that cosmeceuticals should not and may not claim to cure, nor treat pathological skin conditions. Such cosmeceuticals should, instead, focus on functional skin repair purposes, including anti-ageing, anti-wrinkling and skin hydration. This

stipulation poses a profound challenge to research and development scientists to formulate clinically effective cosmeceutical products, without crossing the therapeutic-pharmacological action boundary (European Commission, 2009:59; Nohynek *et al.,* 2010:251; Wunderlich, 2011a:5).

The labelling of formulations as *natural* products does not necessarily warrant their safety, nor efficacy. A de-oxyribonucleic acid (DNA) barcoding study, using short genetic markers in organisms' DNA for identifying and classifying particular species, was conducted on natural, or herbal products. Approximately 60% of those natural products contained plant ingredients, other than those stated on the label, such as substituted plant ingredients, or additional filler plant materials (Newmaster *et al.*, 2013: 1). Such unidentified and unanticipated materials may interfere with other medicines, herbs, or supplements, also taken by the user and possibly trigger adverse effects, or toxic reactions, or reduce their clinical efficacy. Inadequate information on natural, bio-active ingredients and/or unsubstantiated label claims represent misinformation that may cause harm to the consumer. Apart from the obvious importance of having to scientifically investigate natural plant materials, it is also important to safeguard consumers by ensuring that currently perceived perceptions and the high value that is generally placed on natural products, are substantiated, whereas a general distrust in the natural ingredient market should be prevented (Brandt *et al.*, 2011:141; Gagliardi *et al.,* 2007:45; Newmaster *et al.*, 2013: 1; Nohynek *et al.,* 2010:240, 252).

Plants of the *Rosaceae* family are frequently used in the food, pharmaceutical, cosmetic and personal care industries (Barros *et al.*, 2011:2233). *Rosaceae* is a versatile plant family and all of the components of the rosehips, seeds, petals, flowers and fruits at different stages of their development can be used, according to the phytochemical contents of these plant segments (Barros *et al.*, 2011:2234).

For the purpose of this study, *Rosa rubiginosa* (rosehip) seed oil was investigated and included in a new cosmeceutical topical formulation, which was then subjected to accelerated stability testing, topical skin delivery assessments and clinical efficacy studies.

2.2 Botanical and geographical distribution of *Rosa rubiginosa*

Rosa rubiginosa belongs to a sub-specie of the *Rosaceae* family, which was originally endemic to Europe, but now also thriving in various areas in Chile, Spain, Argentina, New Zealand, Australia and Africa (Buzunova & Zieliński, 2011:99; Nowak, 2005:229).

Rosehips are the fruits of the wild growing rose plants and a valuable source of naturally derived raw materials mostly used in the food industry (Adamczak *et al.*, 2011:55; Barros *et al.*, 2011:2235). Isolated rural communities harvest organically grown African rosehips by hand and

then sell them to production companies. While rosehip seeds are often regarded as a waste material by the food industry, the extracted vegetable oil is an inexpensive, natural raw material that is effectively used in the cosmetic industry (Adamczak *et al.*, 2011:60; Aquirre *et al.*, 2009:184; Del Valle *&* Uquiche, 2002:1261; Franco *et al.*, 2007b:3511; The Rosehip Company, 2010; Vermaak *et al.*, 2011:921).

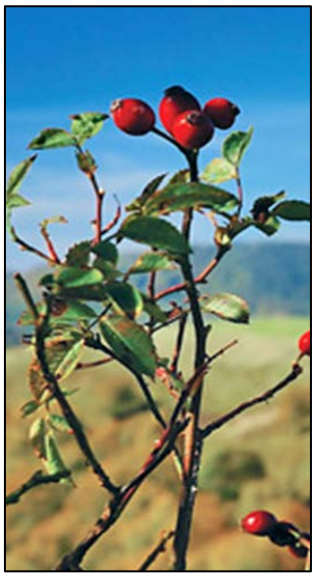

Figure 2.1: *Rosa rubiginosa* (therosehipcompany.com, 2014).

Figure 2.2: Rosehip fruits and seeds (Tenenbein, 2013).

In terms of the Conservation of Agricultural Resources Act of South Africa (Act 43 of 1983), *R. rubiginosa*, also known as *R. eglanteria, R. mosqueta*, or sweet briar (common name), is declared a Category 1 invader (noxious weed) and is it therefore not allowed to be planted, nor harvested in South Africa. This invader species grows rapidly and spreads effortlessly to cover large ecological areas. *R. rubiginosa* adapts with ease to new ecological regions and exhibits high invasive abilities. If the harvesting of *R. rubiginosa* rosehips and other plant materials are,

however, well managed and controlled, it retain a positive impact on ecosystems from a conservation point of view. Rosehips are therefore relevant, non-timber forest products that positively contribute towards the sustainability of various rural communities' economies (Aquirre *et al.,* 2009:183, 184; Vermaak *et al.*, 2011:92; Zimmermann *et al.*, 2010:445).

2.3 Stage 1: Cosmeceutical formulation development: Pre-formulation research

When initiating the development of a new cosmeceutical product, it is important to first identify the therapeutic objective of the new formulation, its target area and the intended label claims, before commencing with the product design (Woodruff, 2010:14).

Pre-formulation fundamentals that were considered during this study:

- Identification of the therapeutic purpose,
- Identification of the target area,
- Pharmacological, pharmacokinetic and/or physicochemical evaluation of the active ingredient,
- Identification of a suitable dosage form,
- Evaluation of the physicochemical properties of the delivery vehicle (Barry, 2009:596; York, 2009:5).

These important elements of the pre-formulation research process are individually discussed in the following sections.

2.3.1 Pre-formulation: Identification of the therapeutic purpose

The determination of a therapeutic goal at the onset of the formulation design process is imperative to the successful completion of a formulation project. Cosmeceutical formulators should establish the aim and goals which are intended with the development of a particular therapeutic product and identify suitable formulation ingredients (Abbott, 2012:217; Barry, 2009:596; Guesnet *et al.*, 1994:65; Mitsui, 1997:321).

Rosehip seed oil is utilised in numerous cosmetic products and is it considered as a safe, effective and non-invasive ingredient, capable of healing damaged skin. Product label claims include the improvement of skin stretch marks and hydration, the restoration of elasticity, damage from overexposure to the sun, eczema and psoriasis, the smoothing of wrinkles, the prevention of premature skin ageing, hyper-pigmentation, dermatitis, scars and age spots, and

the repair and treatment of psoriasis (Franco *et al.,* 2005:444; Franco *et al.,* 2007a:150; Franco *et al.*, 2007b:3506; Olivier, 2013).

The aim and therapeutic purpose of the planned cosmetic product during this study were to develop a semi-solid topical formulation, suitable for investigation of the skin hydration, anti-ageing and anti-inflammatory properties of rosehip seed oil. A formulation vehicle therefore had to be designed, or chosen that would to the least possible extent attribute towards the bio-active properties of the researched active ingredient, rosehip seed oil.

2.3.2 Pre-formulation: Identification of the target area (deposition site)

The skin is a complex organ, consisting of a large surface area that represents a significant potential route for drug administration. Pharmaceutical and cosmeceutical topical product design usually follow different aims and approaches. Pharmaceutical formulations may require active ingredients to pass through the stratum corneum to reach the viable epidermis bloodstream (circulatory system), whereas cosmeceuticals usually are required to remain in the upper skin layers, or to at least not penetrate into the viable epidermis. Therefore, topical delivery results in the accumulation of active ingredients, or permeant molecules in the top skin layers, an effect that is typically desired for cosmetic formulations, whereas the opposite is required with regards to systemic, or transdermal drug delivery (Izquierdo, 2008:174; Wiechers, 2008a:10).

For the planned rosehip cosmeceutical product design, the identification of the anticipated target area included consideration of consumers' intended application site (i.e. the face, hands or body), the skin's condition (normal, oily, dry, or damaged), age (baby, young adult, mature) and skin type (Barry, 2009:596; Guesnet *et al.*,1994:65; Mitsui, 1997:321).

2.3.2.1 Topical and transdermal delivery

Skin permeation and the dynamics that influence this process, determine whether an active ingredient would reach the actual intended target site, or deposition area. The general transport process and the biological factors that influence active ingredient delivery are hence discussed in this section.

The skin constantly maintains its barrier function, primarily to protect the body against external environmental factors and possible permeants. While healthy skin barrier function intends to predominantly keep external substances out, transdermal delivery aims at accomplishing the exact opposite, namely the transport of molecules from the outer skin surface into either the deeper skin layers, or into the systemic circulation (WHO, 2006:23).

Topical formulations are applied to the skin and aims for the active ingredient, or the permeant, to move through the various skin layers to either reach the viable epidermis, the dermis capillary system, or the lymphatic system (WHO, 2006:23; Williams, 2013:676). Permeation, or the transport of the molecule through the stratum corneum is mainly achieved through diffusion, with no active transport processes involved. The lipophilic stratum corneum skin barrier seems to act as the rate limiting factor which hinders hydrophilic molecule diffusion, whereas the hydrophilic epidermis and dermis layers are the rate limiting factors during lipophilic molecule transport. Lipophilic molecule permeation decreases if the stratum corneum is damaged. Consequently, if the stratum corneum's integrity has been compromised, or if the upper skin layers have been damaged, molecules will be able to directly diffuse into the hydrophilic epidermal and dermal layers (WHO, 2006:23).

Three main routes which facilitate active ingredient delivery are suggested, i.e. transcellular, inter-cellular and appendageal penetration. Transcellular penetration requires substances to continuously diffuse through corneocyte membranes, whereas the inter-cellular route suggests that substances would diffuse through the lipid matrix, in between the packed corneocytes. Shunts of hair follicles, and sebaceous and sweat glands facilitate the appendageal route (WHO, 2006:17).

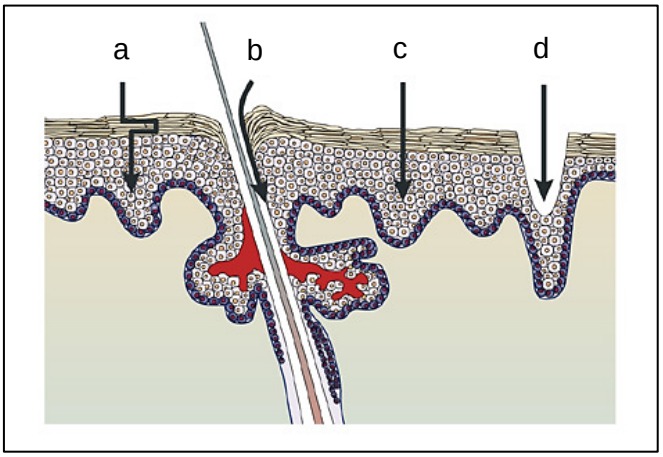

Figure 2.3: Schematic representation of the transdermal delivery routes: (a) transcellular, (b) appendageal, (c) inter-cellular penetration and (d) diffusion directly into the hydrophilic epidermal and dermal layers (Prausnitz *et al.*, 2004: 119).

To illustrate the processes of permeant diffusion and transdermal penetration, mathematical models, such as Fick's laws of diffusion are applied (Izquierdo, 2008:174; Wiechers, 2008a:10). This model assumes that the permeant would move in one direction only, i.e. from the outer skin surface into the deeper skin layers and tissues, as a function of the rate of the concentration change over time, proportional to the rate of the concentration gradient change at a specific point. The chemical potential gradient (concentration gradient) for diffusion across skin creates a transdermal flux, causing molecules to diffuse from a higher to a lower concentration, with the

concentration gradient being the primary driving force for transport. As a result, if the permeant concentration is increased, transdermal penetration will also increase (Wiechers, 2008b:83, 88; Williams, 2013:676, 681).

Fick's first law of diffusion describe passive diffusion of a permeant, during which the diffusion process is unaffected by any possible limitation, or by the active ingredient's physicochemical, or dermal properties. Fick's diffusion law assume that the skin has equal structural and diffusional properties in all directions, which is not entirely accurate (Williams, 2013:676, 681).

Transdermal diffusion, however, is not such a straightforward process, as is suggested by Fick's laws. Firstly, various biological factors, such as skin structure, micro-circulation, barrier function and skin hydration levels, as well as active ingredient and vehicle properties influence this process. Biological factors, active and vehicle ingredient properties are therefore discussed in the next sections.

2.3.2.2 Skin structure

Skin structure influences dermal penetration by potentially restraining an active ingredient from reaching its target site. The skin is the largest organ of the body and covers a surface area of approximately 2 m^2 in the average adult and it comprise of approximately 15% of the total body weight. The skin is a complex living organ that constantly adapts according to external and internal response signals, to offer continuous protection to the inner body by facilitating defence and repair mechanisms, by regulating the body temperature, by controlling trans-epidermal water loss (TEWL) and by maintaining its barrier function (Lai-Cheong & McGrath, 2013:317; WHO, 2006:10).

Skin permeation varies according to biological variables, such as age, race, sex and anatomical site. Different anatomical locations vary in skin thickness, appendageal distribution, stratum corneum lipid composition, epidermal capillary networks and thus skin structure, which significantly influence active ingredient delivery (Riviere, 1993:115; WHO, 2006:18, 23; Wiechers, 2008a:3, 18).

Epidermis lipid composition is a major determinant of the extent of absorption and the permeation rate of molecules through the skin. Lipid composition regulates the partitioning of an active ingredient from the application site, into the lipid matrix and corneocytes, by controlling the kinetics of permeation. An important function of the lipid matrix is to prevent TEWL and salt loss, thereby hindering water soluble substances from penetrating through the epidermal layers and contributing towards strong corneocyte cohesion. Deficiencies in the stratum corneum lipid composition would result in skin barrier function abnormalities, which in turn would affect skin barrier permeability (Baroni *et al.*, 2012:259; Menon *et al.*, 2012:6, WHO, 2006:14). Epidermis

lipid composition of the lipid matrix is species dependant, which explains the variations in active ingredient penetration properties, as observed among different living beings (Riviere, 1993:123).

Altered epidermal lipid composition, either due to dermatological diseases, or nutritional fatty acid deficiencies, would disrupt the healthy skin barrier function and hence inter-cellular molecule permeation (Riviere, 1993:123).

2.3.2.3 Micro-circulation

Micro-circulation of the viable epidermal and dermal capillaries is significantly influenced by external trigger factors, such as changes in ambient temperature. The capillary network in the viable epidermis changes the blood flow rate markedly in response to thermo-regulatory requirements. Cutaneous blood flow is increased when the ambient temperature exceeds body temperature and the capillary veins will dilate to facilitate heat loss through the skin. If the ambient temperature exceeds 43°C, normal blood flow can be increased ten-fold (WHO, 2006:23). The opposite occurs when ambient temperatures fall and is blood flow decreased, or even shunted in certain areas to prevent heat loss. Micro-circulation and capillary uptake will limit transdermal penetration, when the rate at which permeant molecules are transported through the stratum corneum layer exceeds clearance into the capillary vessels. If micro-circulation is insufficient, the permeant will accumulate in the viable epidermis, dermis, or body tissue (Riviere, 1993:118; WHO, 2006:23).

2.3.2.4 Skin barrier function

Skin barrier function is directly dependent upon the integrity of the stratum corneum. Healthy skin, with an intact stratum corneum exhibits optimal skin barrier protection function, thereby limiting topical delivery and transdermal penetration. Once an active ingredient molecule achieves penetration through the upper layer of the stratum corneum, diffusion within the stratum corneum will be the subsequent rate limiting penetration factor (Riviere, 1993:116; Wiechers, 2008b:92; Williams, 2013:677).

As mentioned before, transdermal penetration is increased when the barrier function is disrupted, or when the stratum corneum layer is damaged. Disruption of the barrier function is brought about by various extrinsic factors, such as temperature, humidity, mechanical, chemical, microbial, or sun damage and nutritional component deficiencies, or by intrinsic factors, such as skin diseases (Riviere, 1993:115; WHO, 2006:19; Williams, 2013:677).

2.3.2.5 Skin hydration level

The stratum corneum layer contains approximately 5 – 20% of water. Relative humidity and ambient temperature changes affect the skin hydration levels, which in turn significantly alter molecule penetration. A topically applied formulation vehicle would increase skin hydration by either providing water directly to skin surface, or due to occlusion, by preventing TEWL to entrap water within the stratum corneum. A higher water content may increases overall thickness of the epidermis, due to corneocyte swelling, which subsequently increases the rate and extend of percutaneous absorption, by altering the partitioning and concentration gradient of the penetrating active ingredient molecules (Riviere, 1993:117, 118; WHO, 2006:20; Zatz, 1993a:128).

Consideration of all of the above factors emphasise the importance of having to be aware that skin condition may affect the active ingredient molecule penetration rate and penetration level. Cosmetic products should only to be applied to healthy skin and should they not be utilised for the treatment of pathological skin conditions. Cosmetic product active ingredients should not reach the viable epidermis and possible systemic availability and therapeutic action thereof should be prevented (Brandt *et al.*, 2011:141; Nohynek *et al.,* 2010:251).

2.3.3 Pre-formulation: Pharmacological, pharmacokinetic and/or physicochemical evaluation of the active ingredient

It is not merely the skin structure, or dermal penetration pathways that are responsible for active ingredients to reach their target deposition area. The physicochemical properties of the active ingredient molecules are very important limiting factors in managing drug transport into and/or through the skin. To therefore assist with a transport feasibility assessment, the general permeant properties, responsible for influencing skin penetration, are discussed in the following sections (Williams, 2013:680).

Another motivation for investigating the properties/characteristics of active ingredients is based upon the regulatory requirement of having to protect and ensure consumer health. According to European Union (EU) legislation and Scientific Committee on Consumer Safety (SCCS), the marketing of no cosmetic product may commence without a Product information file (PIF), which should include a Cosmetic product safety report (CPSR) that evaluates the chemical structures of the formulation ingredients, toxicity profiles and exposure patterns to establish safety-in-use (SCCS, 2012:1, 9). The safety evaluation of final cosmetic products should include an assessment of all of the cosmetic ingredients included in the formulation. The assessment should identify the physical form, molecular weight, chemical identity and characterisation, stability, solubility, partition coefficient, function and therapeutic use, pharmacokinetic information, as well as data obtained from toxicity or literature studies. The gathered original

analytical data and literature information are then reviewed in order to characterise the cosmetic ingredients. All of the gathered data is used to compile a cosmetic product dossier, which is then submitted for evaluation by the appropriate regulatory authorities (SCCS, 2012:12).

An evaluation of rosehip's physicochemical and pharmacological properties would therefore firstly denote whether the target area is reached and indicate whether the rosehip seed oil shows the necessary potential for achieving the intended beneficial purpose. Secondly, together with a review of the information obtained with regards to the formulation vehicle's ingredients, the data may be utilised to perform a cosmetic product safety-in-use assessment.

According to the literature, rosehip seed oil contains vitamin C, tocopherols, phytosterols, β-carotene, various bioflavonoids, all-*trans*-retinoic acid (tretinoin) and relatively high concentrations of saturated and unsaturated fatty acids (Aquirre *et al.*, 2009:184; Barros *et al.*, 2011:2235; Nogala-Kalucka *et al.*, 2010:1485). Its anti-oxidant activity had been investigated through determination of the content of phenolic compounds (natural anti-oxidants) in seed lipids and it was established as 140.5 mg/100 g of dry matter. Phytochemical analyses indicated that whole rosehips, derived from the *R. rubiginosa* plant material growing in Poland, contained an average flavonoid content (expressed as quercetin equivalent) of 72.0 mg/100 g of dry mass (Adamczak *et al.*, 2011:107).

Since the preceding information is not merely enough to decide as to whether a cosmeceutical product, containing rosehip seed oil, would be regarded effective, or safe, the following sections describe the physicochemical properties and bio-active functions of the abovementioned constituents, present in this oil.

2.3.3.1 Molecular size of the active ingredient

Molecular size is indicative of the skin's permeation potential of a drug, where permeation and maximum flux decrease exponentially with an increased molecular weight. Molecular weight increases concomitantly with lipophilicity. Ideally, to increase a drug's permeation potential, a molecule should have the lowest possible molecular weight, preferably less than 500 Da. As per Table 2.1, all of the rosehip constituents have molecular sizes below 500 Da and should it therefore be able to permeate the skin satisfactorily (WHO, 2006:27; Wiechers & Watkinson, 2008:63; Williams, 2013:680). However, in order to predict possible skin permeation of a drug, all of the physicochemical characteristics of a penetrant must be considered together and not in isolation.

Table 2.1: Molecular weight of *Rosa rubiginosa's* constituents (IUPHAR/BPS.org, 2014; PubChem.gov, 2014; Sigma-Aldrich.com, 2014a, b, c)

Tocopherols	Dalton (Da)
γ-Tocopherol	416.68
α-Tocopherol	430.71
β-Tocopherols	416.68
δ-Tocopherols	402.65
Phytosterols	
Sitosterol	414.71
Campesterol	400.68
Stigmasterol	412.69
Avenasterol	412.69
Fatty acids	
Palmitic acid	256.42
Stearic acid	284.48
Oleic acid	282.46
Linoleic acid	280.45
Linolenic acid	278.43
Tretinoin	300.21

2.3.3.2 Octanol-water partition coefficient

The octanol-water partition coefficient (Log P, or Log $P_{oct/wat}$) describes the molecular dispersal of an active ingredient from one formulation phase into another. Log P indicates the degree of distribution of a molecule in the octanol and water phases, and is it therefore indicative of how well a molecule would distribute through the stratum corneum lipids and the hydrophilic water component (Williams, 2013:677). Transdermal delivery is optimised for molecules that exhibit a balanced lipophilicity and a log P of approximately 2 – 3. Hydrophilic molecular structures usually have a negative log P value, whereas, lipophilic molecules exhibit a positive log P. Higher log P values are indicative of an increased lipophilicity. Highly lipophilic molecules, like rosehip seed oil and its constituents (Table 2.2), would be trapped in the stratum corneum, which generally is the required target area of cosmetic applications (EC, 2004:5; Gaisford, 2013:368; Karande & Mitragotri, 2009:2363; Wiechers & Watkinson, 2008:63; Wiedersberg & Guy, 2013:150; Williams, 2013:676, 686).

Table 2.2: Log P values for rosehip's constituents (IUPHAR/BPS.org, 2014; PubChem.gov, 2014; Sigma-Aldrich.com, 2014a, b, c)

Tocopherols	Log P
γ-Tocopherol	10.3
α-Tocopherol	10.7
β-Tocopherols	10.3
δ-Tocopherols	10.0
Phytosterols	
Sitosterol	9.3
Campesterol	8.8
Stigmasterol	8.6
Avenasterol	8.6
Fatty acids	
Palmitic acid	6.4
Stearic acid	7.4
Oleic acid	6.5
Linoleic acid	6.8
Linolenic acid	5.9
Tretinoin	5.0

2.3.3.3 Physical state of the active ingredient

The physical form of the active ingredient influences its skin permeation ability. A low melting point increases the likelihood that a solid state active ingredient would be transformed into a liquid state at room temperature. Consequently, dissolved particles would diffuse through skin layers more readily than solid particles, resulting in an increased molecule permeability (Cleary, 1993:209; Wiechers & Watkinson, 2008:63).

Table 2.3: Melting point values for rosehip's constituents (IUPHAR/BPS.org, 2014; Parchem.com, 2015; PubChem.gov, 2014; Sigma-Aldrich.com, 2014a, b)

Active ingredient	Melting point
Tocopherols	2.5 – 3.0°C
Phytosterols	135.0 – 145.0°C
Fatty acids	
Palmitic acid	62.5°C
Stearic acid	69.3°C
Oleic acid	16.3°C
Linoleic acid	-6.9°C
Linolenic acid	-10.6°C
Tretinoin	181.0°C

Although rosehip seed oil is a liquid, none of its constituents naturally occur in the solid state. As a result, neither the physical form, nor the melting points of the constituents of this active ingredient should limit their skin permeation.

According to the Scientific committee on consumer safety (SCCS), the typical physical and chemical data set aspects of rosehip seed oil that should be reviewed include boiling point, flashpoint, density and viscosity information (SCCS, 2012:18). According to various material safety data sheet (MSDS) information, rosehip oil has a boiling point above 100°C at 760 mm Hg, a flashpoint of above 300°C, a density at 25°C of 0.90 – 0.92 g/ml, and is it insoluble in water and slightly viscous, i.e. 77 cP at 20°C (Jeen® International Corporation, 2013:1; MakingCosmetics Inc., 2012:1; Martinez, 2006:1).

2.3.3.4 Bio-active functionality

To establish whether the intended biological purpose will be achieved once the active ingredient reaches its target area, it is important to investigate the anticipated bio-active functions each of the constituents of the active ingredient, in this case, the rosehip oil (Wiechers, 2008a:2, 18).

2.3.3.4.1 Tocopherols

Rosehip seeds contain tocopherols (vitamin E) in total amounts of approximately 15.66 mg/100 g dry mass that predominantly comprise of γ-tocopherol (14.22 mg/100 g dry mass), but have α-, β- and δ-tocopherols also been identified (Nogala-Kalucka *et al.*, 2010:1485; Oláh *et al.*, 2011:711). Tocopherols, especially γ-tocopherol, demonstrate anti-oxidant, cell protection and anti-inflammatory properties (Oláh *et al.*, 2011:711). It has been observed that α-tocopherol, in concentrations of up to 5% in topical formulations, reduces skin erythema conditions (Schneider, 2008:467). Tocopherols also react with free radicals in the cell

membrane to protect poly-unsaturated fatty acids from oxidative degeneration (Dintcheva *et al.,* 2014:15).

2.3.3.4.2 Phytosterols

Phytosterols (2.44 mg/g fat), sitosterol, campesterol, stigmasterol and avenasterol were identified, with sitosterol (2.00 mg/g fat) being found the most prominent phytosterol in rosehip seed oil (Nogala-Kalucka *et al.*, 2010:1485). Phytosterols have shown the potential for healing damaged skin when applied topically (Puglia & Bonina, 2008:223). It is, however, noteworthy that phytosterols are highly susceptible to oxidation and should they be protected accordingly, when utilised in topical formulations (O'Callaghan *et al.*, 2014:787).

2.3.3.4.3 Triglycerides and fatty acids

Rosehip seed oil is a valuable source of triglycerides (see Figure 2.4) and free fatty acids (saturated and unsaturated) (Franco *et al.*, 2007b:3506). The high concentration of fatty acids in rosehip seeds contributes towards the significant utilisation of rosehip seed oil in the cosmeceutical product market (Ercisli, 2007:1379; Nogala-Kalucka *et al.*, 2010:1483; Vermaak *et al.*, 2011:922).

There are large variations in the literature regarding the measured quantities of fatty acids in rosehip seed oil. The fatty acid composition depends upon the maturity of the rosehip, or seed, various environmental factors (light, temperature, availability of water, soil and atmospheric elements) and the extraction method followed (Adamczak *et al.*, 2011:555-62; Barros *et al.*, 2011:2235; Concha *et al.*, 2006:771-775; Da Silva *et al.*, 2008:1489; Nowak, 2005:233). Cold-processing extraction methods may reduce fatty acid degradation, or lipoperoxidation. The grinding of the rosehip seeds during their preparation for extraction reduces the rosehip seeds' particle sizes, resulting in higher quantities of oil being extracted (Concha *et al.*, 2006:771; Del Valle & Uquiche, 2002:1264).

Rosehip contains a high concentration of unsaturated fatty acids, comprising approximately 58 – 79% of poly-unsaturated fatty acids (PUFA). The PUFA content was determined by adding the amount of the relative content of linoleic- and linolenic acid in the rosehip oil (Adamczak *et al.*, 2011:555-62; Barros *et al.*, 2011:2235; Concha *et al.*, 2006:771-775; Da Silva *et al.*, 2008:1489; Nowak, 2005:233).

Table 2.4: Average fatty acid composition in rosehip seed oil and whole rosehip plant material, following the conventional cold-processing method, without using assisted solvent, nor enzyme extraction (Adamczak *et al.*, 2011:57; Barros *et al.*, 2011:2235; Concha *et al.*, 2006:773; Nowak, 2005:232)

Acid	Seed oil (average %)	Whole rosehip (average %)
Palmitic (C16:0)	4 – 10	2.0
Stearic (C18:0)	2 – 5	1.5
Oleic (C18:1)	14 – 19	11.0
Linoleic (C18:2n-6)	47 – 54	37.0
Linolenic (C18:3n-3)	14 – 26	26.0

Fatty acids exist in nature, where they either function as *building blocks* to form complex molecules, such as triglycerides, fixed oils, fats and waxes, or as isolated substances (Brondz, 2002:3). Generally, natural fatty acids have a chain of 4 to 28 carbons (usually unbranched and even numbered), which may be saturated, or unsaturated (IUPAC, 1997). These fatty acids are important biological components in every living organism. Essential fatty acids are long chain poly-unsaturated fatty acids which cannot be synthesised and are obtained through diet (Brondz, 2002:3; Ercisli, 2007:1379).

Triglycerides are derived from esterified free fatty acids, held together by a glycerol *spine*. The glycerol (1, 2, 3-propantriol) *spine* has three functional alcohol groups and three fatty acids, identical or not, which must react to form a triglyceride molecule. Triglycerides are large molecules with a high molecular weight (Figure 2.4) (Franco *et al.*, 2007b:3506; IUPAC, 2014; Ophardt, 2003).

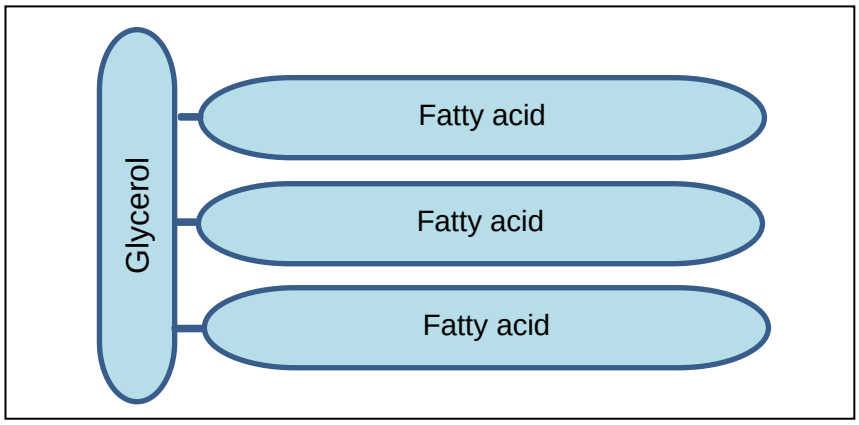

Figure 2.4: Schematic representation of the triglyceride fatty acid structure.

Unsaturated fatty acids, the building blocks of the rosehip triglycerides, possess valuable free radical scavenging properties that protect the skin against dehydration, while also moisturising it

(Sharafzadeh, 2013:234). Fatty acids are also known for rapid topical and transdermal absorption and fast penetration properties which are valuable to pharmaceutical carrier applications (Franco *et al.,* 2005:443).

Also, the topical application of linoleic acid stabilises the skin's fatty acid balance, which prevents skin lipid ceramide deficiency and hence facilitates the regeneration of the skin barrier function. Topical application bypasses metabolic conversion in the liver, causing linoleic acid to be more readily available for use in the skin, than when taken through diet. Topically applied linoleic acid has shown to decrease trans-epidermal water loss (TEWL) and wrinkle formation (Regiert, 2008:357).

Furthermore, during a clinical study performed by Regiert (2008:356), it was established that nutritional linoleic acid deficiency may have caused skin diseases, such as eczema, during which oleic acid had been incorporated into ceramides, instead of into linoleic acid. Subsequently, the skin's topography changed into a scaly, dry skin surface, with increased TEWL and skin damaged barrier function.

2.3.3.4.4 Tretinoin (all-*trans*-retinoic acid)

According to the literature, rosehip seed oil contains biologically active tretinoin (all-*trans*-retinoic acid) (Aquirre *et al.*, 2009:184; Concha *et al.*, 2006:771; Franco *et al.*, 2007b:3506; Ioele *et al.*, 2005:251). Tretinoin is one of the retinoid isomers that is frequently incorporated in cosmetic formulations (Brand *et al.*, 2011:142).

Analyses of rosehip seed oils indicated that different extraction methods had influenced the concentration of the tretinoin present in the oil and did its concentration range between 0.051 - 0.324 mg/l (Concha *et al.*, 2006:772).

Tretinoin is a naturally occurring derivative of vitamin A that binds to various isoforms of retinoic acid receptors, responsible for cell reproduction regulation, proliferation and differentiation, and therefore used to treat various skin disorders, including acne, ultra-violet (UV) damage and keratinisation disorders, such as ichthyosis and keratosis follicularis (IUPHAR/BPS, 2014). Retinoids are known to promote collagen and elastin production and anti-ageing properties through enhanced epidermal proliferation (Rosetti *et al.,* 2011:63).

Following the literature review of the constituents found in rosehip seep oil and the evaluation of their potential bio-active benefit, it was decided to proceed with this project. The next step was to identify a suitable formulation vehicle.

2.3.4 Pre-formulation: Identification of a suitable dosage form

According to Barry (2009:597), choosing an appropriate type of formulation is essential, as it functions as the *delivery vehicle* of the active ingredient. According to EU regulation, cosmetic products are broadly divided into six groups according to functionality, i.e. to perfume, to clean, to change appearance, to protect, to keep in good condition and to correct body odours (Woodruff, 2010:4). A cosmeceutical product vehicle type should be designed to achieve the intended purpose, by utilising compatible and functional active and vehicle ingredients (Woodruff, 2010:40).

Taking into consideration rosehip seed oil's physicochemical properties, it was decided to formulate an oil in water (o/w) emulsion. An emulsion is a multi-phasic liquid system, defined as liquid droplets and/or fluid crystals, dispersed within a liquid. The dispersed phase, also known as the internal phase, is evenly dissolved into the external, or continuous phase. An o/w emulsion is created when a lipophilic internal phase is dispersed within an external hydrophilic aqueous phase. Contrary, a water in oil (w/o) emulsion is formed when a hydrophilic, aqueous internal phase is dispersed into a lipophilic external phase. Emulsions exhibit liquid, or semi-solid consistencies. The emulsion formulation type was selected, because of its significant role in incorporating the active ingredient, but also to assist with an effortless topical application design that would not leave any oil residue (Buchmann, 2001:149).

Emulsions generally are thermo-dynamically unstable systems and therefore emulsifiers, such as surfactants and various viscosity enhancers are included in these formulations. Modification of the dispersion media, by forming a gel structure, for example, would contribute towards an increased stability character of such an unstable emulsion system (Lerche & Sobisch, 2011:1800; Traynor *et al.,* 2013:2173). Hence for this study it was decided to prepare an o/w emulsion within a stabilising gel-dispersion system.

2.3.5 Pre-formulation: Evaluation of the physicochemical properties of the delivery vehicle

As mentioned and as evident from the evaluation of the active ingredient's physicochemical properties, the formulation vehicle's properties should likewise be assessed. This is necessary, firstly, to predict whether the intended target area would be reached by the delivery vehicle and secondly, to assist with the final product's safety-in-use review. Hence, for a cosmeceutical formulation to achieve the planned therapeutic action, it is essential to take into account the physicochemical properties of the vehicle ingredient, in addition to those of the active ingredients being utilised (Guesnet *et al.,* 1994:65, Mitsui, 1997:321).

Although the physicochemical properties of the chosen vehicle should aid the transdermal penetration and skin permeability of the drug, the vehicle's influence is not constant. Topical delivery and transdermal penetration tend to be much more dependent upon the physicochemical properties of the active ingredient (Wiechers, 2008b:83, 88). General vehicle character affects transdermal permeation and the extent thereof is discussed in the following sections (Guesnet *et al.*, 1994:65;, Mitsui, 1997:321; Williams, 2013:686).

2.3.5.1 Solvent properties

Poor drug release from a topical formulation limits its topical and transdermal transport. Although the vehicle should allow for moderate solubility, the vehicle's solubility should not be too high, as it may result in the non-release, or entrapment of the active ingredient molecule within the vehicle (Williams, 2013:680, 694). While the active ingredient should therefore have maximum solubility within the formulation, this must be lower than the solubility of the active ingredient in the stratum corneum. A maximum partition coefficient will further increase the release of the active ingredient molecules from the formulation into the skin (Otto *et al.,* 2009:9; Wiechers, 2008a:11).

The polarity nature (polar, or non-polar) of the carrier influences the solubility factor of the active ingredient molecules. Since a molecule dissolves best in a solvent with similar chemical properties than itself, polar molecules will dissolve best in polar solvents, or non-polar molecules in non-polar solvents (Wiechers & Watkinson, 2008:63). Partitioning into the skin will be enhanced if the polarity differences between the active ingredient molecules and the formulation are increased, which means that the solubility of the active ingredient molecule will subsequently be decreased (Otto *et al.,* 2009:9). Polar solvents may induce a di-pole in the dissolved molecule and increase its solubility, whilst non-polar carriers generally increase penetration (EC, 2004:5; Gaisford, 2013:372). Non-ionic surfactants exhibit relatively low dermatologic irritation potential and assist with penetration enhancement to increase the absorption of the active ingredient molecules (Zatz, 1993b:151). The pH of the formulation is important to ensure a non-ionic, or neutral molecule structure and dissociation constant (pK_a). The vehicle's pH can also alter active ingredients' aqueous solubilities, if the dissolved molecules have a relevant pK_a value (EC, 2004:5; Gaisford, 2013:368; WHO, 2006:28; Williams, 2013:694).

2.3.5.2 Transdermal penetration enhancers

Topical formulation vehicles may alter skin barrier effects by removing, or damaging the stratum corneum layer that would subsequently increase dermal penetration of the hydrophilic molecules. Penetration enhancers are included in topical formulations to single purposely increase the dermal delivery of active ingredients. Mechanisms include the modification of the

stratum corneum's integrity and inter-cellular lipid organisation changes through lipid extraction, polarity alteration, phase separation, or fluidisation (Izquierdo, 2008:174; Williams, 2013:695).

2.3.5.3 Vehicle occlusion effect

Topical formulation vehicles may increase skin hydration levels directly, or indirectly. The water content present in the water phase of a topical emulsion may directly cause an increase in the level of skin hydration, whereas the occlusion effect of the oil phase may indirectly increase skin hydration. Occlusion prevents, or improves dry skin conditions by decreasing TEWL from the stratum corneum into the atmosphere to subsequently increase skin hydration levels. Topical and transdermal flux is higher through hydrated skin than through dry tissue, resulting in an increase in dermal transport. Again, it is important to remember that active ingredient penetration is primarily dependent upon the physicochemical properties of the permeant and occlusion may not attribute to the transport effect of all molecules, as is the case with hydrophilic substances (Otto *et al.*, 2009:19; Williams, 2013:687, 694).

2.4 Stage 2: Cosmeceutical formulation development: Sample preparation

Product formulation can commence, once the literature research on the active and vehicle ingredients has been completed. Cosmetic product formulae should be kept simple and preferably no additional ingredients should be added, if not for the purpose of adding value to the formulation's stability or functional properties. Preparation methods should be clear-cut, without placing strain on the ecological system, or increasing production costs, e.g. cold-process methods should be used, instead of temperature dependant processes, where possible (Woodruff, 2010:30).

Select active and vehicle ingredients that comply with the Cosmetic ingredient review (CIR) expert panel list. Take into consideration cosmetic regulations and the permitted, or restricted ingredients lists, relevant to the applicable regulatory authority (Woodruff, 2010:25, 47).

Therefore, when designing a suitable vehicle system, contemplate the required pH (mostly ranging between 4 – 6), the solubility of the active ingredient, the required skin penetration level, vehicle functionality (e.g. moisturisation of the emulsion system with additional emollients to soften the skin, a formulation with cleanser abilities, or a sunscreen with protective, or water-resistant properties), formulation rheological modifier ingredients, an effective preservative system and if necessary, fragrance ingredients (Woodruff, 2010:56).

It is furthermore important to prepare cosmetic products that are aligned to the International conference on harmonisation (ICH) tripartite guidelines concerning Good manufacturing practice (GMP), to ensure that all formulations are safe to use and consistently manufactured according to universally accepted good quality standards (ICH-Q7A, 2000:1).

2.5 Stage 3: Cosmeceutical formulation development: Stability testing

According to the SCCS's (2012:74) guidance on the testing of cosmetic substances and their safety evaluation, the physical stability of a cosmetic formulation should be established to ensure that no changes in its physical state occur during the transport, storage, or handling of the product. Cosmetic formulations that exhibit physical state, or chemical changes when exposed to variations in temperature, humidity or UV light, may become ineffective and/or present with possible health risks. Stability tests are therefore essential to ensure that cosmetic formulations remain both safe and effective for consumer use (SCCS, 2012:74).

Stability assessments should include the evaluation of various relevant parameters, such as a formulation's physical state, pH, droplet size distribution and rheological property changes, when exposed to various accelerated storage conditions. Such stressful storage conditions are also successfully used to predict the long-term physical stability of formulations, as well as any changes in their consistency over time (Tadros, 2004:227).

2.6 Stage 4: Cosmeceutical formulation development: Clinical efficacy evaluation

Cosmetic formulations should not contain any active ingredients that would therapeutically alter the biological skin function of the consumer and must they only include cosmetically approved ingredients, used in concentrations permitted for cosmetic use. Cosmetic products should be deemed safe for use and should any possible adverse reactions at the most comprise of irritation, or sensitisation reactions. To date, no uniform, nor globally accepted position on safety and efficacy testing of cosmetic products have been determined and does each country currently regulate cosmetic product import, labelling and trading according to enforcement by each individual government's regulatory requirements. Currently, the FDA requires no pre-marketing safety testing of cosmetic products and are no regulatory laws enforced. The only prerequisite for selling cosmetic products requires that all ingredients must be utilised in accordance with the FDA's approved CIR list (Schwarcz, 2014:1).

Conversely, the EU Cosmetics Directive, No 76/768/EEC, has been replaced by Regulation No 1223/2009 (Cosmetics Regulation) and enforced since 11 July 2013, with the sole purpose of meeting more stringent safety regulation procedures with regards to cosmetic products, destined for sales in the EU. In accordance with this regulation, a PIF should be compiled and it must include all of the necessary particulars relating to the identity, quality, safety to human health and the effects claimed for the cosmetic product. Manufacturers, or cosmetic final product owners must therefore prepare, as discussed above, a CPSR, documenting that a safety assessment has been conducted prior to placing a cosmetic product on the EU market. Safety dossiers, or PIF's, should typically include (depending on the formulation type) *in vivo*

clinical trial outcomes regarding the evaluation of skin sensitisation, or irritation potential, photo-toxicity, or -sensitisation potential and if applicable, ocular-irritation.

Another reason for performing clinical trials is to substantiate label claims. The evaluation and testing of a cosmetic formulation's intended purpose is hence to validate cosmetic label claims, mainly used for advertising and marketing purposes. During recent years, there has been increased pressure on cosmetic companies to provide solid evidence in support of cosmetic formulation label claims. Statements on a topical formulation label, such as natural, organic, or healing, does not necessarily indicate that the formulation's ingredients are safe and/or effective. *In vivo* clinical safety and efficacy trials are designed to scientifically evaluate cosmetic formulations, to therefore ensure clinically tested cosmetic products that are truly safe and effective to use (Brandt *et al.*, 2011:141; Gagliardi *et al.*, 2007:45; Nohynek *et al.*, 2010:240, 252).

2.6.1 Application of Good Clinical Practice principles during cosmetic clinical trials

To investigate and quantify various skin conditions, non-invasive *in vivo* bio-engineering techniques have been developed. These techniques, if used in alignment with these ICH-GCP principles, are effectively used to assess cosmetic product safety, or to substantiate cosmetic claims in an objective and unbiased way (Beradesca, 2011:89).

Good clinical practice (GCP) is an international ethical and scientific quality standard, comprising of all clinical trial related aspects. GCP includes all of the processes involved in clinical trial design, ranging from the initial protocol design, volunteer screening and recruitment, data collection methods, statistical and data management, through to the final report compilation process. On 17 July 1996, the International Conference on Harmonisation's (ICH) first approved Topic E6, ICH Guideline for GCP, which was accepted to ensure standardised clinical trial principles and procedures, the generation of reliable results, whilst simultaneously protecting the rights, well-being and safety of trial participants (Vijayananthan & Nawawi, 2008:1).

According to current South African regulation, it is not mandatory for cosmetics efficacy laboratories to comply with ICH-GCP Guidelines to the same extent as is required for clinical trials on pharmaceutical formulations. However, evidence that cosmetic clinical trials have been performed in accordance with ICH-GCP Guidelines ensures credible results, which could be constructively used during multi-centre research and the development of future cosmeceutical formulations (Hughes-Formella, 2011:27).

ICH-GCP principles, as per Topic E6 (R1) of the ICH Guideline for GCP, are broadly described as follows (Berardesca, 2011:89; Hughes-Formella, 2011:27):

- Clinical trials should be performed in accordance with the ethical principles, as described by the Declaration of Helsinki and should only be performed when the anticipated benefits justify the risk. The rights, safety, and well-being of the trial participants should always be considered as the most important aspect of clinical trial design. It is important to understand that signed informed consent, obtained during enrolment procedures, may be withdrawn at any stage during the clinical trial.

- Investigational cosmetic products (ICPs) that are tested should meet regulation standards, be deemed safe to use and may not contain any prohibited cosmetic ingredients. An Investigator's brochure (IB) should be compiled by the product sponsor and contain an ingredient list, the application schedule and relevant literature information.

- Clinical trials should be performed in strict compliance with a protocol, pre-approved by the sponsor, the clinical research organisation (CRO) representative and the institutional review board (IRB), or independent ethics committee (IEC).

- Any adverse events should be examined and reported.

- Clinical team members should be adequately informed and qualified to perform their expected duties.

- Standard operating procedures (SOPs) should describe all actions taken with regards to the preparation and initiation, during and at the closing of clinical trials.

- The privacy and confidentiality of participant data and information must be maintained.

- ICPs should be handled and stored in accordance with the sponsor’s special handling, or storage condition instructions.

In addition, ICH-GCP E9, which relates to the statistical principles of clinical trials and ICH-GCP E3, with regards to the structure and content of clinical study report guidelines, are also essential guidelines during clinical trials. The alignment of clinical trial design to these guidelines will ensure universally accepted, accurate, validated data management, results interpretation and a credible clinical reporting structure.

2.6.2 Variables affecting *in vivo* clinical data of cosmetic products

Non-invasive measurements that utilise specialised, bio-engineering instruments, should be performed in a consistent and standardised manner. Hence, the limitation and control of possible variables, or sources of error during clinical efficacy trails, are essential in ensuring the validity of measured data being generated (Berardesca, 2011:90; Wunderlich, 2011b:70). The main sources responsible for these variations are described in the following sections.

2.6.2.1 General environmental variables

All measurements should be performed in climatically controlled conditions, at regulated ambient temperature and relative humidity (RH). Ambient temperature should be held within a range of 21 ± 1°C and the relative humidity within 50 ± 10% RH. Bio-engineering instruments are designed to detect extremely small changes that occur in the skin's condition and would measurements thereof be largely affected by undesirable ambient temperature and relative humidity changes. For example, when changes in relative humidity occur, significant variation and inconsistent skin capacitance measurements are revealed. Also, increased ambient temperatures trigger sweating, which results in unreliable and much higher TEWL values. To therefore minimise environmental response changes in skin condition, sweat gland activity, skin temperature, or micro-circulation, participants must acclimatise for at least 20 – 30 minutes, before any measurements are taken (Berardesca, 1997:129; Berardesca, 2011:91, 92, 95).

Airflow disturbances and uncontrolled air circulation should furthermore be limited. Non-GCP compliant air conditioning systems should be checked and should breathing over measurement areas be prevented. Intense light sources that are too close to the skin measurement areas alter ambient, probe and/or skin surface temperatures and should they not be used (Berardesca, 1997:129; Berardesca, 2011:91, 92, 95; Wunderlich, 2011b:70).

Seasonal variations also significantly affect skin condition. Increased cold weather conditions cause dry, flaky skin, which in turn decreases skin barrier function, alters topography image analysis data and hence results in data variations. Clinical studies should therefore be planned in order to finish them in the same season, as started (Berardesca, 2011:92).

2.6.2.2 Volunteer related variables

2.6.2.2.1 Anatomical location

Different anatomical skin areas exhibit significant variations in skin structure composition, micro-circulation, stratum corneum thickness, skin lipid concentration and connective tissue components. All related measurements should consistently be taken at the same skin location (Berardesca, 2011:91, 94). In addition to the variations among different skin locations of any

individual, other significant differences should also be considered when assessing the same anatomical site of different individuals (WHO, 2006:18, 23).

2.6.2.2.2 Circadian rhythm

Non-invasive bio-engineering measurements are influenced by the human circadian rhythm. Clinical efficacy studies that investigate stratum corneum hydration and TEWL are reported to be affected by this phenomenon. To limit this variable, measurements should be taken at a fixed time schedule for each volunteer. The actual time of day and date of each measurement should be recorded accordingly (Berardesca, 2011:95; Callaghan, 2008:325; Darlenski *et al.,* 2011:129, 135; Lodén, 2011:289; Sotoodian & Maibach, 2012:301).

2.6.2.2.3 Autonomic nervous system

Autonomic nervous system responses, including psychological stressors, cause variations in measurement values. Participants should therefore be allowed to rest emotionally and physically during the acclimatisation period and should the clinical environment also encourage such a relaxed emotional state (Callaghan & Wilhelm 2008:325; Darlenski & Fluhr, 2011:129; Lodén, 2011:289; Sotoodian & Maibach, 2012:301).

2.6.2.2.4 Age

Skin ageing is a complex biological process that involves genetic pre-disposition (intrinsic) and environmental factors (extrinsic) that have been well documented in research literature. Ageing skin exhibits various significant structural changes, which include the following (Berardesca, 2011:93, 94; Choi *et al.*, 2013:351; Darlenski *et al.*, 2011:149; WHO, 2006:18):

- A decrease in dermal thickness.
- A decrease in stratum corneum hydration.
- A decrease in sebaceous gland activity.
- A decrease in stratum corneum lipids.
- Atrophy of the capillary network that causes a reduction in the micro-circulation function of the epidermis.
- An uneven distribution of water content.
- A decrease in baseline TEWL.
- An increase in corneocyte size.

- An increase in wrinkles.
- A loss of elasticity properties, due to a decrease in dermal collagen and elastin fibres.
- A decrease in glycosaminoglycans.

It is therefore essential to record the participant's age during the clinical trial screening process and to specify pre-determined population inclusion and exclusion criteria.

2.6.2.2.5 Ethnicity

Significant physiological variations exist among the skins of different human races and should this therefore be considered during protocol design (Berardesca, 2011:93; Warrier *et al.*, 1996: 239).

2.6.2.2.6 Skin condition

Changes in skin condition, resulting from skin diseases, cause variations in measured data. As discussed under the section on Skin structure and function, a disruption of the normal skin structure would decrease healthy skin barrier function and may result in significant changes, such as increased TEWL, erythema and blood flow. Whereas low TEWL values correlate with the stratum corneum hydration levels when measured in healthy skin, this does not apply to damaged skin (Berardesca, 2011:95; Darlenski & Fluhr, 2011:129). Therefore, and since cosmetic products may not claim to cure pathological skin conditions, only healthy participants should be included in clinical efficacy studies for cosmetics.

2.6.2.2.7 Gender

Certain structural skin variations correspond to gender. Dermal thickness, for example, generally is much higher among men than in woman. Interestingly, no significant differences between men and women are found with regards to the measurement of stratum corneum hydration, or TEWL (Berardesca, 2011:93, 94; Darlenski & Fluhr, 2011:135).

To conclude from the above discussions, because of the existence of many possible volunteer related variables, the *in vivo* clinical trial protocol design should include detailed inclusion and exclusion criteria, such as gender, age and specific ethnic, or skin type volunteer groups (Berardesca, 2011:93, 94). Furthermore, it is important to strictly adhere to protocol and time scheduling procedures, to limit any possible variations that may result from individual stressors and circadian rhythm.

2.6.2.3 Removal of remaining test product from the skin prior to measurements

Applied test products should be removed before performing any measurements. To prevent variations in measurements, care must be taken not to use cleansing agents that would alter the skin surface, or the skin composition. It is important to strictly follow protocol and where test products are removed, the exact same technique should be consistently employed to all participants and with each measurement (Berardesca, 2011:93).

2.6.2.4 Instrument related factors

Measuring instruments must be maintained and calibrated routinely in accordance with SOPs and good laboratory practice (GLP) to prevent possible result variations during clinical efficacy trials. Pre-trial laboratory preparation SOPs should include calibration checks of all equipment, prior to each set of measurements (Berardesca, 2011:90; Wunderlich, 2011b:71).

The correct handling and use of all measuring equipment in accordance with SOPs and GLP are extremely important during any clinical study. Laboratory equipment should be used in accordance with manufacturer approved instructions in climate controlled conditions. Closed chamber devices would theoretically be more insensitive to ambient air flow than open chamber methods, but ambient air flow should nevertheless be limited in all circumstances (Berardesca, 2011:90, 91). Various instrument operator variables may also occur. Some instruments are sensitive to the pressure of the probe on the skin surface and can measurements be affected if the investigator does not maintain a consistent probe pressure. Skin-probe contact may further cause an occlusive effect if the skin contact time is too long. When evaluating all of the possible variables, it is understandable that the investigator should be trained in correct measurement principles, measurement value ranges and basic device design, firstly to be able to detect questionable results. Secondly, measurements should be performed in a consistent manner and consecutive measurements should preferably be performed by the same investigator to produce reproducible, validated results (Berardesca, 2011:90, 91; Khazaka, 2011:86; Lodén, 2012:289; Sotoodian & Maibach, 2012:301; Wunderlich, 2011b:72).

2.6.3 Clinical efficacy trials to investigate skin moisture levels

For many consumers, topical moisturising forms a fundamental part of their daily skin routines, or for managing skin conditions, such as dry skin, or atopic dermatitis. Skin moisturising label claims are commonly used by cosmetic product owners. For that reason, it is important to investigate the clinical efficacy and moisturising effect of a topical preparation, by measuring the stratum corneum hydration levels and/or the improvement thereof (Berardesca, 1997:126, Darlenski & Fluhr, 2011:124, 126, 127; Lodén, 2012:286).

Stratum corneum water content is measured by assessing the capacitance, resistance and impedance properties of the skin. Impedance depends upon the resistance (or ease) that an electric current experiences when flowing through the skin. An instrument, such as the Corneometer® CM 825, measures the change in the di-electric constant. Water content has an effect on the capacitance values and even sub-clinical skin hydration changes are detected with a precision capacitor. The Corneometer® CM 825 measurements are then converted into arbitrary units, representing relative stratum corneum water content (Berardesca, 1997:126; Darlenski & Fluhr, 2011:128).

In view of the fact that human skin is a complex, constantly adapting biological system, topical formulation moisturisers influence skin hydration through various mechanisms. These mechanisms involve the stratum corneum brick–and-mortar structural organisation, natural moisturising factors, sebum secretion and several other mechanisms furthermore responsible for maintaining skin barrier function. An increase in the stratum corneum water content may even result from a repaired skin barrier function, which would decrease TEWL, cause less water to evaporate from the skin surface and consequently increase the measured stratum corneum water content. Therefore, to objectively assess moisturising label claims and to guard against the misinterpretation of results, simultaneous evaluation of skin barrier function (e.g. by measuring TEWL), skin topography (e.g. through surface evaluation of the living skin (SELS) parameters) and the visco-elastic properties of the skin should also be considered (Berardesca *et al.*, 1997:131; Darlenski & Fluhr, 2011:124, 127, 140; Lodén, 2012:292).

2.6.4 Clinical efficacy trials to investigate skin visco-elastic properties

Skin mechanical properties are evaluated to investigate any dermal connective tissue changes, as revealed by ageing skin. Both the skin's ability to recover from deformation and the skin's elasticity decrease as the ageing process takes place and these mechanical property changes are assessed by using various non-invasive bio-engineering instruments (Ahn *et al.*, 2007:283; Callaghan & Wilhelm, 2008:331). Non-invasive measuring instruments apply torsion, or suction chamber methods, indentation, shear wave propagation, or skin stretching to quantify changes in the skin's mechanical properties (Darlenski *et al.,* 2011:149).

Biomechanical parameters, based upon skin deformation, have been developed. The Cutometer® (Courage & Khazaka, Cologne, Germany) is a non-invasive, suction skin elasticity meter, designed to measure the visco-elastic properties of the epidermis (upper skin layer) (Courage & Khazaka electronic GmbH, 2015b; Ahn *et al.*, 2007:283; Callaghan, 2008:331). The objective of measuring the visco-elastic properties with the Cutometer® is to support cosmeceutical claims of firmness and skin tone (Callaghan & Wilhelm, 2008:331; Courage & Khazaka electronic GmbH, 2015b). The Cutometer® MPA 580 (Courage & Khazaka Cologne, Germany) deforms the upper layer of the skin mechanically by applying a negative pressure of

400 mbar for 2 sec to pull the upper skin layer into the 2 mm probe aperture. A non-contact optical measuring system then calculates the penetration depth of the skin inside the probe (Courage & Khazaka electronic GmbH, 2015b). The Cutometer® MPA 580 software analyses the measured deformation-relaxation properties of the skin, whereafter the R-parameters are calculated. The R-parameters are used to evaluate skin firmness and elasticity. R0 represents skin distensibility, R2 is a relatively elastic parameter representing gross elasticity, R6 is reflective of the skin's stretch capacity, R7 illustrates the degree of elastic recovery, whereas R8 represents the skin's total recovery (Courage & Khazaka electronic GmbH, 2015b; Ohshima *et al.*, 2013:240).

2.6.5 Clinical efficacy trials to investigate skin barrier repair

The quantity of water that evaporates through the epidermis (stratum corneum) into the surrounding air is expressed in terms of TEWL values. TEWL provides a validated, non-invasive solution to evaluate skin barrier function and skin permeability (Callaghan, & Wilhelm 2008:325; Danby *et al.*, 2013:44; Sotoodian & Maibach, 2012:301). TEWL *in vivo* measurements are performed through open, closed, or ventilated chamber approaches (Sotoodian & Maibach, 2012:301).

TEWL values are reliable and accurate if the known method limitations are taken into consideration when performing these measurements (Farahmand *et al.*, 2009:396). Open chamber methods are susceptible to inaccurate measurements, when climatic conditions and air movement are not controlled. Closed chamber methods limit continuous measurement, because the air inside of the chamber may reach a saturated state and thus prevent further evaporation from the skin. Ventilated chamber devices conduct measurements in a closed chamber micro-environment, but differ from closed chamber devices in that the water that evaporates from the skin surface is captured into a carrier gas that passes through the chamber, which is then measured (Sotoodian & Maibach, 2012:302). The ventilated chamber allows for continuous evaporation from the skin surface and the carrier gas may cause extrapolated evaporation from the skin surface, due to the carrier gas never reaching the saturated state (Sotoodian & Maibach, 2012:301).

The Tewameter® (Courage & Khazaka, Cologne, Germany) measures water evaporation from the skin by applying the open chamber approach. A hollow cylinder contains two pairs of hygro-sensors (sensitive to temperature and humidity), which are placed on the skin. Continuous measurements are possible and computer software analyse the measured data. The TEWL evaporation rate is expressed as g/h/m^2 (Courage & Khazaka electronic GmbH, 2015f).

The closed chamber principle is demonstrated by using the Vapometer® (Delfin Technologies Ltd., Delfin, Finland). The evaporation rate is calculated according to the increase in relative

humidity in a closed off measurement area. The angle at which the probe is positioned on the skin must ensure full enclosure of the skin area. Continuous measurements are therefore not possible, but the obvious benefit is that the closed micro-environment is not directly influenced by any ambient air flow (Delfin Technologies Ltd., 2014).

Because insufficient skin barrier function will firstly influence skin permeability, the penetration of severe irritants and allergens through the epidermis would increase. Secondly, impaired skin barrier function will exhibit significantly higher TEWL values, compared to healthy skin (Callaghan & Wilhelm, 2008:325; Danby *et al.*, 2013:43, 45, 48; Sotoodian & Maibach 2012:301). Low TEWL values are therefore indicative of intact, healthy skin, capable of restricting uncontrolled water loss through evaporation *via* the stratum corneum. An intact skin barrier is responsible for sustaining skin hydration levels and for preventing exacerbation of dermatological disease conditions, such as atopic dermatitis, or dry skin (Dal'Belo *et al.*, 2006:241).

Consequently, formulations that restore skin barrier function reduce TEWL values, which would result in the stratum corneum water content (hydration) being maintained (or even increased) (Callaghan & Wilhelm, 2008:325; Darlenski & Fluhr, 2011:128; Farahmand *et al.*, 2009:392; Sotoodian & Maibach, 2012:301).

When evaluating the clinical efficacy of cosmeceutical formulations, it would be considered clinically significant to include an investigation method that would assess whether, or not the test product preserves and/or improves skin barrier function. TEWL values that are interpreted in conjunction with other clinically significant parameters, such as skin moisture, would thus assist in objectively evaluating clinical efficacy and skin barrier integrity (Darlenski & Fluhr, 2011:129; Dal'Belo *et al.*, 2006:242). When performing TEWL measurements, it is important to consider that TEWL is influenced by body temperature, specific anatomical site, autonomic nervous system (including psychological stressors), circadian rhythms and blood flow to the skin (Darlenski & Fluhr, 2011:129; Lodén, 2012:289; Sotoodian & Maibach, 2012:301).

2.6.6 Clinical efficacy trials to investigate skin surface topography

Skin surface analyses are used to evaluate skin condition and any changes thereof. Various methods have been developed through which skin surface properties can be investigated by using non-invasive, digital imaging, the analysis of skin replicas, counter-replicas of skin surface, or additionally, through the examination of the skin surface's scaliness by employing adhesive tape-stripping methods (Darlenski & Fluhr, 2011:129; Lévêque, 1999:104, 105).

The Visioscan® VC 98 (Courage & Khazaka, Cologne, Germany) instrument applies digital imaging to evaluate skin topography. The Visioscan® VC 98 emits a ultra-violet A (UVA) light

and records high resolution (256 grey pixel level) skin surface images. Light, or bright pixels that are visible on these images reveal skin scaliness, whereas dark areas depict wrinkles. Images are analysed by computer software, whereafter the skin surface condition is quantified in terms of various parameters, i.e. SELS, roughness, texture, volume and surface (Courage & Khazaka electronic GmbH, 2015e).

2.6.7 Clinical efficacy trials to investigate skin surface pH

Healthy skin has an acidic mantle, with pH values ranging between 4 – 6 (Ali & Yosipovitch, 2013:261). The acidic surface mantle is maintained by the stratum corneum and an intact skin barrier function to help prevent skin infections (Baroni *et al.,* 2012:259). Skin hydration, sweat, sebum, anatomical skin site, genetic pre-disposition, age, or topical products that have been applied to the skin will influence skin pH measurements (Baroni *et al.*, 2012:259). Flat glass electrodes are used to measure skin surface pH, but would the validity of the results and the interpretation thereof be limited, if the probe is too dry, or too wet, and not regularly calibrated (Ali & Yosipovitch, 2013:266).

Skin surface pH is altered through the pathogenesis of skin diseases, dermatitis (contact or atopic), acne vulgaris, or fungal skin infections (such as *Tinea pedis* or *Candida albicans*) that cause dry, inflamed, scaly, or flaky skin conditions. Changes in the acid mantle pH is noticeable when the skin barrier function is compromised and pH values may clinically assist with evaluating the skin barrier function (Ali & Yosipovitch, 2013:265; Baroni *et al.,* 2012:259).

2.7 Conclusion

The pre-formulation literature research study included the identification of the therapeutic purpose, the intended target area, the pharmacologic, pharmacokinetic and/or physicochemical evaluations of the constituents in rosehip seed oil, the identification of the formulation type and the physicochemical evaluation of the vehicle (excipients) as suggested by Barry (2009:596; York, 2009:5).

The physicochemical properties of rosehip seed oil indicated that it would be possible to successfully utilise it in cosmetic formulations.

The lipophilic nature of this oil would ensure its entrapment within the stratum corneum, as is typically required of cosmetic formulations, and the fatty acid component may assist with successfully delivering the cosmetic formulation's active ingredient molecules at the intended target area (Gaisford, 2013:368; Karande & Mitragotri, 2009:2363; Wiechers & Watkinson, 2008:63; Wiedersberg & Guy, 2013:150; Williams, 2013:676, 686).

With regards to the bio-active constituents found in this oil, typical limitations should be taken into consideration to prevent any possible constraints of the formulated cosmetic application. According to the literature study, the constituents that are found in the rosehip seed oil are very sensitive to degenerative reactions, such as oxidisation and photo-isomerisation. Tretinoin, for example, is extremely susceptible to photo-isomerisation, which would consequently affect the clinical efficacy of the final formulation (Brisaert *et al.,* 2000:50; Ioele *et al.*, 2005:252).

Since cosmeceutical formulations that contain rosehip seed oil claim cell regeneration capabilities, the restoration of skin elasticity, hydration, scar repair, the removal of dark spots being caused by photo-damage, and acting as an anti-ageing agent that smoothes wrinkles, their clinical efficacy should be investigated (Aroma alternatives® Ltd. Co., 2014:1; Olivier, 2013:1). To objectively investigate these label claims and to also guard against the misinterpretation of test results, simultaneous evaluation of various skin condition parameters should be assessed. Therefore, when investigating skin moisturising claims, apart from the apparent water content evaluations, skin barrier function, skin topography and the visco-elastic properties of the treated skin should also be assessed (Berardesca *et al.*, 1997:131; Darlenski & Fluhr, 2011:124, 127, 140; Lodén, 2012:292).

Following an evaluation of the literature research findings concerning rosehip seed oil, the potential benefits of utilising this oil in cosmetic formulations seemed obvious. Commercially available oil that is sold to cosmeceutical manufacturers is not yet standardised. Reliable information with regards to commercially acquired rosehip seed oil is also scarce. Additionally, arguable variations were found in various accredited and peer reviewed literature sources regarding issues, such as the physicochemical and bio-active properties, and the constituents of this oil. This study was therefore conducted to further investigate the properties and possible advantages of this naturally derived ingredient for possible use in topical formulations.

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Chapter 3

Preparation of a cosmeceutical formulation containing Rosa rubiginosa (rosehip) seed oil

3.1 Introduction

All conventional skin care products that are externally applied, were traditionally categorised as cosmetic products. However, such formulations could be further defined, in accordance with the Federal Food, Drug and Cosmetic Act 1 (FD&C Act, sec. 201(i)), in terms of their intended use to cleanse, to beautify, or to promote attractiveness. This classification would therefore include any products that are used for their perceived ability to change the appearance of human skin. Modern cosmetic products, called cosmeceutical formulations, contain bio-active ingredients that influence the biological function of the skin, such as correcting non-pathological variations of normal skin, but with insufficient therapeutic action to avoid the risk of being classified as pharmaceuticals (Brandt *et al.*, 2011:141; Nohynek *et al.*, 2010:251).

Cosmeceuticals may not claim to cure, nor treat pathological skin conditions, but their label claims must focus on functional skin repair properties, such as anti-ageing, anti-wrinkling, or the improvement of skin hydration (Nohynek *et al.*, 2010:251). During this study, a cosmeceutical oil-in-water (o/w) emulsion, within a stabilising gel structure, were formulated and prepared.

3.2 Formulation development process of topical cosmeceutical products

3.2.1 Pre-formulation studies

Pre-formulation research forms a fundamental part of cosmetic formulation design. The pre-formulation process should include (Barry, 2009:596; York, 2009:5):

- The identification of the intended purpose of the planned formulation.
- The identification of the preferred target site, or deposition area.
- An assessment of the pharmacological, pharmacokinetic and physicochemical properties of the bio-active ingredients.
- The identification of the formulation type (delivery vehicle, or dosage form).
- An evaluation of the properties of the delivery vehicle ingredients.

Pre-formulation studies should precede large scale formulation production, to ensure that all formulation batches would be chemically and physically stable, hence exhibiting unvarying characteristics for each manufactured trial batch (Barry, 2009:596; York, 2009:5).

3.2.1.1 Identification of the intended purpose of the planned formulation

This process includes the identification of the therapeutic aims and goals that must be achieved when developing a new cosmeceutical formulation. Formulators should take into account the anticipated clinical action, or properties that the manufacturer desires, such as anti-ageing, the reduction, or softening of wrinkles, or dark spots. During this process, formulators must apply their knowledge of transdermal transport, formulation design and the complexity of skin science, to ensure clinically effective product innovation (Abbott, 2012:217; Barry, 2009:596; Guesnet *et al.*, 1994:65; Mitsui, 1997:321).

3.2.1.2 Identification of the formulation type

Multi-phase formulation systems, such as emulsions, are frequently used in cosmetic formulations (Barry, 2009:596). Emulsions are multi-phasic formulation systems that are defined as liquid droplets and/or fluid crystals, dispersed in a liquid phase, according to the International Union of Pure and Applied Chemistry (IUPAC). The dispersed phase, also known as the internal phase, is evenly dispersed within the external, or continuous phase. An o/w emulsion is formed when the internal lipophilic phase is dispersed within an external hydrophilic aqueous phase, whereas the opposite occurs when forming a water in oil (w/o) emulsion, i.e. the internal hydrophilic aqueous phase is dispersed into the external lipophilic phase (Attwood, 2013:87).

During topical cosmetic formulation design, it is inadequate to merely consider the properties of the bio-active ingredients, but must the properties of the ingredients in the delivery vehicle and their influence on the skin also be assessed (Barry, 2009:597; Guesnet *et al.*, 1994:65; Mitsui, 1997:321; Otto *et al.*, 2009:1). Alternatively, a cosmetic formulation vehicle may also be incorporated to perform an additional function, or to have a specific effect on the skin, such as cleansing, decoration, care, hydration, or protection (Buchmann, 2001:146).

The type of emulsion mainly determines the skin-feel properties, or the perceived effect on the skin, following topical application. W/o emulsions have an occlusive tendency and greasy feel, whereas o/w emulsion type formulations are characterised by a light feel, good spreadability, additional skin hydration potential and a cooling effect, resulting from the evaporation of the externally applied water phase. O/w emulsion vehicle systems show a higher consumer acceptance and are they widely used in the cosmetic industry (Buchmann, 2001:152; Izquierdo, 2008:173; Otto *et al.*, 2009:1). Hence, an o/w emulsion, dispersed within a stabilising gel

structure, was chosen as the preferred vehicle system for use in the planned rosehip seed oil formulation.

3.2.2 Early formulation

To prepare a mathematical model that would predict the characteristics of an optimised cosmetic formulation, is difficult when formulating with natural ingredients, because not all of the required data for such models are known. As a result, the purpose of cosmetic pre-formulation studies may not necessarily be to conclude an exact formula for a natural product, but to limit the collective sum of trials during the development process (Guesnet *et al.*, 1994:65; Mitsui, 1997:321). Formulation development during this particular study included the use of the hydrophilic-lipophilic balance (HLB) system, to assist in the development of a stable o/w emulsion formulation and to determine the optimal surfactant composition (Otto *et al.*, 2009:5).

For the purpose of this study, it was decided to use a cold process system, in which no methods, nor ingredients were used that would require the application any heat to prepare the final product. After formulating and evaluating several trial o/w emulsions, one base formulation was chosen, based upon the satisfactory outcomes of its texture, visual appearance and spreadability properties.

3.2.3 Final formulation

For the purpose of this study, two o/w emulsions (hereinafter referred to as emulsion(s)), each containing 20% (w/w) of *Rosa rubiginosa* (rosehip) seed oil, were prepared. The one emulsion comprised of rosehip seed oil, spiked with tretinoin, and was it subjected to accelerated storage conditions and stability testing over a period of 6 months (discussed in Chapter 4). The second emulsion was exactly the same as the first, but without any tretinoin. This formulation was used to perform clinical efficacy studies on a cosmeceutical formulation that contains rosehip seed oil (discussed in Chapter 6 and Appendix C).

3.3 Formulation of an o/w emulsion

3.3.1 Characteristics and benefits of an o/w emulsion

An emulsion has the capability of solubilising both hydrophilic and lipophilic substances, which, together with it being a versatile cosmetic formulation vehicle type, contributes towards emulsions being commonly used in the cosmetic formulation industry (Buchmann, 2001:149). Also, because o/w emulsion formulations exhibit such good skin spreadability and light feel properties when applied topically, altogether contributed towards the decision for employing this vehicle system during this study (Buchmann, 2001:152; Izquierdo, 2008:173; Otto *et al.,* 2009:1).

3.3.2 Main ingredients of an o/w emulsion

Basic cosmetic emulsion formulations comprise of a water phase and an aliphatic hydrocarbon, or lipophilic phase, stabilised by surfactant molecules. Additional components are incorporated to preserve, or characterise the cosmetic formulation function, such as emollients, humectants, preservatives and anti-oxidants. All ingredients should be non-toxic, non-irritant and non-sensitising when used in the concentrations permitted for cosmetic formulations (CIR, 2010:4; Izquierdo, 2008:172).

It is not only the biological activities of the active ingredient that are responsible for achieving the planned cosmetic effect, but also the ingredients of the delivery vehicle being utilised. Cosmetic formulations contain multi-functional vehicle ingredients, like emulsifiers, for example, for the purpose of providing additional skin moisturising benefits, or to increase the viscosity of the formulation, or for functioning as penetration enhancers. Although typical cosmetic emulsion ingredients are described separately in the sections below, vehicle ingredients may be purposely incorporated for performing more than one function (Buchmann, 2001:146).

3.3.2.1 Emulsifiers

Two immiscible liquids that are mixed together will form a thermo-dynamically unstable, temporary emulsion. The large increase in surface area of the dispersed phase droplets results in increased inter-facial free energy of the emulsion system. Droplet coalescence decreases the surface free energy that results in phase separation. To prevent coalescence of the dispersed droplets, inter-facial surface-active ingredients, known as emulsifiers, are incorporated. Emulsifiers, also called surfactants (neutral, cationic, or anionic), decrease the inter-facial tension between the immiscible internal and external emulsion phases to form an inter-facial film. This film acts as a mechanical, or electro-static barrier that decreases, or prevents droplet coalescence. Electro-static barrier function increases by introducing electro-static or steric repulsive forces, thereby increasing droplet-droplet repulsion by counteracting Van der Waal's forces of attraction. Emulsifiers are essential components of a cosmetic emulsion, serving to stabilise and prevent phase separation, due to dispersed phase droplet coalescence. The type of emulsion formed and its classification depend upon the relative quantities of lipophilic, or hydrophilic phases and the type of emulsifiers used (Attwood, 2013:87; Eccleston, 2013:442; Izquierdo, 2008:172; Otto *et al.*, 2009:5).

To increase the stability of a formulation, emulsifier blends, or multiple emulsifiers are incorporated into the immiscible oil and water phases. Various types of surfactants are chosen, depending upon the surfactant characteristics and the preferred emulsion type. O/w emulsions favour the use of hydrophilic polar emulsifier agents, whilst w/o emulsions favour non-polar emulsifiers to form the inter-facial barrier. Droplet charges, as provided by the emulsifier inter-

facial film barrier, influence emulsion dispersion state stability. Non-ionic surfactants are less toxic and less sensitive to pH and electrolyte variations than ionic surfactants (Attwood, 2013:88; Buchmann, 2001:152; Otto *et al.*, 2009:6).

For the purpose of this study, two emulsifier components, both suitable for use in cold process emulsion preparation methods, were chosen. Lipophilic Labrafac™ WL 1349 (Gattefossé, Saint-Priest, France), consisting of medium chain fatty acid triglycerides (HLB = 1), was dispersed in the oil phase (Gattefossé.com, 2010). Tween® 80 (Sigma Aldrich), a non-ionic polyethylene sorbitol ester, containing natural fatty acids (oleic acid), was utilised as a hydrophilic surfactant and dispersed in the water phase (HLB = 15) (Sigma-Aldrich.com, 2014).

3.3.2.2 Emollients

In cosmetic formulations, emollients are mostly used to soften and protect the skin from dryness, to improve the sensory feel and to assist with the spreading of the emulsion, following topical application (Buchmann, 2001:152; Otto *et al.*, 2009:9). Emollients have additional formulation applications, where an appropriate emollient combination system can be successfully utilised, if required to develop optimised delivery formulations, firstly, by accommodating active ingredient solubility in the formulation and secondly, by adjusting formulation solubility to increase the partitioning coefficient value (Otto *et al.*, 2009:9).

3.3.2.3 Humectants

Humectants are included in cosmetic formulations, with the functional clinical objective of increasing the hydration state of skin, or additionally to reduce the evaporation of water from the emulsion during storage (Buchmann, 2001:152; Eccleston, 2013:442). No ingredient, other than the rosehip seed oil that was utilised as the oil phase component in the o/w emulsion, was incorporated for the purpose of increasing skin moisture levels.

3.3.2.4 Viscosity increasing agents

Emulsifiers that do not primarily participate in the formation of an inter-facial barrier film, but that are incorporated for the purpose of increasing the viscosity of the continuous external phase, to thus prevent the movement of the dispersed droplets, are called co-emulsifiers, or viscosity enhancers. The chosen viscosity enhancer that was employed during this study to increase the viscosity of the external (hydrophilic) phase in the o/w emulsion formulation to the required levels (Buchmann, 2001:152; Eccleston, 2013:442), was an anionic alkyl acrylate cross-polymer thickening agent, Carbopol® Ultrez 21 polymer (Lubrizol). The formulations were then neutralised to pH 6 - 7 to thicken and increase their viscosities. Carbopol® Ultrez 21 polymers swell when neutralised and hydrated, and do the swollen polymers cross-link to create a tightly

packed, gel dispersion medium (Lubrizol Advanced Materials, Inc., 2002:3; Vintiloiu & Leroux, 2008:187).

3.3.2.5 Preservatives

The presence of water in the o/w emulsion's continuous phase provides ideal conditions for the growth of microbial organisms. Preservatives are routinely added, anti-microbial chemicals that prevent bacterial, or fungal micro-organisms from growing in cosmetic formulations. Microbial contamination causes organo-leptic alterations in formulations that may cause changes in their viscosity, colour and/or texture, and may such affected products also be harmful to consumers, if used. W/o emulsions are less susceptible to micro-organism growth, due to the dispersed water droplets being protected by the surrounding continuous oil phase (Buchmann, 2001:152; Eccleston, 2013:441). Parabens are a group of *p*-hydroxybenzoic acid (PHBA) esters that are either used in combinations (max. 0.8%), or as a single paraben (max. 0.4%), to serve as preservatives in cosmetic formulations. Various conflicting and scrutinising reports are available regarding the use of parabens, but according to a report, reviewed by the Cosmetic Ingredient Review Expert Panel, parabens are both non-irritating and non-sensitising, when used in permitted concentrations in cosmetic formulations and are they therefore regarded as safe for human use (CIR, 2008:1). Methylparaben and propylparaben were utilised as the two preservatives in this study.

3.3.2.6 Anti-oxidants

Anti-oxidants are useful in cosmetic formulations for protecting the product (not the skin) and the formulation ingredients against oxidative deterioration, due to possible chemical reactions with oxygen. Butylated hydroxytoluene (BHT) is an example of a chain breaking anti-oxidant that donates a hydrogen atom to a lipid radical to form a stable, anti-oxidant free radical, incapable of continuing the oxidation process. BHT is effective at low concentrations and is frequently used in cosmetic formulations in the permitted concentrations, ranging from 0.0002% to a maximum of 0.5000% (Eccleston, 2013:442; CIR, 2002:19, 20).

3.3.2.7 Perfumes and colouring agents

Certain additives are used purely to enhance the aesthetic appeal of cosmetic formulations, by improving the product fragrance, or colour (Buchmann, 2001:152). Since not all colour and fragrance ingredients are inert, or because they may cause skin sensitising and skin irritation reactions, no colorant, nor fragrant ingredients were utilised during this study.

3.3.2.8 Active ingredients or bio-active substances

Cosmeceutical formulations are similar to conventional, or traditional cosmetic formulations, except that bio-active ingredients are added to cosmeceuticals to deliver a specifically intended biological function (Brandt *et al*., 2011:141; Nohynek *et al.*, 2010:251). The clinical efficacy of o/w emulsions that contain commercially acquired rosehip seed oil, were investigated during this study (see Chapter 6 and Appendix C), without adding any additional bio-active substances.

3.3.3 Hydrophilic-lipophilic balance system

Because of the hydrophilic and lipophilic structures of surfactants, non-ionic surfactants contain a hydrophilic-lipophilic balance (HLB) value between 0 and 20. Higher HLB values (HLB > 10) suggest increased hydrophilic properties, whereas lower values (HLB < 10) indicate a more lipophilic character. The HLB system determines the suitable surfactant proportions that should be used in emulsion formulations (Uniqema Ltd., 2005:1).

According to the HLB system, the oil components that are used in the emulsion's oil phase possess a required HLB (rHLB) value, which is used to select an appropriate surfactant combination. Vegetable oils have an rHLB value, ranging between 6 and 8 and does rosehip seed oil have an rHLB value of 7. Since no other oil constituent was added to the oil phase of the o/w emulsion, prepared in this study, to emulsify rosehip seed oil, a surfactant blend, containing an HLB value of ± 7 would suffice. The HLB value of a surfactant blend varies according to the relative proportion of each surfactant added and is the HLB system applied to determine the quantities of each surfactant needed to formulate a stable dispersion system (Meher *et al.*, 2013:1540; Sevcíková *et al.*, 2010:131; Sigma-Aldrich, 2014).

3.4 Formulation of a cosmeceutical o/w emulsion containing rosehip seed oil

3.4.1 Ingredients used in the o/w emulsion

A semi-solid o/w emulsion, containing 20% of rosehip seed oil (w/w) was prepared, using the constituents as per Table 3.1 and Table 3.2.

Table 3.1: Ingredients being used to prepare the o/w emulsion during this study

Ingredient	Supplier	Batch nr
Rosa rubiginosa (rosehip) seed oil	The Rosehip Company	N/A
Labrafac™ WL 1349	Gattefossé	000132642
Tween® 80	Merck (Pty) Ltd	001042689
Carbopol® Ultrez 21	Lubrizol	101014179
Butylated hydroxytoluene (BHT)	Merck (Pty) Ltd	K42115274
Methylparaben	Merck (Pty) Ltd	K42067957
Propylparaben	Merck (Pty) Ltd	K41917527
De-ionised water	MilliQ® water filter system with Millipak® 40 Millipore (0.22 µm)	

3.4.2 Formulation used to prepare the o/w emulsion

Table 3.2: Formulation used to prepare a 20% rosehip seed oil o/w emulsion (v/v)

Ingredient	Percentage	Function
Dispersed oil phase (A)		
Rosa rubiginosa (rosehip) seed oil	20.00	Oil (bio-active component)
Labrafac™ WL 1349	6.15	Emulsifier
Butylated hydroxytoluene (BHT)	0.50	Anti-oxidant
Propylparaben	0.04	Preservative
Continuous water phase (B)		
Tween® 80	3.85	Surfactant
Carbopol® Ultrez 21	0.60	Thickening agent
Methylparaben	0.20	Preservative
De-ionised water	68.66	Solvent

3.4.3 Preparation method

3.4.3.1 Rosehip seed oil preparation

As discussed earlier, tretinoin spiked rosehip seed oil was utilised to prepare the o/w emulsion that would be subjected to accelerated stability testing (Chapter 4), whilst a second o/w emulsion formulation was prepared, by using rosehip seed oil without added tretinoin, to evaluate the clinical efficacy of a rosehip seed oil formulation (Appendix C).

Figure 3.1: a) Rosehip seed oil solution with added tretinoin stirred on a magnetic stirrer plate for 24 h; b) rosehip seed oil and tretinoin solution ready for centrifugation for 20 min; c) two test tubes illustrating the differences in clarity of the rosehip seed oil and tretinoin solution before and after centrifugation; d) rosehip seed oil and tretinoin solution being filtered to remove excess insoluble tretinoin particles.

Tretinoin was added to commercially acquired rosehip seed oil, because no traceable quantities of tretinoin had been detected during analysis with the validated HPLC method, as described in Appendix A. It was hypothesised that tretinoin might have been present at the time of extraction, but that it had possibly degraded to undetectable levels, due to its heat and light lability character (Tashtoush *et al.*, 2007:859). To determine the rate of tretinoin breakdown in a natural carrier oil, also known to contain tretinoin, the rosehip seed oil was spiked with tretinoin (BASF, Ludwigshafen, Germany) (Concha *et al.*, 2006:772). The process of dissolving tretinoin in the rosehip seed oil is illustrated below in Figure 3.1.

It was found that tretinoin had not readily dissolved in rosehip seed oil and was approximately 500 ml of the oil (The Rosehip Company, Mohale's Hoek, Lesotho) solution, containing 5 g of tretinoin (BASF, Ludwigshafen, Germany), therefore stirred for 24 h, using a Labcon® MSH10 magnetic stirrer plate. No external heat was applied during stirring (see Figure 3.1.a).

After 24 h, the rosehip seed oil-tretinoin solution was placed in an Eppendorf® 5804R centrifuge, set at 1,100 relative centrifugal force (RCF) at 25°C for 20 min (see Figure 3.1.b). The centrifuged solution was filtered using Whatman® glass micro-fiber binder free filters (0.7 µm particle size retention in liquid) to remove any excess insoluble tretinoin particles (see Figure 3.1.d). The filtered solution was analysed and a tretinoin assay performed, using the HPLC validated method, as described in Appendix A.

3.4.3.2 Rosehip seed oil o/w emulsion preparation

Both Mixtures A (dispersed oil phase) and B (continuous water phase) were prepared by weighing all ingredients as per Table 3.2 and were they employed, as diagrammatically summarised in Figure 3.2. Mixtures A and B were placed separately on Labcon® MSH10 magnetic stirrer plates for 10 min to ensure the proper wetting and mixing of all ingredients. Since a cold process method was followed, no external heat was applied during the emulsion preparation process.

Following the wetting and dissolving of all ingredients, Mixtures A and B were individually homogenised at 13,500 rpm for 5 min, utilising a Heidolph Diax 600 homogeniser (Labotec, Halfway House, SA). Mixture B was kept in the homogeniser, while Mixture A was added very slowly to B. Following the complete addition of Mixture A to B, the final emulsion was obtained after homogenising it for another 10 min at 13,500 rpm.

The formulation's pH was adjusted to 7.4, using 2 M NaOH (8% m/v) for the o/w emulsions used during the transdermal diffusion studies and for those batches that were subjected to accelerated storage conditions (Chapters 4 and 5). The pH was adjusted to approximately 5.5 for those o/w emulsions that were prepared for use during the clinical efficacy testing (Chapter 6

and Appendix C). During adjustment of the pH, the mixtures were continuously stirred, using a homogeniser at 8,000 rpm.

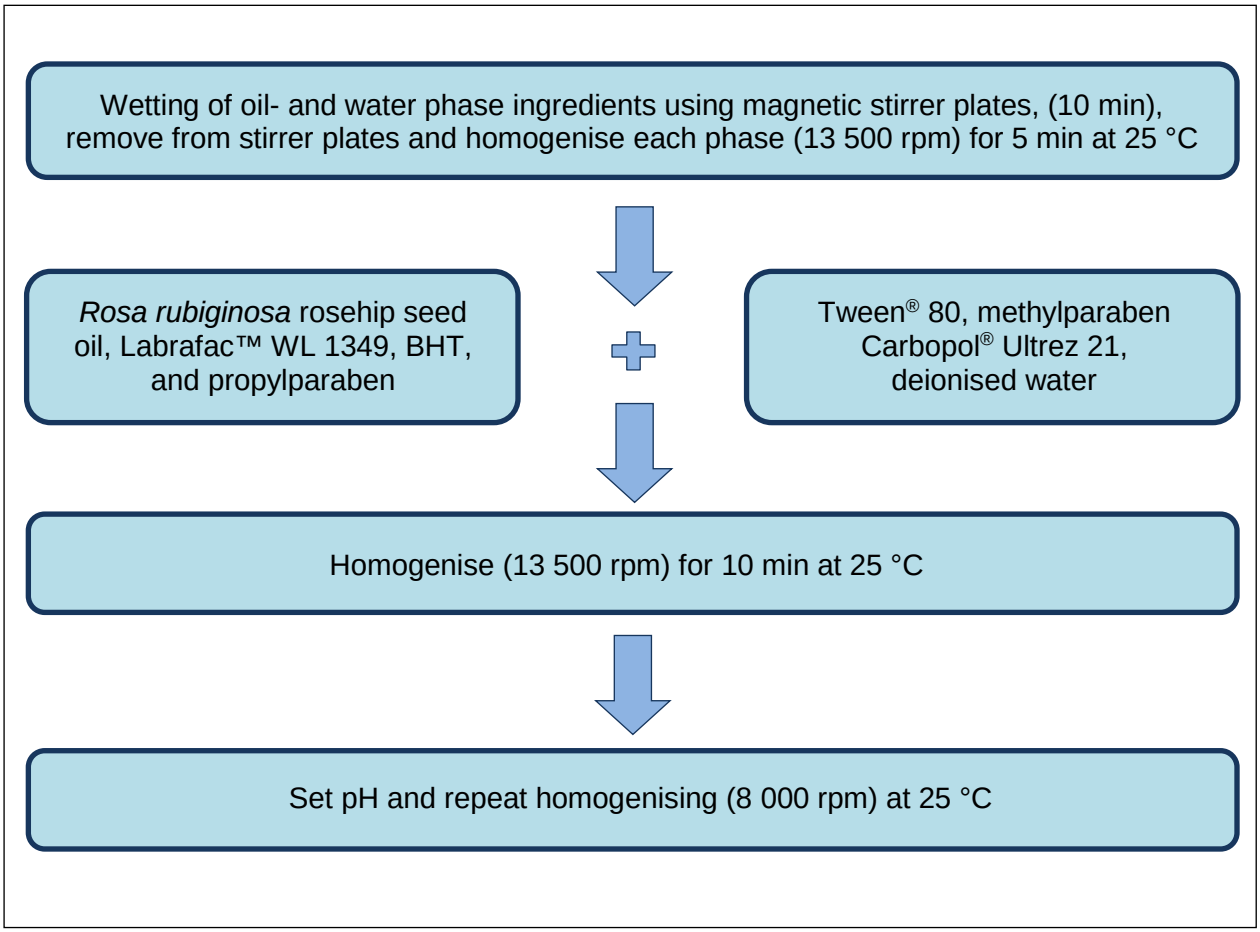

Figure 3.2: Diagrammatic outline of the preparation process of the o/w emulsion, containing rosehip seed oil.

3.4.4 Outcome

The prepared o/w emulsion had a silky feel when applied to the skin, and a smooth and uniform texture, with a light yellow colour and no distinct smell.

3.5 Conclusion

This chapter described the preparation of the two final o/w emulsions during this study, i.e. the rosehip seed oil formulation, spiked with tretinoin, and another without.

Adequate amounts of these formulations were prepared for use in the stability testing and for performing the diffusion studies (rosehip seed oil, spiked with tretinoin), as described in Chapter 4 and 5. Several new batches of rosehip seed oil (without added tretinoin) were prepared for clinical efficacy testing, as described in Chapter 6 and Appendix C.

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Chapter 4

Stability testing of the new cosmeceutical formulation containing *Rosa rubiginosa* (rosehip) seed oil

4.1 Introduction

The stability testing of cosmetic formulations is a broad concept and could be applied to cover an extensive range of stability issues. Stability testing should involve all of the applicable ingredients, including the initial raw materials used during the formulation and/or manufacturing processes and those present in the final product. Stability testing procedures also represent the transport and storage conditions to which final products will be subjected following manufacturing, i.e. during distribution, storage and finally, when bought and used by the consumer. During the manufacturing process, accelerated stability testing is performed to predict possible changes that may affect the final product's appearance and efficacy. The maintenance of the physical and chemical characteristics of all raw materials and final products, their functionalities and aesthetics, when stored at different temperatures and relative humidity (RH) conditions, should therefore be assessed. The information obtained through stability testing is further utilised to determine the optimal storage conditions of a formulation and to establish a final product's shelf-life (Barry, 2009:595; York, 2009:5).

Emulsions are important delivery systems that are commonly used in the cosmetic, pharmaceutical and food industries. Emulsions are typically bi-phasic dispersion systems, containing a water and an oil phase to accommodate carrier properties of both lipophilic and hydrophilic active ingredients. These two immiscible phases generally are thermo-dynamically unstable (Lerche & Sobisch, 2011:1800; Traynor *et al.*, 2013:2173).

Formulators purposely manipulate the state of dispersion and the stability thereof, by using emulsifiers and surfactants to lower the droplet interface tension, or to utilise ingredients, such as viscosity enhancers, to physically stabilise such a dispersion system. Emulsions, with the ability to resist formulation phase changes over time, are deemed stable when the state of dispersion remains uniform throughout the entire formulation. No creaming, sedimentation, coalescence, flocculation, agglomeration, Ostwald ripening, nor phase inversion should hence alter the state of dispersion over a pre-determined period of time. The characterisation of the emulsion's dispersion state and the changes thereof are therefore directly indicative of the stability of such a formulation (Lerche & Sobisch, 2011:1800; Traynor *et al.*, 2013:2173).

According to the International Conference on Harmonisation (ICH) Guidelines Q1A(R2), significant physical and/or chemical formulation changes should be assessed during **long-term** (25 ± 2°C/60 ± 5% RH), **intermediate** (30 ± 2°C/60 ± 5% RH) and **accelerated** (40 ± 2°C/75 ± 5% RH) storage conditions. Significant change, according to the ICH Guideline, is defined as a 5% change from baseline value, and/or additional failure to meet pre-determined acceptance criteria, such as changes in appearance (i.e. colour changes) and physical attributes (i.e. phase separation), unacceptable pH changes, or degradation product levels that exceed the pre-determined acceptance values (ICH, 2003:3, 9).

For the purpose of this study, approximately 2,000 ml of an o/w emulsion, containing 20% of rosehip seed oil (w/w) that had been spiked with tretinoin (BASF, Ludwigshafen, Germany), was prepared, according to the method as described in Chapter 3.4. This batch was packaged in suitable, smaller containers, for the purpose of testing its stability over a period of 6 months at the three storage conditions. The bulk emulsion was evenly mixed each time before filling the next 50ml glass container, having tight closing screw caps, containing polyvinylidene chloride (PVDC) liners that are impermeable to water. The aim of this study was to evaluate the stability of the prepared formulation, containing 20% of rosehip seed oil (w/w), over a period of 6 months. Samples were subjected to accelerated, intermediate and long-term storage conditions, and was stability testing performed at four time intervals, i.e. at T0 (initial value immediately after preparation), T1 (after 1 month), T2 (after 2 months), T3 (after 3 months) and T4 (after 6 months).

The following assessments, as discussed in the sections below, were performed:

- Assays of active ingredients and excipients.
- pH.
- Viscosity properties.
- Conductivity.
- Dispersion phase particle size distribution.
- Creaming index.
- Microscopic particle size examination.
- Visual appearance: macroscopic examination.

4.2 Materials and methods

4.2.1 Assays of active ingredient and excipients

The validated single HPLC method, as described in Appendix B, was employed to analyse the concentrations of the active ingredient (tretinoin) and of the excipients (methylparaben, propylparaben and BHT) in the formulation vehicle. These four ingredients' concentrations were quantified and were the mean percentages, relative to the initial assay results of each component, assessed.

Approximately 1 g of each rosehip seed oil o/w emulsion test sample, representative of each storage condition, was weighed in triplicate and transferred into a 50 ml volumetric flask each. Each volumetric flask was filled to volume with tetrahydrofuran (THF), whereafter each dissolved sample was filtered and transferred into an amber HPLC auto-sampler vial and analysed.

4.2.2 pH

A Mettler Toledo SevenMulti™ pH/conductivity meter (Schwerzenbach, Switzerland), equipped with a glass Mettler Toledo InLab® 410 electrode (Schwerzenbach, Switzerland) was used to measure the pH of each test sample. Before a set of pH measurements was performed, electrode check calibration was conducted, using Mettler Toledo pH buffer solutions at pH 4.01, 7.00 and 10.01. Baseline (T0) pH values were determined directly after preparation of the formulations. All pH measurements were performed in triplicate for each test sample at all test intervals.

Figure 4.1: Mettler Toledo SevenMulti™ pH/conductivity meter.

4.2.3 Viscosity

The viscosity, or the formulation's resistance to flow, was measured by using a Brookfield DV2T Digital Viscometer (Stoughton, Massachusetts, USA) at pre-set shear rates (Brookfield Engineering, 2014). Approximately 15 ml of each formulation sample was transferred into a cylindrical small sample chamber. This sample chamber, enclosed in a sample adapter and flow jacket and connected to a water bath system, was set at 25°C. A cylindrical type spindle, SC4-25, was inserted into each sample for measurement.

Figure 4.2: Brookfield DV2T Digital viscometer and water bath system.

After 1 h, the spindle speed was set to a rotation speed of 2.0 rpm (rotation per minute). Viscosity readings, measured in centipoise (cP), were recorded at room temperature (25°C). Multi-point data collection was set at an interval of every 10 sec for a total time of 5 min. An average of 32 viscosity measurements was hence taken per sample. The percentage torque was recorded at approximately 50%.

4.2.4 Conductivity

Conductivity is the ability of a solution to conduct the flow of charged ions (electricity) and are the outcomes indicative of the ease with which a medium allows an electrical current to flow. Conductivity measurements are used to assess a formulation's character and would maintained values throughout the duration of a stability study be indicative of a formulation's stability. Conductivity is associated with the presence of charged ions. Lower conductivity values are recorded for w/o emulsions than for o/w emulsions, since water, being the continuous phase, conducts electricity significantly better than when water droplets are being dispersed within an oil phase (Ferreira *et al.,* 2010:1385; Lamba *et al.*, 2015:719).

Conductivity, measured in Siemens per centimeter (S/cm) was performed, using a Mettler Toledo SevenMulti™ pH/conductivity meter, equipped with a glass Mettler Toledo InLab® 731 electrode (Schwerzenbach, Switzerland). Electrode check calibration was performed at the start of each new test interval, using Mettler Toledo 1413 µS/cm and 12.88 mS/cm conductivity standard solutions. The electrode was rinsed with de-ionised water and carefully dabbed dry, prior to each subsequent measurement. All measurements were performed in triplicate.

Figure 4.3: Glass Mettler Toledo InLab® 731 electrode.

4.2.5 Particle size distribution of dispersion phase

Important differences exist between different emulsion systems. Emulsion classification, when merely identified according to droplet diameter size, may be misleading. It is therefore necessary to provide more details that would further distinguish between emulsions, micro-emulsions and nano-emulsions. Generally, emulsion dispersed droplet diameters range between 0.5 and 10.0 µm. Uncertainty may arise, since droplet sizes that range between 10.0 and 150.0 nm are regarded as micro-emulsions, whilst droplet sizes smaller than 250.0 nm and hence bigger than those of micro-emulsions, are grouped as nano-emulsions (Buchmann, 2001:156; Chena *et al.,* 2006:52; Izquierdo, 2008:173; Lopes, 2014:53). Furthermore, micro-emulsions and emulsions are dissimilar disperse systems. Apart from the obvious smaller droplet particle sizes, are micro-emulsions transparent, thermo-dynamically stable fluid disperse systems, opposed to the milky, thermo-dynamically unstable fluid, or semi-solid emulsion system. Micro-emulsions require larger surfactant concentrations than emulsions and will they spontaneously form with very little, or no additional energy input. Nano-emulsions differ from micro-emulsions with regards to their larger dispersed droplet sizes, less surfactant being utilised, whilst higher energy input is required to create nano-emulsions. Nano-emulsions are thermo-dynamically unstable, whereas micro-emulsions, with their smaller dispersion phase droplet sizes, than both emulsions and nano-emulsions, show the best stability properties (Lopes, 2014:53, 54; Schneider, 2008:469).

Emulsion instability, or breakdown can be revealed though the identification of changes in the dispersed droplet particle diameter sizes, measured over time. Various emulsion breakdown processes (as a result of flocculation, Ostwald ripening, or coalescence) are revealed through observed changes in the particle size distribution of an emulsion. Observed changes in

dispersed droplet mean diameters, such as an increased droplet size, suggest droplet growth, therefore signifying a loss of emulsion stability. Changes in droplet size can potentially alter the viscosity of a formulation, with bigger droplets obstructing formulation particle movement (Tadros, 2004:228, 248; Traynor *et al.*, 2013:2173). Particle size distribution and the changes thereof were hence investigated during this study.

For the purpose of this study, the Malvern Mastersizer 2000 (Malvern Instruments Ltd., Worcestershire, United Kingdom), equipped with a wet cell disperse unit, Hydro 2000 MU (laser diffraction system) that measures particle size distributions over a range of 0.020 to 2,000 µm, was used. Sample material refractive indexes (RI) of cod liver oil (1.481) and dispersant (water) (1.330), with enhanced sensitivity measurements, a beam length of 2.35 m and a stirring speed of 2,000 rpm were programmed for all assessments done. Particle size distribution results were evaluated from the Malvern Mastersizer 2000 MAL1007548 Ver. 5.60 computerised reports.

Figure 4.4: The Malvern Mastersizer 2000 with Hydro 2000 MU.

Approximately 0.05 ml of each formulation sample was mixed with 5 ml of de-ionised water. Each mixed solution was further diluted by adding drops thereof into a laboratory beaker, containing approximately 500 ml of de-ionised water. Measurements were performed once obscuration values of approximately 10% were reached.

The changes over time of the dispersion median particle size volume distribution diameters (D(0.5)), were recorded. D(0.5) is described as the average particle size, or median diameter, where 50% of the particles are found larger than and 50% of the particles sizes are smaller than D(0.5). Mean D(0.5) baseline values, measured directly after emulsion preparation, were compared to mean D(0.5) values that were obtained from each subsequent measurement. Particle size distribution measurements were performed in triplicate for each sample.

4.2.6 Creaming index

Phase separation occurs in de-stabilised emulsions. Driven by the earth's gravity forces, phase separation may lead to sedimentation, flotation, or creaming of the dispersed particles. Creaming may occur more frequently than sedimentation, due to the dispersed oil phase densities generally being much lower than those of the continuous aqueous phase. Emulsion phase separation can be accelerated by adding centrifugal stress, thus increasing the applied physical forces at a specified time and speed. To determine the possibility of de-stabilisation, or emulsion breakage of the prepared formulations during this study, the creaming index (CI) was investigated (Lerche & Sobisch, 2011:1801; Tadros, 2004:228).

Approximately 10 ml of each formulation sample was transferred into screw cap glass test tubes that were placed in an Eppendorf® 5804R centrifuge (Eppendorf AG, Hamburg, Germany) and centrifuged at 1,100 RCF for 30 min at 25°C. The heights of the emulsion and creaming layers were measured and the CI calculated, using Equation 4.1 (Ferreira *et al.*, 2010:1384; Korać *et al.,* 2014:270; Ushikubo & Cunha, 2014:147).

CI = (height of creaming layer / total height of emulsion) x 100% **Equation 4.1**

Figure 4.5: Eppendorf® 5804R centrifuge.

4.2.7 Microscopic assessment

Visual inspection of the dispersion phase particle sizes, to determine the possible occurrence of any visible changes, was performed by means of light microscopic images that were taken at each test interval. An aliquot of each sample was placed on a glass microscope slide, spread out and covered with a glass cover slip. Each slide was mounted on a Motic microscope (Motic, Hong Kong), equipped with a Moticam 3 camera (Motic, Hong Kong), using Motic Images Plus software.

Figure 4.6: Motic microscope, equipped with a Moticam 3 camera that uses Motic Images Plus software.

4.2.8 Visual appearance assessment

Macroscopic analyses, to evaluate the possible occurrence of any visible instabilities, such as colour change, phase separation, or any other detectable visible changes, were performed. Sensory assessments (i.e. smell and feel) were also performed. The sample appearances were visually inspected and photographic images taken with a Canon Power Shot SX50 HS (Canon Inc., Japan) digital camera. Subsequent images were compared to those taken of the formulation, immediately following production. Visual variations were recorded at every stage.

4.2.9 Statistical analysis

The mean percentage (%) stability parameter change, relative to the initial values (T0), was used to determine whether any *significant change*, defined by a 5% change from baseline values, as per the ICH Guidelines, had occurred, by using Equation 4.2.

((Tx-T0) / T0) x 100% **Equation 4.2**

Where:

T0 = Baseline measurement

Tx = Subsequent stability measurement value at pre-determined test intervals.

4.3 Results and discussion

4.3.1 Concentrations of active ingredient and excipients

The mean percentage concentration changes of all of the ingredients, in all samples at all storage conditions over the duration of the stability test period, relative to their baseline measurements, were calculated. Of all of the outcomes, only methylparaben, stored at 40 ± 2°C/75 ± 5% RH, had completely remained within the acceptable 5% variation limit (ICH Guidelines Q1A (R2)). All assay results being generated during the 6 months of stability testing are summarised in Table 4.1, with all outcomes that had not complied with the acceptable 5% variation limit, indicated in red.

The significant variations among the assay results could have been partly attributed to possible active ingredient instabilities, but also to the various challenges that had been encountered during the HPLC analyses.

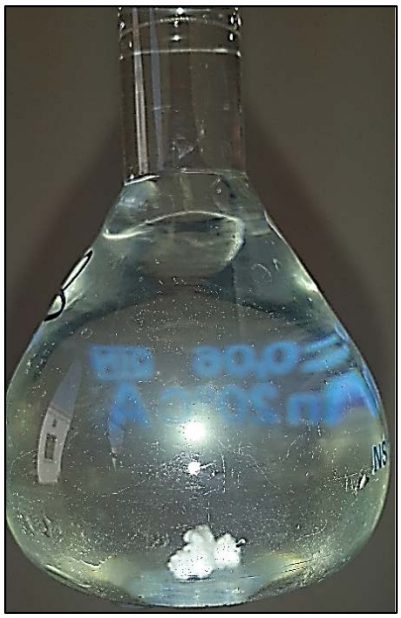


Figure 4.7: Illustration of a test sample that incompletely dissolved in THF.

Firstly, during the validation studies (as described in Appendix B), the test samples had completely dissolved in the solvent (THF), but during the stability studies, random test samples

at random time intervals failed to completely dissolve. Additional sonication and vigorous shaking of the assay sample solutions were carried out (up to 25 min), but without success. Visible lumps of test sample remained in those flasks, as illustrated in Figure 4.7. No possible reason could be identified for those sporadic occurrences.

Secondly, during HPLC analyses, predominantly from T2 onwards, additional peaks eluted after approximately 8.3 and 8.6 min. The degradation of tretinoin caused isomerisation that resulted in the additional peaks detected on the HPLC chromatograms. The peak that eluted after 8.6 min, the larger of the two additional peaks, was identified as the tretinoin isomer, isotretinoin (Bagatin *et al.,* 2015:87; Lehman & Malany, 1989: 597; Ourique *et al.*, 2011:97). Irrespective, the validated elution times of all of the ingredients remained unchanged, without any interference by these peaks.

Table 4.1: Mean concentration (%) of each formulation ingredient at each stability test interval, relative to T0

25 ± 2°C/60 ± 5% RH					
	Initial (T0)	**Month 1 (T1)**	**Month 2 (T2)**	**Month 3 (T3)**	**Month 6 (T4)**
Methylparaben	100.00	98.69	105.70	113.61	100.20
Propylparaben	100.00	88.54	107.26	92.69	102.09
BHT	100.00	104.31	99.29	118.54	124.94
Tretinoin	100.00	62.08	71.58	69.60	84.13
30 ± 2°C/60 ± 5% RH					
Methylparaben	100.00	99.00	106.15	102.42	99.06
Propylparaben	100.00	88.42	113.55	80.79	99.75
BHT	100.00	103.87	99.28	115.45	123.46
Tretinoin	100.00	54.09	69.54	60.35	77.42
40 ± 2°C/75 ± 5% RH					
Methylparaben	100.00	96.69	104.95	104.39	98.88
Propylparaben	100.00	87.03	99.34	81.41	101.75
BHT	100.00	104.49	100.00	115.77	123.81
Tretinoin	100.00	60.84	62.52	54.53	61.04

As anticipated, the highest concentration change was recorded for tretinoin, because of its high UV light sensitivity. During the stability assays, it was observed that UV light and temperature significantly altered the generated results over time. Although none of the assay procedures were performed in direct sunlight and with the laboratory lights switched off, UV light exposure had occurred. As a result, and as discussed before, visible tretinoin isomerisation was observed and two additional tretinoin isomer peaks depicted on the chromatographs at

approximately 8.3 and 8.6 min. These outcomes emphasised the importance of employing extreme caution when handling tretinoin, especially by limiting UV light exposure (Ioele *et al.,* 2005:256).

4.3.2 pH

The pH value, calculated by the concentration and ratio of the hydrogen [H^+] and hydroxide ions [OH^-], should remain constant in a stable solution (Hach Company, 2010:6). No significant changes, in accordance with the ICH Guidelines Q1A (R2), were revealed, following the calculation of the mean percentage changes, relative to the baseline measurements, for all of the test samples at all storage conditions. The average pH values of the test samples were found to have remained within the range of 7.00 - 7.36.

Alternatively, the pH is determined by utilising a logarithmic function, according to which a change of one pH unit represents a ten-fold change in concentration of the hydrogen ion (Hach Company, 2010:6). Although no significant changes in accordance with the ICH Guidelines were revealed, the pH of all of the test samples had generally decreased over time, suggesting that the [H^+] balance of the formulation had been disturbed, by releasing more [H^+], possibly due to the breaking of the hydrogen bond of the gel dispersion structure.

The release of free water (H_2O) from the gel dispersion system, as represented by the condensation (water drops) on the inside the container lids (see Table 4.7), possibly contributed towards the variation of both the [H+] and [OH^-] concentrations, as revealed by the decrease in the measured pH values over time (Hach Company, 2010:4).

During the formulation preparation process, the pH of the freshly prepared formulation was measured between 3 and 4, which was demonstrative of a very low viscosity character. To increase the formulation's viscosity, 2 M of sodium hydroxide (NaOH) (8% m/v), which is commonly utilised as a pH neutraliser in formulations that contain Carbopol® Ultrez 21 (Lubrizol Advanced Materials, Inc., 2002:3), was added sparingly. The NaOH neutraliser added [OH^-] directly to the dispersion system, which consequently increased the formulation's pH to the measured baseline value (Hach Company, 2010:5). It was unclear, whether the increase in the [OH^-] being added to the formulation system, had initiated a cascade of events that had contributed towards the instability of the formulation.

Table 4.2: pH values of a 20% rosehip seed oil (w/w) o/w emulsion, stored at different stability test conditions and measured at pre-determined time intervals

25 ± 2°C/60 ± 5% RH					
	Initial (T0)	**Month 1 (T1)**	**Month 2 (T2)**	**Month 3 (T3)**	**Month 6 (T4)**
pH	7.36	7.08	7.09	7.01	7.04
	7.42	7.11	7.11	7.04	7.06
	7.30	7.10	7.12	7.05	7.06
Mean	7.36	7.10	7.11	7.03	7.05
SD	0.06	0.02	0.02	0.02	0.01
%RSD	0.82	0.22	0.21	0.30	0.16
Mean % pH change relative to T0[a]					
		T0 - T1	**T0 - T2**	**T0 - T3**	**T0 - T4**
		-3.58	-3.44	-4.44	-4.17
30 ± 2°C/60 ± 5% RH					
pH	7.36	7.09	7.23	7.14	7.03
	7.42	7.17	7.21	7.16	7.03
	7.30	7.18	7.2	7.21	7.03
Mean	7.36	7.15	7.21	7.17	7.03
SD	0.06	0.05	0.02	0.04	0.00
%RSD	0.82	0.69	0.21	0.50	0.00
Mean % pH change relative to T0[a]					
		T0 - T1	**T0 - T2**	**T0 - T3**	**T0 - T4**
		-2.90	-1.99	-2.58	-4.48
40 ± 2°C/75 ± 5% RH					
pH	7.36	7.22	7.04	7.13	6.97
	7.42	7.17	7.05	7.17	7.01
	7.30	7.17	7.06	7.19	7.01
Mean	7.36	7.19	7.05	7.16	7.00
SD	0.06	0.03	0.01	0.03	0.02
%RSD	0.82	0.40	0.14	0.43	0.33
Mean % pH change relative to T0[a]					
		T0 - T1	**T0 - T2**	**T0 - T3**	**T0 - T4**
		-2.36	-4.21	-2.67	-4.94

a Determined using Equation 4.2

4.3.3 Viscosity

Significant changes in the viscosity of the formulation occurred over time, when measured against the ICH Guidelines Q1A (R2). The calculated mean percentage viscosity changes at each test interval at all storage conditions, relative to the baseline measurements, are depicted in Table 4.3, with the significant variations shown in red.

A possible explanation of the observed viscosity changes may have been attributed to the anionic acrylates/C10-30 alkyl acrylate cross-polymer, Carbopol® Ultrez 21 (Lubrizol) that had been utilised as thickening agent, in combination with the non-ionic surfactant, polyethylene sorbitol ester, Tween® 80 (Sigma Aldrich). Such polymer-surfactant organisation and the maintenance thereof are influenced by a variety of factors, generally ranging from the ionic character, the degree of hydrophobicity, or the chain length of the polymer and of the non-polar tail of the surfactant, to the extent of salts and additives found in the formulation system (Barreiro-Iglesias *et al.*, 2003:165).

Formulations that contain the acidic Carbopol® Ultrez 21 polymer should be neutralised to a pH, ranging between 6 and 7, so as to thicken and increase their viscosities. The increased pH state triggers repulsion among the polymer carboxylic groups, which then cause swelling of the polymers in an aqueous medium. These swollen polymers cross-link to create a tightly packed, gel-like dispersion structure, which in turn leads to an increased formulation viscosity (Barreiro-Iglesias *et al.*, 2003:166).

Furthermore, weak hydrogen bonding of the hydroxyl groups between polymers and the inter-molecular Van der Waal's forces are also responsible for the arrangement and maintenance of the gel dispersion structure (Barreiro-Iglesias *et al.*, 2003:175; Lubrizol Advanced Materials, Inc., 2002:3; Vintiloiu & Leroux, 2008:187). The interactions occurring between the anionic polymer thickening agent and the non-ionic surfactant, as a result of the hydrogen bond formation between these components, may have affected the stability of the gel structure.

The addition of ionic molecules increases the ionic strength of the continuous phase, which in turn causes the gel structure to shrink and to release water from the gel system. This event is known as the salt effect (Korać *et al.*, 2014:268). The release and leakage of water, as a result of the salt effect, cause the formulation's viscosity to decrease (Lamba *et al.,* 2015:718). It is important to note that for formulations that contain Carbopol® Ultrez 21 as the thickening agent, the addition of mono-valent ions would also decrease their viscosities (Lubrizol Advanced Materials, Inc., 2002:4). To therefore limit the unnecessary addition of charged ions that would possibly cause a viscosity change, de-ionised water and a non-ionic surfactant were used during the formulation process. Although NaOH is a well accepted and widely used neutraliser in formulations that include Carbopol® Ultrez 21, it was uncertain whether the NaOH, used

during the emulsion neutralisation process, had possibly contributed towards the addition of mono-valent ions to the formulation dispersion system, causing the salt effect (Lubrizol Advanced Materials, Inc., 2002:4). Incidentally, water droplets from condensation were observed on the inside of the tightly closed glass container lids, as are visible on the images to the far right of Table 4.7.

Table 4.3: Viscosity values (cP) of a 20% rosehip seed oil (w/w) o/w emulsion, stored at different stability test conditions and measured at pre-determined time intervals

25 ± 2°C/60 ± 5% RH					
	Initial (T0)	**Month 1 (T1)**	**Month 2 (T2)**	**Month 3 (T3)**	**Month 6 (T4)**
Mean (cP)	132335.81	117509.39	118008.55	109776.70	101490.26
SD	612.89	288.79	624.06	1931.64	324.87
%RSD	0.46	0.27	0.53	1.76	0.32
Mean % viscosity change relative to T0[a]					
		T0 - T1	**T0 - T2**	**T0 - T3**	**T0 - T4**
		-11.20	-10.83	-17.05	-23.31
30 ± 2°C/60 ± 5% RH					
Mean (cP)	132335.81	114667.55	114951.03	118209.55	112102.42
SD	612.89	252.62	849.07	2435.77	1241.47
%RSD	0.46	0.24	0.74	2.06	1.11
Mean % viscosity change relative to T0[a]					
		T0 - T1	**T0 - T2**	**T0 - T3**	**T0 - T4**
		-13.35	-13.14	-10.67	-15.29
40 ± 2°C/75 ± 5% RH					
Mean (cP)	132335.81	110322.72	105995.52	124371.00	108271.19
SD	612.89	593.01	2078.80	1615.96	593.44
%RSD	0.46	0.59	1.96	1.30	0.55
Mean % viscosity change relative to T0[a]					
		T0 - T1	**T0 - T2**	**T0 - T3**	**T0 - T4**
		-16.63	-19.90	-6.02	-18.18

a Determined using Equation 4.2

It remained uncertain as to what the exact mechanisms were that had been responsible for the viscosity changes. It was hypothesised that the revealed decreases in the viscosity could have mainly been attributed to the gradual disintegration of the gel structure, because of the breakage of the hydrogen bonding, which led to the de-stabilisation of the gel dispersion system (Behera *et al.*, 2015:259).

4.3.4 Conductivity

Significant changes were revealed between the calculated mean percentage conductivity changes, relative to the baseline measurements, when compared to the ICH Guidelines Q1A (R2), as depicted in red in Table 4.4.

Table 4.4: Conductivity values of a 20% rosehip seed oil (w/w) o/w emulsion, stored at different stability test conditions and measured at pre-determined time intervals

25 ± 2°C/60 ± 5% RH					
	Initial (T0)	**Month 1 (T1)**	**Month 2 (T2)**	**Month 3 (T3)**	**Month 6 (T4)**
µS/cm	1500	1757	1761	1713	1703
	1498	1753	1804	1722	1712
	1491	1795	1785	1739	1710
Mean	1496	1768	1783	1725	1708
SD	4.73	23.18	21.55	13.20	4.73
%RSD	0.32	1.31	1.21	0.77	0.28
Mean % conductivity change relative to T0[a]					
		T0 - T1	**T0 - T2**	**T0 - T3**	**T0 - T4**
		18.18	19.18	15.26	14.17
30 ± 2°C/60 ± 5% RH					
µS/cm	1500	1689	1670	1686	1712
	1498	1667	1658	1688	1710
	1491	1687	1656	1686	1711
Mean	1496	1681	1661	1687	1711
SD	4.73	12.17	7.57	1.15	1.00
%RSD	0.32	0.72	0.46	0.07	0.06
Mean % conductivity change relative to T0[a]					
		T0 - T1	**T0 - T2**	**T0 - T3**	**T0 - T4**
		12.34	11.03	12.72	14.35
40 ± 2°C/75 ± 5% RH					
µS/cm	1500	1719	1677	1546	1634
	1498	1695	1610	1565	1626
	1491	1732	1609	1575	1622
Mean	1496	1715	1632	1562	1627
SD	4.73	18.77	38.97	14.73	6.11
%RSD	0.32	1.09	2.39	0.94	0.38
Mean % conductivity change relative to T0[a]					
		T0 - T1	**T0 - T2**	**T0 - T3**	**T0 - T4**
		14.64	9.07	4.39	8.75

a Determined using Equation 4.2

Fluctuation in conductivity measurements were revealed over time, for all of the samples tested. Based upon the above discussion about the breakage of the hydrogen bonding that had caused the dissociation of the surfactant, as a function of the water content, which had led to free water being released from the gel structure, it was presumed that the conductivity may have also been affected by this event, and also because larger quantities of free water would bring about higher conductivity measurements (Behera *et al.*, 2015:259; Korać *et al.,* 2014:272; Ngawhirunpat *et al.*, 2013:800).

Additionally, the release of electrolytes, although it was uncertain as to whether this effect had taken place, may have also increased electrical conductivity (Korać *et al.,* 2014:272; Lamba *et al.*, 2015:719; Lubrizol Advanced Materials, Inc., 2002:4).

4.3.5 Particle size distribution of dispersion phase

The particle size distributions were determined on the initial formulations (T0), as well as at long-term, intermediate and accelerated storage conditions after 1 (T1), 2 (T2), 3 (T3) and 6 (T4) months of storage. The results obtained for D(0.5) dispersed droplet mean diameter (µm) and percentage changes are summarised in Table 4.5. A particle size distribution graph, generated following an assessment of the formulation test sample at the final measurement point (T4), after 6 months of storage at 40 ± 2°C/75% ± 5% RH, is illustrated in Figure 4.8.

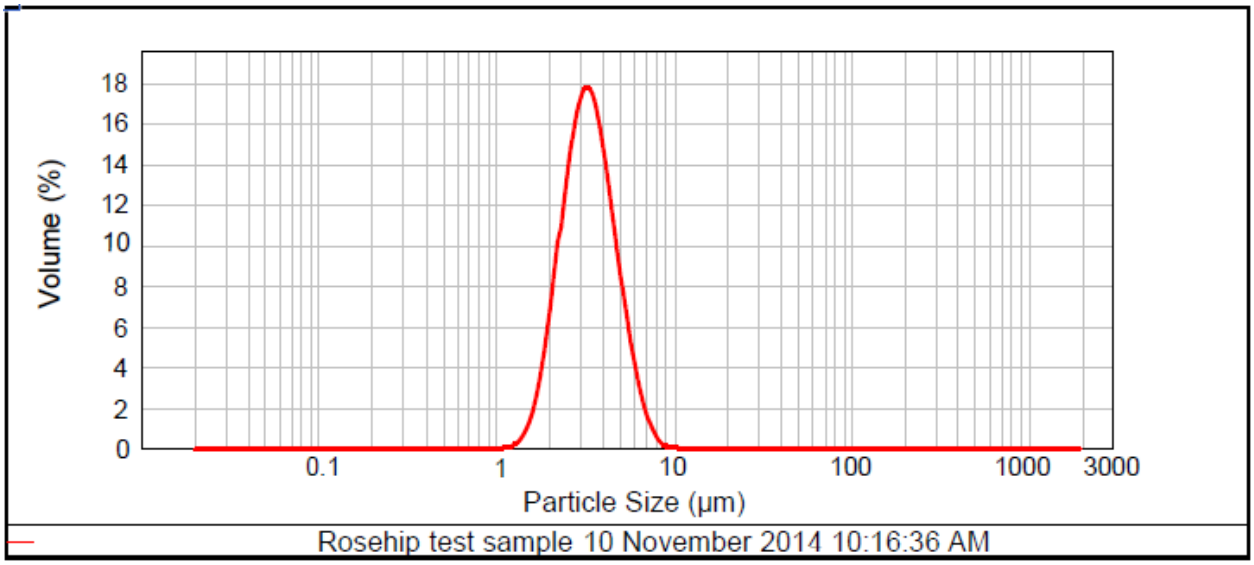


Figure 4.8: Particle size distribution at 6 months of the test sample stored at 40 ± 2°C/75 ± 5% RH (image adapted from the Malvern Mastersizer 2000 with Hydro 2000 MU).

Table 4.5: D(0.5) dispersed droplet mean diameter (µm) values of a 20% rosehip seed oil (w/w) o/w emulsion, stored at different stability storage conditions and measured at pre-determined time intervals

25 ± 2°C/60 ± 5% RH					
	Initial (T0)	**Month 1 (T1)**	**Month 2 (T2)**	**Month 3 (T3)**	**Month 6 (T4)**
D(0.5) µm	3.310	3.291	3.267	3.309	3.312
	3.307	3.286	3.333	3.315	3.298
	3.375	3.268	3.254	3.406	3.293
Mean	3.331	3.282	3.285	3.343	3.301
SD	0.038	0.012	0.042	0.054	0.010
%RSD	1.154	0.369	1.290	1.626	0.298
Mean % D(0.5) droplet diameter change relative to T0[a]					
		T0 - T1	**T0 - T2**	**T0 - T3**	**T0 - T4**
		-1.47	-1.38	0.38	-0.89
30 ± 2°C/60 ± 5% RH					
D(0.5) µm	3.389	3.281	3.324	3.391	3.383
	3.377	3.284	3.322	3.384	3.305
	3.373	3.283	3.246	3.639	3.317
Mean	3.380	3.283	3.297	3.471	3.335
SD	0.008	0.002	0.044	0.145	0.042
%RSD	0.246	0.047	1.349	4.184	1.259
Mean % D(0.5) droplet diameter change relative to T0[a]					
		T0 - T1	**T0 - T2**	**T0 - T3**	**T0 - T4**
		-2.87	-2.44	2.71	-1.32
40 ± 2°C/75 ± 5% RH					
D(0.5) µm	3.303	3.299	3.359	3.354	3.319
	3.295	3.291	3.359	3.262	3.328
	3.292	3.28	3.264	3.260	3.322
Mean	3.297	3.290	3.327	3.292	3.323
SD	0.006	0.010	0.055	0.054	0.005
%RSD	0.172	0.290	1.648	1.631	0.138
Mean % D(0.5) droplet diameter change relative to T0[a]					
		T0 - T1	**T0 - T2**	**T0 - T3**	**T0 - T4**
		-0.20	0.93	-0.14	0.80

a Determined using Equation 4.2

During storage, no significant changes, when compared to the ICH Guidelines Q1A (R2), were revealed, following calculation of the mean percentage viscosity changes, relative to baseline measurements. The mean D(0.5) droplet diameter of freshly prepared emulsions was 3.331 µm, which remained within the acceptable limit of 5% for all of the emulsions, stored at

the various storage conditions. It was therefore assumed that no droplet coalescence, nor Ostwald ripening had occurred during any stage of sample storage (Traynor *et al.*, 2013:2173).

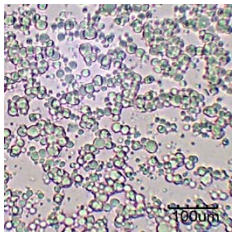
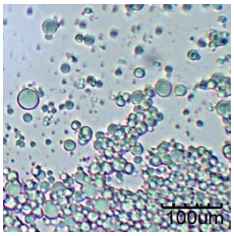
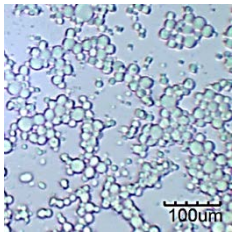
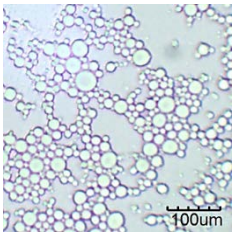
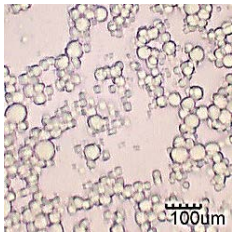
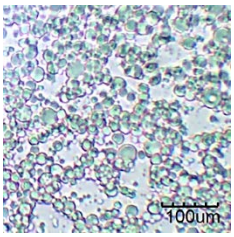
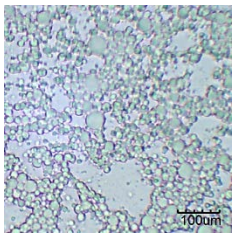
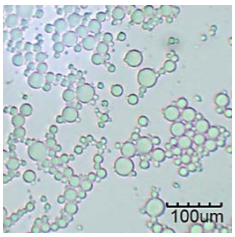
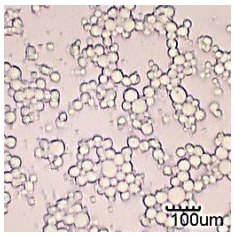
4.3.6 Creaming index

Creaming is a frequently observed form of emulsion instability, which is characterised by evident phase separation (Lamba *et al.*, 2015:720). The methods that were followed for evaluating the possible occurrence of creaming, is described in Section 4.2.6 above.

No visible phase separation had occurred by adding centrifugal stress, hence by increasing the applied physical gravitational forces. Neither creaming, nor sedimentation was revealed at any of the test intervals for any of the tested formulation samples.

4.3.7 Microscopic assessment

Table 4.6: Microscopic images taken at each stability time interval

Initial	Month 1	Month 2	Month 3	Month 6
25 ± 2°C/60 ± 5% RH				
				
	30 ± 2°C/60 ± 5% RH			
				
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	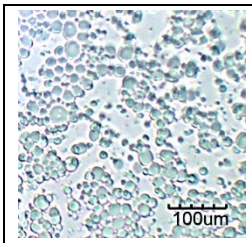	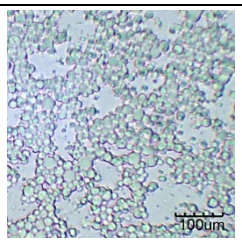	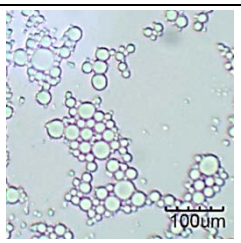	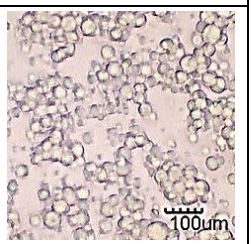

Further analysis of the test samples, by applying polarisation microscopy methods, did not reveal substantial information regarding the gel structure, nor about any o/w emulsion changes over time.

Surfactants, the amphiphilic molecules that contain both hydrophilic and hydrophobic components to lower the surface tension among the two immiscible liquid phases and the emulsifiers, as utilised in this study, were found to attain consistent, uniform formulations over time. Following visual inspection of the microscopic images, it was concluded that the formulation system was protected against coalescence, since no droplet growth was observed.

Flocculation had occurred to some extent, as was visible on the images. However, as discussed in Section 4.3.5 above, since particle size distribution had not significantly increased, nor changed over time, the possible de-stabilisation of the formulation structure could not have been attributed to flocculation (Lamba *et al.*, 2015:712).

4.3.8 Visual appearance assessment

Table 4.7: Photographic images taken at each stability time interval, with images of the glass containers in the first five columns and the lids in the last column to the right

Initial	Month 1	Month 2	Month 3	Month 6	Month 6
25 ± 2°C/60 ± 5% RH					
					
	30 ± 2°C/60 ± 5% RH				
					
	40 ± 2°C/75 ± 5% RH				
					

Freshly prepared emulsions were smooth, soft, semi-solid, slightly yellow, gel-like formulations, with a very slight, but not unpleasant, nor distinct smell. Visual inspection revealed no visible phase separation, nor texture changes throughout the full duration (6 months) of the study. The slight smell decreased over time until none was detected after 6 months. No phase separation, creaming, nor cracking was observed in all test samples for the duration of the study. Water droplets from condensation were observed on the inside of the container lids of all samples at all storage conditions, indicating that this aqueous based product had the potential for water evaporation and water loss from the final product. A slightly lighter yellow colour was observed for samples after storage for 6 months.

4.4 Conclusion

In accordance with the ICH Guidelines Q1A (R2) definition of a significant change being a 5% change from baseline value, significant changes were revealed during this study with regards to the assay outcomes of the formulation ingredients, the viscosity results and the conductivity measurements.

According to the ICH Guidelines Q1A (R2), additional failure to meet pre-determined acceptance criteria, such as changes in appearance (i.e. colour changes) and physical attributes (i.e. phase separation), unacceptable pH changes, or degradation product levels that exceed the pre-determined acceptance levels, should also be considered (ICH, 2003:3, 9). During this study, significant changes were revealed with regards to the pH value changes, particle size distributions and creaming index outcomes.

Tretinoin had exhibited the largest concentration changes among all samples at all storage conditions and at all test intervals. These outcomes emphasised the sensitivity and susceptibility of tretinoin to isomerisation from UV light exposure and the significance of maintaining good manufacturing practises, including the upkeeping of optimal storage conditions. The possibility of inconsistent mixing of the bulk emulsion before packaging, which may have resulted in the ingredient concentrations having differed among the containers from which samples were taken, can also not be excluded.

Significant viscosity character and conductivity changes had occurred in all of the test samples. The reduced test sample viscosities and the increases in the conductivity measurements were believed to have been caused by the gradual disintegration of the gel structure, due to the breakage of the polymer hydroxyl group being linked through hydrogen bonding. The de-stabilised polymer-surfactant hydrogen bonds had consequently caused the release of free water from the gel system, which resulted in increased conductivity measurements over time. The disintegration of the emulsion stabilising gel system therefore also caused the formulation viscosity to decrease (Behera *et al.*, 2015:259; Korać *et al.*, 2014:272; Ngawhirunpat *et al.*, 2013:800).

Formulation viscosity changes affect the dispersed droplet mobility. Liquid o/w emulsions present with a low stability character, which, due to the high mobility of dispersed droplets, may set off events of coalescence, flocculation, or sedimentation. The viscosity character of the test sample had not changed over time, as the stability support being offered by the gel structure had assisted in maintaining the emulsion droplet organisation. In this study, the viscosity changes, measured over 6 months, had not influenced the emulsion droplet character, as was concluded from evaluating the particle size distribution and creaming index outcomes (Ushikubo & Cunha, 2014:145).

Formulation pH adjustment influences viscosity. However, it is doubtful that the pH variations, as summarised in Table 4.3, had been responsible for the revealed test samples' viscosity changes. The reasons are, firstly, although all of the tested samples' pH values had changed over the course of the stability study, no significant pH changes for any test sample were observed (Table 4.3). Secondly, for Carbopol® Ultrez 21 containing emulsions, maximum viscosity is achieved at a pH, ranging between 6 and 7. If the test formulation's pH state had indeed influenced the viscosity of the formulation, it should have increased concurrently over time, but instead, the viscosity measurements had decreased. In this study, therefore, the possibility of any pH changes being the cause of the significant viscosity changes, could be excluded (Lubrizol Advanced Materials, Inc., 2002:4). It was furthermore hypothesised that the de-stabilised polymer-surfactant hydrogen bonds had contributed towards the observed pH changes through the modification of the hydrogen availability in the formulation system.

According to the ICH Guidelines Q1A (R2), a 5% water loss after 3 months at accelerated storage conditions, is considered as a significant change for a product, packaged in semi-permeable containers, unlike the ones used during this study. All samples during this study were stored in tightly closed 50 ml glass containers, with tight closing screw caps, containing polyvinylidene chloride (PVDC) liners that are impermeable to water. Since water permeation wasn't a possibility, the mass change could not be investigated (ICH, 2003:10). The water droplets from condensation that were observed on the inside of the container caps may have contributed towards the increases in the concentrations of the ingredients over time. For future studies, test samples should be stored in semi-permeable containers that would allow the possible evaporation of ingredients/water from the formulation, to determine the effect of water loss and to calculate the effects of increased temperature conditions on the test formulations more extensively.

Given that the oil phase was mainly composed of a non-standardised natural ingredient, the physicochemical effects of the rosehip seed oil constituents could not be fully quantified. Natural products are generally chemically and physically unstable once removed from their natural sources. Due to the challenges involved, it is not always entirely possible to fully characterise the ingredients present in natural products. In this study, the rosehip oil itself may have contributed towards, or influenced the character of the formulation in a number of ways, which would require further investigation (Lamba *et al.,* 2015:722).

Although no observed changes had altered the general visual, or sensorial acceptance criteria of this test formulation, the mere fact that the viscosity, the concentrations of the ingredients and the conductivity measurements had not remained within the required ICH specifications, it was concluded that this formulation could not be considered stable, nor suitable for manufacturing purposes.

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Chapter 5

Vertical Franz cell type permeation studies

5.1 Introduction

According to the Organisation for Economic Co-operation and Development (OECD) guidance on dermal absorption, No.156, "*in vitro* methods are designed to measure the penetration of chemicals into the skin and their subsequent permeation across the skin into a fluid reservoir, as well as to determine the partition into the different skin layers and possible deposition therein." *In vitro* diffusion studies were hence performed during this study, to determine the transdermal delivery of the active pharmaceutical ingredient (API), tretinoin, and to calculate its systemically available concentrations, following the topical application thereof (Guth *et al.*, 2015:113; OECD, 2004:13; OECD, 2011:28).

Since metabolic activity is not required in the investigation of passive diffusion, the *in vitro* vertical Franz cell method, utilising excised human skin and tape stripping techniques, were used to determine the topical and transdermal delivery of tretinoin from the emulsion delivery vehicle and through the skin (OECD, 2004:16). Prior to the skin diffusion assessments, membrane release studies, using the same vertical Franz diffusion method, but with synthetic membrane filters instead, were performed to determine the release of the API from the test formulation.

In order to review the extent of skin permeation of an API, all of the physicochemical characteristics of a penetrant must be considered, which during this study included:

- Tretinoin has an aqueous solubility below 0.1 g/100 mL.
- Tretinoin has a log P between 5.0 and 6.3.
- Tretinoin has a molecular weight of approximately 300 Da (PubChem.com; 2016).

From the low aqueous solubility of tretinoin, it is evident that it is practically insoluble in water, which may limit its transdermal delivery (PubChem.com; 2016).

The log P values indicate the degree of distribution of a molecule in the octanol and water phases, and is it therefore indicative of how well a molecule would distribute through the stratum corneum lipids and the hydrophilic water component (Williams, 2013:677). Transdermal delivery is optimised for molecules that exhibit a balanced lipophilicity and a log P of approximately 2 – 3. Hydrophilic molecular structures usually have a negative log P value, whereas, lipophilic molecules exhibit a positive log P. Higher log P values are indicative of an

increased lipophilicity. Highly lipophilic molecules, such as tretinoin, may be trapped within the stratum corneum, which would further hinder its transdermal potential (Gaisford, 2013:368; Karande & Mitragotri, 2009:2363; Wiedersberg & Guy, 2013:150; Williams, 2013:676, 686).

Molecular size is indicative of the skin's permeation potential of a drug, where permeation and maximum flux decrease exponentially with an increased molecular weight. Molecular weight increases concomitantly with lipophilicity. Ideally, to increase a drug's permeation potential, an API should have the lowest possible molecular weight, preferably less than 500 Da (WHO, 2006:27; Wiechers & Watkinson, 2008:63; Williams, 2013:680). The permeation of an API through the skin is hence a multi-faceted process, which is also influenced by the formulation vehicle utilised (OECD, 2011:42; Sigma-Aldrich, 2015b; Williams, 2003:28).

5.2 Methods

Apart from the physicochemical properties of an API, a number of additional factors can potentially affect the dermal absorption of an API, such as the study type (*in vivo* / *in vitro*), the skin type (human / test animal), the test conditions (temperature, skin thickness, receptor fluid), the anatomical site on the skin, the skin's condition, the vehicle type (composition, liquid, solution, etc.), the applied dose (amount, single, multiple, finite, infinite), the skin surface area, the duration of exposure (contact time, occlusion, evaporation, etc.) and the environmental conditions (Williams *et al.,* 2011:121). All of these conditions are discussed in the following sections, followed by a description of the test methods.

5.2.1 Test formulations

Two semi-solid test formulations, i.e. two o/w emulsions, were prepared for both the membrane release and *in vitro* diffusion studies, as described in Chapter 3, section 3.4.3.

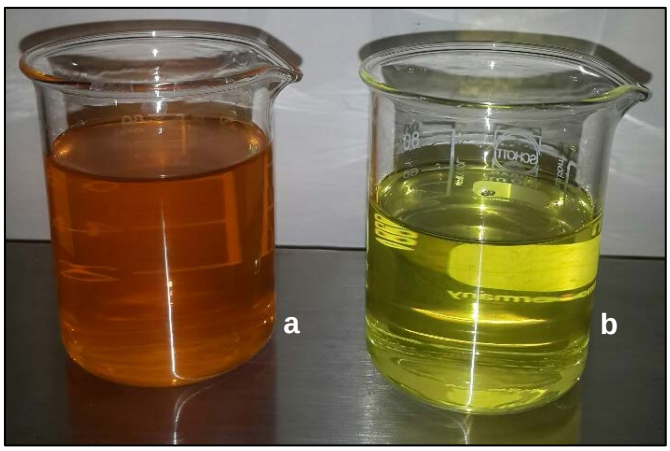

Figure 5.1: a) *Rosa rubiginosa* seed oil, spiked with tretinoin and (b) *Rosa canina* seed oil, without tretinoin.

The test formulation, containing 20% of *R. rubiginosa* (sweet briar) seed oil, spiked with tretinoin, was prepared for the investigation of the dermal permeation capabilities of tretinoin. Tretinoin, a yellow to light-orange crystalline powder, also known as retinoic acid, vitamin A acid, *trans*-retinoic acid and all-*trans*-retinoic acid, was dissolved in *R. rubiginosa* rosehip seed oil, as described in Chapter 3, section 3.4.3.1. The placebo, or control formulation that was used, was prepared containing 20% *R. canina* (dog rose) seed oil, without any added tretinoin (Figure 5.1).

5.2.2 Preparation of the receptor phase solution

The functionality of a receptor phase solution firstly depends upon skin compatibility and secondly, but most importantly, upon the solubility of the API in the receptor solution. It is also important that the receptor solution does not interfere with the subsequent analytical method that is employed (OECD, 2004:15). The receptor solution that is utilised during the *in vitro* studies should serve the same role as the blood, during *in vivo* analyses (OECD, 2011:29).

Table 5.1: Receptor phase preparation of PBS:ethanol (50:50, v/v) (BP, 2015b:1; OECD, 2011:30)

Solution	Ingredients		Preparation method
	Solids	Liquids	
A	6.8050 g of potassium dihydrogen orthophosphate (KH_2PO_4)	250.0 ml of dH_20	Dissolve KH_2PO_4 in dH_2O
B	1.5736 g of sodium hydroxide (NaOH)	393.4 ml of dH_20	Dissolve NaOH in dH_2O
C	Mix solutions A and B		
D		250.0 ml of absolute (99%) ethanol (C_2H_5OH)	
E	Mix equal parts (250.0 ml) of solution C and D		
			Adjust pH to 7.4 with 2 M of hydrochloric acid (HCl) (7.3% m/v)
			De-gas and filter the receptor phase solution immediately prior to commencing with testing

* dH_2O = deionised water.

The solubility of an API in the receptor phase solution should be demonstrative thereof that it is not a rate limiting factor (EFSA, 2012:8). Tretinoin has a very poor aqueous solubility (< 0.1 g/100 mL), it is slightly soluble in ethanol and have an estimated log P value of 5.0 to 6.3 (BP, 2015a:1; PubChem, 2016). To prevent the insolubility of tretinoin in the receptor phase

solution from being the rate limiting factor during the skin absorption process, the selected receptor phase should have an adequate capability to solubilise the tretinoin. Since ethanol is frequently used as the co-solvent for topical formulations that contain tretinoin, equal amounts of phosphate buffer solution (PBS) and ethanol, adjusted to pH 7.4, were chosen as the components of the receptor phase solution (BP, 2015b, 1; EFSA, 2012:8; OECD, 2004:15; Ourique *et al.*, 2011:96). Fresh receptor phase batches were prepared, as summarised in Table 5.1, 24 h prior to the diffusion studies.

5.2.3 HPLC analysis of tretinoin

The HPLC analysis of tretinoin was performed following the sampling of the receptor phase solution by employing the validated HPLC method, as described in Appendix A, to determine the concentration of the tretinoin that had been delivered to and transported through the skin (and membrane).

The tretinoin content was quantified at 349 nm, using an HP 1100 series HPLC system, equipped with a pump, auto-sampler, UV detector and Chemstation Rev. A.10.03 data acquisition and analysis software (Agilent Technologies, Palo Alto, CA). The column that was used was a Venusil XBP $C_{18(2)}$ (150 x 4.6 mm, 5 µm, Agela Technologies, Newark, DE). The mobile phase comprised of acetonitrile containing 0.5% of glacial acetic acid (v/v). The flow rate was set at 1.0 mL/min and the tretinoin eluted after approximately 5.2 to 5.5 min. A standard calibration curve was generated from the analysed standard solution outcomes. Calibration graphs were plotted according to linear regression analysis, which had a correlation coefficient value (R^2) of 0.998. Standard solutions were also injected, as reference, after every twenty sample runs. 50 µl of each sample solution was injected in duplicate into the HPLC for analysis.

5.2.4 Membrane release studies: vertical Franz cell method

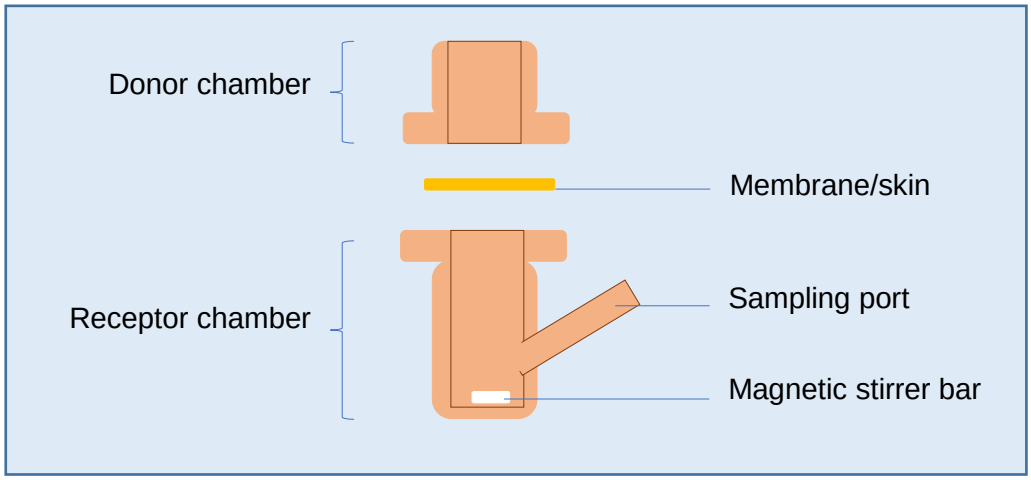

Figure 5.2: Schematic representation of a Franz type cell diffusion system (image adapted from PermeGear, Inc., 2015).

5.2.4.1 Components of a vertical Franz cell system

Polyvinylidene fluoride (PVDF) membrane filters (Pall® Life Sciences, Michigan, USA) were utilised during the membrane release studies. Synthetic membrane release studies neither represent a natural diffusion transport process, nor an active skin barrier function. The use of membranes to determine dermal delivery on its own is therefore inadequate and may generate over-estimated absorption values (EFSA, 2012:7). Vertical Franz cell membrane studies were hence merely performed to establish whether the tretinoin had been sufficiently released from the test formulation and had this test procedure preceded the *in vitro* skin diffusion studies.

A vertical Franz cell consists of a donor (upper) and a receptor (lower) glass chamber, between which a synthetic membrane (or excised skin during *in vitro* studies) is positioned (Figure 5.2). Each Franz cell donor chamber has a surface area of approximately 1 cm^2, whereas the receptor chamber has a receptor solution capacity of approximately 2 mL. To ensure that the two compartments fit and seal securely to prevent leakage of the Franz cell contents, Dow Corning® high vacuum grease (Figure 5.3) was thinly applied on each inter-connecting chamber surface. The membrane was mounted onto the receptor chamber and were the two chambers then carefully assembled to close the Franz cell apparatus. High vacuum grease was also thinly applied on each closed chamber seam. The Franz cell chambers were further securely joined by means of special metal clamps. All components used for the assembly of the Franz cells, were made of inert materials to exclude possible interaction with the API.

Figure 5.3: Dow Corning® high vacuum silicone grease (Sigma-Aldrich, 2015a).

5.2.4.2 Hydration of membranes and membrane-receptor phase equilibrium

To achieve sufficient membrane hydration and to reach membrane-receptor phase equilibrium, twelve assembled Franz cells, each with its mounted synthetic membrane, were filled with 2 mL of pre-heated (37 ± 1°C, representative of the temperature of human blood) receptor phase solution. The temperature in the receptor phase solution was maintained for the duration of the diffusion studies (Lai *et al.*, 2013:106; OECD, 2004:21), by using a pre-heated (37 ± 1°C) water bath. Continued stirring of the receptor phase was achieved by inserting a small magnetic stirrer bar into each receptor chamber (OECD, 2011:28). The Franz cells were placed in a deep tray that was positioned on a magnetic stirrer plate, and the receptor chambers in the tray submerged into the temperature controlled water bath (37 ± 1°C) (Fig. 5.4). A thermometer was placed in the water bath to monitor and control the water bath system temperature (Lai *et al.*, 2013:106; OECD, 2004: 21). After 1 h, the receptor phase of each of the Franz cells was completely withdrawn and discarded.

Figure 5.4: Assembled vertical Franz diffusion cells: (a) placed on a magnetic stirrer plate and b) in a temperature controlled water bath system.

5.2.4.2 Membrane release studies

Following the hydration process, the Franz cell donor chambers were individually filled with 1 ml of pre-heated (32°C) test formulations. Ten Franz cells were filled with the emulsions, containing the 20% *R. rubiginosa* seed oil, spiked with tretinoin, whilst two Franz cells were filled with the placebo formulation, comprising of 100% *R. canina* seed oil. All of the Franz cell receptor chambers were filled with 2 mL of receptor phase solution. It was important for the whole membrane surface to maintain complete contact with the receptor phase solution, until withdrawn. A visual inspection was performed after filling, to confirm that no air bubbles were trapped within the filled receptor chamber.

Following the accurate filling procedures of each Franz cell, the donor chamber opening was sealed with Parafilm®. This was done to avoid possible contamination of the test samples in the donor chamber, as a result of the condensed water droplets that form on the inside of the water bath's metal "roof", and to reduce possible evaporation of the formulation solvents (OECD, 2004:20).

The entire receptor phase solution was extracted at hourly intervals (1, 2, 3, 4, 5 and 6 h) and immediately refilled with fresh, pre-heated receptor phase solution, to maintain sink conditions (Shin *et al.*, 2005: 68). The collected receptor phase samples from the diffusion cells were transferred into amber HPLC auto-sampler vials and analysed directly, without any further preparation.

5.2.5 *In vitro* diffusion study: vertical Franz cell method

5.2.5.1 Skin preparation

Skin from various mammalian species can be used for skin diffusion studies. It is, however, important to take into consideration that the skins from different species differ, but also that the skin, from different anatomical areas of the same species (an individual, or a mammal), differs (OECD, 2004:13; OECD, 2011:27). For the purpose of this study, full-thickness human skin was collected, directly after patients had received cosmetic abdomino-plastic surgery, with the prior, informed consent of all donors. Prior ethical approval for this project for the use of biological material from human subjects in experiments had been obtained from the North-West University Research Ethics Regulatory Committee (Ethics number: NWU-00114-11-A5).

To prepare the skin for the permeation studies, collected skin was sponged with deionised water and dermatomed with a Zimmer™ electronic dermatome, model 8821, to ensure a uniform skin thickness of 400 µm. The dermatomed skin was punched into circles, having a diameter of 15 mm. Each dermatomed skin circle was placed on a Whatman® filter paper with the stratum corneum facing upwards. Air dried dermatomed skin was wrapped in aluminium foil and stored

in the freezer at - 20°C, until utilised. Prior to the diffusion study, frozen dermatomed skin was thawed at room temperature and visually inspected for damage or rupture, before mounting it to the Franz diffusion cells.

Skin barrier integrity of frozen dermatomed skin can deteriorate during storage. Since this effect does not depend on the length of storage, it cannot be predicted (OECD, 2004:17). To therefore try avoiding an over-estimation of the permeability of a test molecule by any skin sample, pre-study skin integrity checks should preferably be performed, prior to the application of the test formulation. Skin integrity tests ensure that the permeation data is obtained by utilising skin with an intact barrier function (OECD, 2004:18). Various recommended methods are described in the literature for performing the evaluations before, during and at the end of a diffusion study. A visual inspection of the skin is performed and are all visibly damaged skin removed and excluded from use during the study. Once the skin is mounted on the Franz cell and the hydration phase has been completed, further pre-study integrity checks may be performed. During pre-study integrity checks, it is suggested that the trans-epidermal electrical resistance (TEER) is measured first. The TEER should remain within the accepted range for the skin type being utilised. Secondly, the trans-epidermal water loss (TEWL) may be measured to determine whether the stratum corneum skin barrier function is within range for the specific skin type and anatomical area. Thirdly, the absorption characteristics of a reference material, such as titrated water, may be performed to determine the trans-epidermal water flux (TWF). It is important that the pre-study integrity checks are completed as quick as possible (Guth *et al.*, 2015:113; OECD, 2004:18; OECD, 2011:27). Skin with unacceptable integrity measurements should be replaced prior to the application of the test formulation (EFSA, 2012:8).

5.2.5.2 Skin diffusion study

In vitro diffusion studies, utilising dermatomed human skin (prepared as described in Section 5.2.5.1), were performed. The same vertical Franz diffusion cell method, as described for the membrane release studies in Section 5.2.4, were employed for the skin diffusion studies, but was excised human skin used for these tests, instead of the synthetic membranes.

Sampling during the diffusion study occurred at hourly withdrawals up and until 12 h. Following extraction, each receptor phase sample was filtered, transferred into amber HPLC auto-sampler vials and directly analysed without any further preparation.

Due to the extremely low tretinoin concentrations that were measured during HPLC analyses, and following an evaluation of the results being generated during the membrane release studies, the diffusion study was repeated, but was only a single sample taken from the receptor phase after 12 h. The extracted samples were immediately analysed on HPLC.

5.2.5.3 Skin fractionation

Skin fractionation is performed to evaluate the localisation of the examined API within the various skin layers, to get an indication of its distribution pattern and bio-availability (OECD, 2004:22). Following the skin diffusion studies, the Franz cells were disassembled. The dermatomed skin circles were pinned to a solid surface. The skin was carefully dabbed dry with dry tissue paper to remove the remaining test formulation, whereafter tape stripping was carried out. Tape stripping is the procedure that is performed at the end of a diffusion study. It involves the sequential application of adhesive tape to the skin areas that were exposed to the test formulations, to purposely remove the stratum corneum-epidermis (SCE) (EFSA, 2012:9; OECD, 2004:22).

3M Scotch® tape was cut into small pieces to fit the skin diffusion areas that had been exposed to the test samples. The diffusion area of each skin sample was clearly visible, due to the indentation caused by the diffusion cells on the skin. The first tape strip for each skin sample was discarded, to prevent possible contamination of the topically applied test formulation and to represent the API that was not bio-available for absorption, due to skin desquamation (or ex-foliation) (EFSA, 2012:10; OECD, 2011:35). Fifteen tape strips (containing the stratum corneum-epidermis / SCE) and the remainder of the skin (epidermis-dermis (ED)), were cut into smaller strips and placed into separate glass vials. The skin sample in each glass vial was covered with 5 ml of solvent (ethanol:THF) (50:50, v/v)), the vials capped and stored for approximately 12 h at 4°C. The SCE and ED samples were filtered, analysed and was the profile of tretinoin, within these dermal layers, determined (EFSA, 2012:9)

5.2.6 Data analysis

For the analysis of the membrane release study outcomes, the average (mean) cumulative concentration of the tretinoin that had diffused through the synthetic membrane into the receptor phase, was plotted against time. The average flux was interpreted by means of the slope of the linear line, between 2 h and 6 h. The maximum average flux was calculated, according to the slope of the linear portion of the absorption:time curve and did it not include the lag phase, or plateau (EFSA, 2012:9). The average percentage (%) of released tretinoin was determined, as well as the average concentration (µg/cm^2.h) of the API that had penetrated through the membrane at hourly intervals, up and until 6 h.

For the diffusion studies, the average percentage (%) of tretinoin that had diffused through the skin, the average cumulative amount per diffusion area (µg/cm^2.h), as well as the average concentration (µg/mL) of the API that had penetrated the skin after 12 h, were calculated.

5.3 Results and discussion

5.3.1 Membrane release study outcomes

During the membrane release studies, newly prepared formulations, as described in Chapter 3.4, that contained approximately 0.5 mg/mL of tretinoin, were subjected to testing to confirm that the API had been released from the formulation.

Upon HPLC analyses of the test samples, an additional peak had eluted that was visible on the chromatograms, and was it identified as the tretinoin isomer, isotretinoin (Bagatin *et al.,* 2015:87; Lehman & Malany, 1989: 597; Manconi *et al.,* 2006; Ourique *et al.*, 2011:97). This isomer did not interfere with the elution of the tretinoin peak and were the outcomes of both the tretinoin and isotretinoin reported. The formation of the isotretinoin was attributed to tretinoin's very high instability when subjected to air, light and/or heat. Due to its highly poor photostability character, a topically applied tretinoin formulation, when subjected to UV irradiation for only 10 min, would result in a mere 30% of the initial tretinoin concentration remaining intact on the skin's surface (Ourique *et al.*, 2011:96). The tretinoin degradation process is responsible for newly formed degradants, such as 13-*cis* (isotretinoin) and 9-*cis* (alitretinoin) isomers, which in turn are responsible for the formation of several additional isomers (Ioele *et al.*, 2005:256). Despite all the precautions taken during the sample preparation and test procedures to limit exposure to direct sunlight (e.g. the use of amber glassware working at low light conditions) and the utilisation of a cold processing preparation method, exposure had unfortunately not been adequately avoided to prevent the degradation of tretinoin (Bagatin *et al.,* 2015:87; Lehman & Malany, 1989: 597; Manconi *et al.,* 2006; Ourique *et al*., 2011:97).

Figures 5.5 and 5.6 graphically represent the measured (HPLC analyses) cumulative concentrations of tretinoin and isotretinoin, sampled from the receptor compartments of the ten Franz cells, at the specified sampling intervals over a period of 6 h, during the membrane release study. The distribution of the data points in these figures is indicative of the variations among the ten samples. The concentrations of isotretinoin among the samples varied more than those of tretinoin. This was attributed to the degradation process not having been a controlled process.

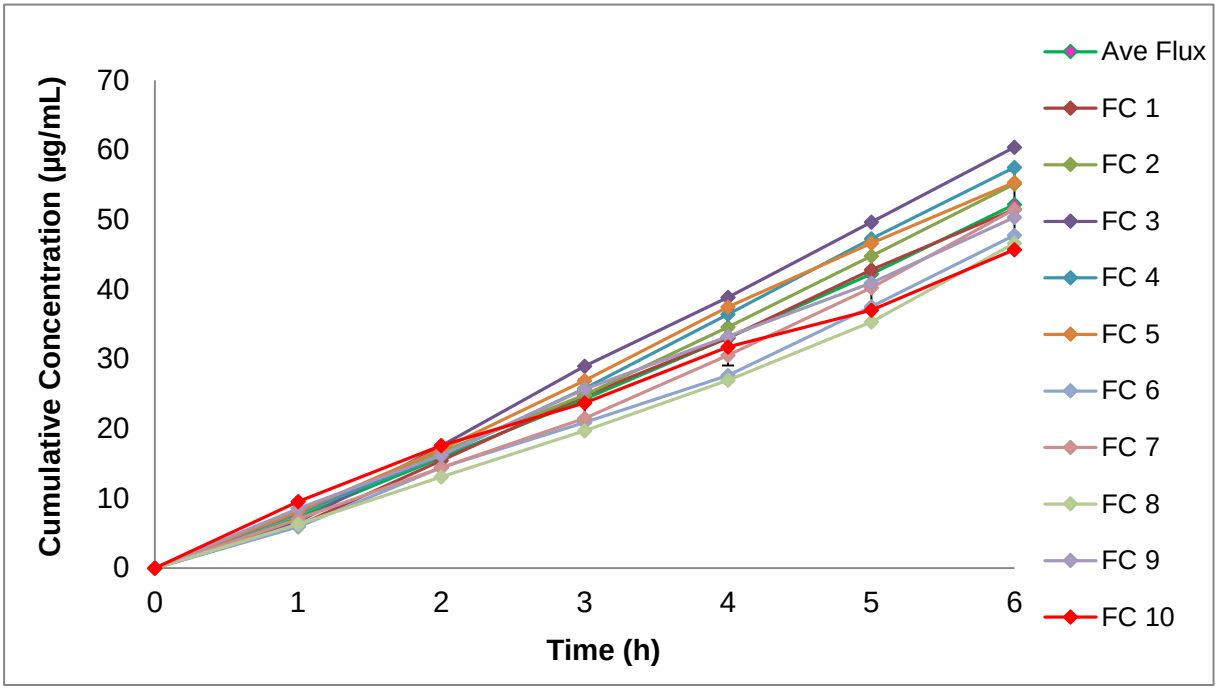

Figure 5.5: Cumulative concentrations (µg/mL) of tretinoin, measured in the withdrawn samples from the receptor compartments of the ten Franz cells, at specified intervals over a period of 6 h.

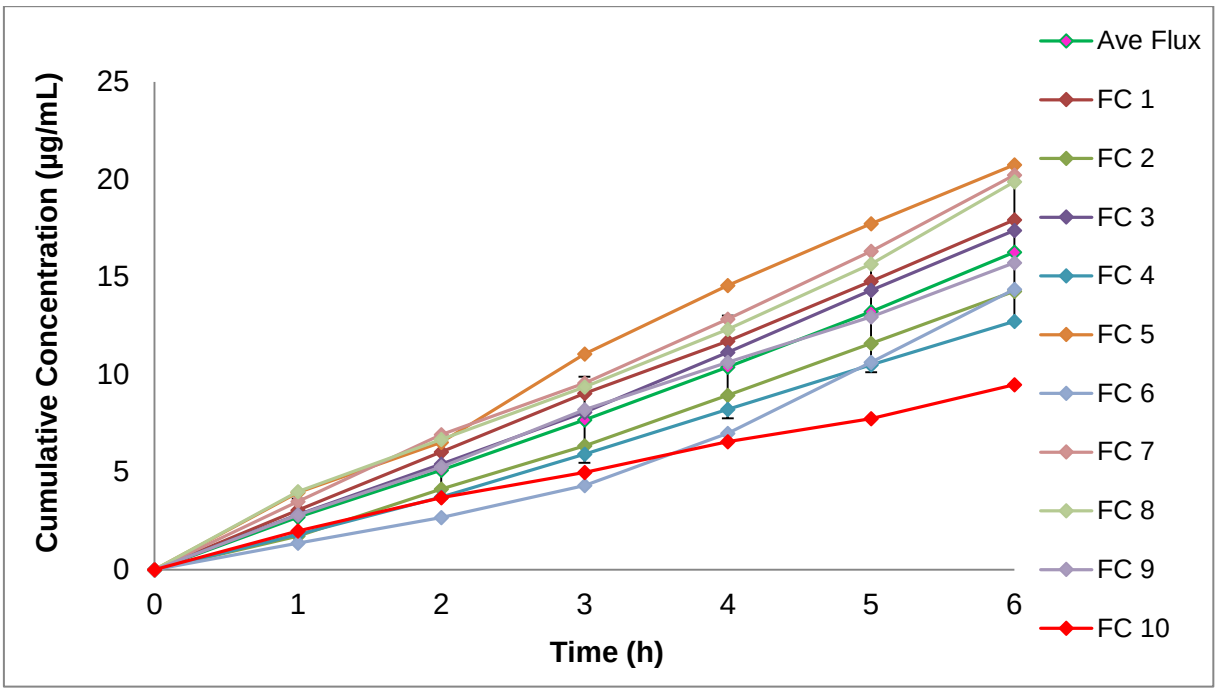

Figure 5.6: Cumulative concentrations (µg/mL) of isotretinoin, measured in the withdrawn samples from the receptor compartments of the ten Franz cells, at specified intervals over a period of 6 h.

Flux (μg/cm^2.h) is defined as the rate of transfer per unit area of surface (Barry 2002:513). The average flux was calculated from the slope of the straight line between 2 h and 6 h.

Figures 5.7 and 5.8 graphically represent the average flux of tretinoin and isotretinoin, at each sampling interval from the receptor compartments of the ten Franz cells, during the membrane release study over a period of 6 h.

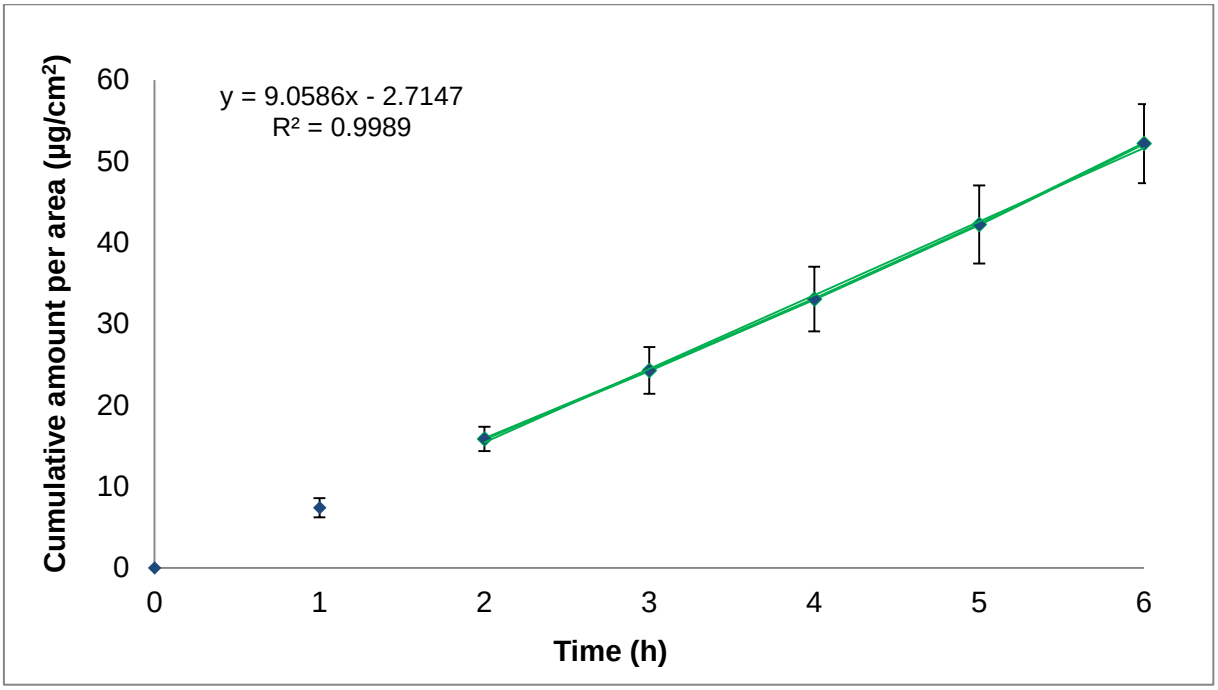

Figure 5.7: Average cumulative amount per area (μg/cm^2) of tretinoin, measured in the withdrawn samples from the receptor compartments of the ten Franz cells, at specified intervals over a period of 6 h.

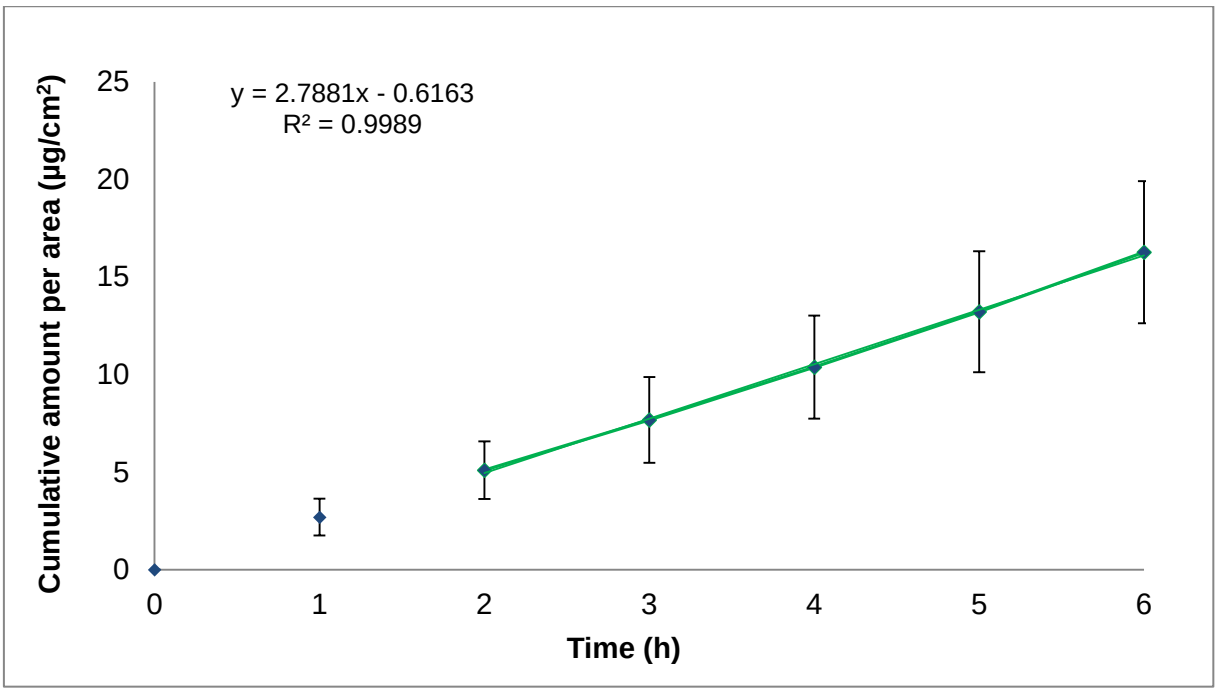

Figure 5.8: Average cumulative amount per area (µg/cm^2) of isotretinoin, measured in the withdrawn samples from the receptor compartments of the ten Franz cells, at specified intervals over a period of 6 h.

The average flux, calculated from the slope of the straight line between 2h and 6 h for tretinoin was 9.0586 µg/cm^2.h, whilst a lower average flux of 2.7881 µg/cm^2.h was obtained for the isotretinoin. Table 5.2 summarises the generated outcomes from the membrane release studies.

Table 5.2: Outcomes from the membrane release studies over a period of 6 h (n = 10)

API and degradant	Average cumulative amount per area (µg/cm²)	Average percentage released (%)	Average flux (µg/cm².h)
Tretinoin	28.060	4.968	9.0586
Isotretinoin	8.753	1.550	2.7881

The low percentage (4.968%) of the released API may have been attributed to the low affinity that a lipophilic molecule, such as tretinoin, has for the hydrophilic character of the o/w emulsion (Otto *et al.*, 2009:9). It was also uncertain to which extent the hydrophilic coating, pore size, membrane thickness, or material type of the PVDF membrane filters being utilised, had influenced the diffusion capabilities of the lipophilic molecules (Ng *et al.*, 2010:210). Although the test results could also be linked to the degree of tretinoin isomerisation (degradation), these membrane release studies confirmed that the API had been released from the emulsion formulation.

5.3.2 Diffusion study outcomes

The tretinoin content in the skin layers and in the Franz cell receptor chambers were quantified by HPLC analysis, as described in Appendix A. During the initial diffusion study, with hourly sampling intervals, the concentrations of the tretinoin and isotretinoin were too low to calculate the flux values (µg/cm².h). As a result, the diffusion study was repeated, but was only one extraction per Franz cell sample done after 12 h. The concentration of the tretinoin that had permeated the dermatomed skin and reached the receptor compartment was within the quantification limit. The average cumulative amount per area (µg/cm^2), the average diffused percentage and the average concentration (µg/ml) of tretinoin and of isotretinoin that had been retained in the receptor fluid, are summarised in Table 5.3.

Table 5.3: The average cumulative amount per area (µg/cm^2), the average diffused percentage and the average concentration of tretinoin and isotretinoin, measured in the diffusion study samples withdrawn after 12 h (n = 10)

API and degradant	Average cumulative amount per area (µg/cm^2)	Average diffused percentage (%)	Average concentration (µg/ml)
Tretinoin	0.673	0.0710	0.362
Isotretinoin	0.090	0.0095	0.049

The concentration of the tretinoin that had diffused into the receptor fluid beneath the dermatomed skin after 12 h, was low at approximately 0.07% of the applied dose. This may have partly been as a result of the degradation of the tretinoin, from sample preparation through to HPLC analyses. According to the literature also, during *in vitro* studies, performed on excised human skin, retinol systemic absorption had varied between a 0.3% to 1.3% recovery of the applied dose after 24 h, depending on the type of vehicle used (Yourick *et al.*, 2008: 119). The emulsion formulation may thus also have influenced the systemic absorption values being achieved. Since the possible impact of the delivery vehicle during this study was not investigated, and because the different vehicle types were not compared, the extent of its possible influence on the diffusion outcomes could not be estimated.

5.3.3 Tape stripping outcomes

The concentration of the API that is present in the skin is measured to determine the extent of total percutaneous absorption (OECD, 2004:24).

The stratum corneum-epidermis (SCE) has a lipophilic nature, whereas the epidermis-dermis (ED) has a more hydrophilic nature. The lipophilic character of the tretinoin suggests that higher amounts of tretinoin would be retaineded within the lipophilic stratum corneum that may produce

a reservoir system within the skin (Bagatin *et al.,* 2015:87; Yourick *et al.*, 2008:120). Lipophilic compounds are therefore understood to mainly penetrate into a skin reservoir, resulting in a much smaller concentration in the receptor fluid (Yourick *et al.*, 2008:120).

According to the tape stripping results of this study (Table 5.4), the average concentration of tretinoin that had been retained in the SCE (tape strips), was 0.019 µg/ml, whereas a slightly higher concentration of 0.027 µg/ml was located within the ED (skin cuttings).

Table 5.4: Average concentration of tretinoin and isotretinoin in the SCE and ED from tape stripping, following the 12 h skin diffusion studies (n = 10)

API and degradant	Average concentration in SCE (µg/ml)	Average concentration in ED (µg/ml)
Tretinoin	0.019	0.027
Isotretinoin	0.005	0.006

During this study, the 0.362 µg/ml of tretinoin that had been recovered from the receptor fluid, was notably higher than the concentrations of 0.019 µg/ml and 0.027 µg/ml found in the SCE and ED, respectively. The results indicated that higher concentrations of the tretinoin had diffused into the receptor fluid, than what had been retained within the skin layers. The emulsion stabilising surfactant, Tween 80®, together with the EFAs present in the rosehip oil, may have solubilised and extracted the inter-cellular stratum corneum lipids, causing a disruption to the skin barrier function, which increased the diffusion of the API molecules (Bigucci *et al.*, 2015:1018, Riviere, 1993:123). Although the magnitude of the penetration enhancement effects of the utilised excipients remained uncertain, they may have contributed towards the measured tretinoin quantities in the receptor solutions of the Franz cell samples. It was further possible that the formulation's water component, which had been directly applied to the stratum corneum-epidermis, may have increased the skin hydration levels. It is known that water causes the swelling of the corneocytes, which then expands the compact structure of the stratum corneum. The hydration effect alters the partitioning and concentration gradient of the penetrating molecules, which also increases skin permeability to enhance API permeation (Riviere, 1993:117, 118; Zatz, 1993:128). The API's permeation may have hence been increased, whilst the normal skin lipid reservoir system may have been disrupted.

5.4 Conclusion

In vitro diffusion through a synthetic membrane is regarded as a suitable method for the assessment of an API's release from a formulation vehicle and is it therefore not a rate limiting step for API penetration and partitioning into the skin (Coneac *et al.,* 2015:901).

The extent of the penetration of tretinoin during the synthetic membrane release studies was, as anticipated, much higher than the results obtained from the skin diffusion studies. Several factors may have contributed towards this. Because no biological diffusion processes are represented by means of the synthetic membrane being utilised, it may lead to an over-estimation of dermal absorption. The membrane release studies gave an indication of the tretinoin concentration that had been released from the formulation vehicle, whereas during the skin diffusion studies, the ability of the delivery vehicle to release the drug at the local site, had been limited by numerous other factors, including drug-skin and vehicle-skin interactions, in addition to the drug-vehicle factors, as exhibited during the membrane release studies (Shin *et al.*, 2005:67). The release of tretinoin from the formulation was confirmed by the outcomes of the membrane release studies.

Due to tretinoin's lipophilic character, it should be artificially retained in the lipophilic stratum corneum layer, even when a high solubility receptor solution is utilised to solubilise the API (Bagatin *et al.,* 2015:87; OECD, 2004:24; OECD, 2011:72). The degree of artificial retention of such a lipophilic test compound in the skin may vary according to the API's physicochemical characteristics (Bagatin *et al.,* 2015:87; OECD, 2004:24; OECD, 2011:72). The deposition of the lipophilic molecules in the stratum corneum and appendices causes the API to be retained within the skin, which would typically decrease its diffusion rate and limit transdermal delivery (OECD, 2011:72), which would then cause typical lipophilic substances to take longer to migrate from a skin layer/depot to the receptor phase solution (OECD, 2011:28). As a result, the highest concentration of tretinoin would be retained within the stratum corneum (Bagatin *et al.,* 2015:87). However, following the assessment of the data obtained from this study, it was revealed that a higher concentration of tretinoin had been present in the receptor phase solution, than in both the SCE and ED. The excipient components that had been utilised in the test formulations may have contributed toward this outcome.

Surfactants solubilise the lipids that are present in the stratum corneum, which would then alter API permeation, where diffusion through the inter-cellular lipid structure is considered the main pathway (Roberts *et al.*, 2002:96-97). Although non-ionic surfactants, such as Tween® 80 (polyethylene glycol sorbitan mono-oleate) exhibit a smaller effect, when compared to the lipid solubilisation effect being demonstrated by ionic surfactants (i.e. sodium lauryl sulphate), such effect may still contribute towards an increase in the diffusion rate (OECD, 2011:42; Sigma-Aldrich, 2015b). Fatty acids are also known to cause alteration of the skin lipids structure.

Incidentally, Tween® 80, which had been utilised as a surfactant in the test formulation, is composed of at least 58% of oleic acid, which had possibly also facilitated and contributed towards a permeant penetration enhancing effect (Sigma-Aldrich, 2015b). As a result, the target deposition area, as required for cosmetic formulations, had not been maintained and had tretinoin not been retained within the stratum corneum reservoir system.

Rosehip seed oil, such as *Rosa rubiginosa*, contains high quantities (approximately 60%) of PUFAs, of which approximately 15% consists of oleic acid (Adamczak *et al.,* 2011:57). It has been established that oleic acid may potentially contribute for up to a twenty-eight-fold increase in the flux (OECD, 2011:41).

However, according to the literature, the penetration enhancer effect had been more extensive for hydrophilic, than for lipophilic permeants (OECD, 2011:41). The degree of the penetration enhancing effect is therefore uncertain. It was possible that, apart from the penetration enhancing effect of the vehicle and the oil phase, a possible synergistic effect may have impaired the barrier protection function of the stratum corneum and increased the diffusion coefficient of the API into the skin (Coneac *et al.,* 2015:902). Additional future studies may be done to investigate the absolute effect of the vehicle in similar emulsion formulations.

Tretinoin concentrations in the SCE and ED represented the extent of its topical delivery, whereas transdermal delivery was defined by the concentrations of the tretinoin being detected in the receptor chambers (Bagatin *et al*., 2015:86). During this study, therefore, transdermal delivery of tretinoin had been achieved and had approximately 0.07% (0.362µg/ml) of the applied dose diffused through the skin, as was measured in the samples from the receptor phase solutions of the Franz cells.

By employing validated methods, *in vitro* skin diffusion studies are used to quantify the penetration of molecules into and their permeation across the skin. *In vitro* study methods are therefore applied to predict *in vivo* API absorption (Brain *et al.*, 2002:198; ICPS, 2006:38, 79). During this study, it was observed that transdermal delivery, which is a known multi-faceted process, had been influenced by several factors. The additional variation and complexity when working with "natural" constituents, may lead to questioning the accuracy of such a permeation prediction, such as had been confirmed during this study (OECD, 2004:24; Williams, 2003:28). During future studies, therefore, *in vitro* diffusion studies that aim at investigating the various significant elements that influence the transdermal delivery process, would add much value.

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Chapter 6

Article for publication: International Journal of Cosmetic Science

Chapter 6 is written in article format for the purpose of its submission for publication in the International Journal of Cosmetic Science. The complete guide for authors of this journal is included in Appendix D.

The formatting of this article chapter is in accordance with the requirements of the guide for authors, except for the paragraphs that were justified for ease of reading and for neatness.

In vivo clinical efficacy investigation of applications, containing *Rosa rubiginosa* rosehip seed oil, sourced from Southern Africa

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ABSTRACT

Objectives: The objectives of this study were to investigate skin moisturisation, elasticity and topography changes, following the application of two investigational products, containing *Rosa rubiginosa* (rosehip) seed oil.

Method: Two investigational cosmetic products (ICPs), i.e. an emulgel, containing 20% (w/w) of *R. rubiginosa* rosehip seed oil and 100% *R. rubiginosa* rosehip seed oil (hereinafter also referred to as rosehip oil) were assessed. *In vivo* clinical efficacy trials included a short-term (4 h), single application pilot study, followed by a longer term (84 days), multiple application study. Non-invasive, bio-engineering methods and instruments were utilised to record any possible changes in the skin parameters.

Results: Compared to untreated skin, the short-term skin moisturising study had achieved a statistically significant difference ($p < 0.05$) for both the ICPs tested. The maximum increase in skin hydration (± 35%) was obtained 60 min after a single application of the pure rosehip seed oil. In the longer term study, statistically significant ($p < 0.05$) skin moisturising effects were observed for both the test products. The maximum moisturising effect by the rosehip emulgel (± 19%) was recorded after 28 days, whereas the maximum hydration increase by the 100% rosehip oil (± 21%) was obtained after 84 days. The changes in skin wrinkles and smoothness were evaluated by means of the wrinkle parameter (SEw) and the smoothness parameter (SEsm), respectively, by using the surface evaluations of the living skin (SELS) method. The change in SEw showed a statistically significant difference ($p < 0.05$) for each ICP, compared to untreated skin areas. For both test products, a maximum wrinkle decrease of approximately 7% was recorded after 56 days. Although the changes in skin smoothness were statistically significantly different ($p < 0.05$) for each test product, compared to untreated skin, the clinical changes being reflected by this parameter did not support a favourable clinical outcome. The visco-elastic property assessment revealed improvement of the skin's stretch capability and was a maximum clinical effect achieved after 84 days.

Conclusion: Both ICPs had significantly improved skin moisture levels and had they furthermore demonstrated beneficial anti-wrinkle and visco-elastic properties. This study demonstrated that *R. rubiginosa* seed oil may be beneficial for the improvement of skin wrinkles and the appearance thereof, when incorporated into cosmetic formulations.

Key words: *Rosa rubiginosa (rosehip seed oil)*; *in vivo*; claim substantiation; skin hydration; skin topography

INTRODUCTION

The utilisation of plant extracts, mixtures of oils and other plant materials for personal care purposes is not uncommon practice. Skin care products had been prepared for ages, long before technical formulation design, clinical efficacy testing, or manufacturing technology were implemented. Traditionally, parts of plants were collected, processed and incorporated into any possible delivery vehicle available, prior to application to the skin.[1], [2]

Members of the *Rosaceae* plant family are versatile plant groups and are the rosehips, seeds, petals, flowers and fruits in various stages of development being sourced, according to the phytochemical content of the plant segment. *Rosaceae* components are frequently used in the food, pharmaceutical and cosmetic industries.[3], [4] The food industry often regards rosehip seeds, from which a vegetable oil is extracted, as a waste material. As a result, this oil that is commonly used in the cosmetic industry, is regarded as an inexpensive, natural raw material.[5], [6], [7]

Rosa rubiginosa (*R. rubiginosa*), also known as *R. eglanteria, R. mosqueta,* or sweetbriar (common name), belongs to the species of the genus, *R. linnaeus*, which is categorised under the *Rosaceae* family [8] These invader species display rapid growth and do they spread effortlessly, covering large ecological areas. *R. rubiginosa* adapts with ease to new ecological regions and exhibits high invasive abilities. Consequently, *R. rubiginosa*, that had originally been endemic to Europe, is now thriving and covering various areas in Chile, Spain, Argentina, New Zealand, Australia and Southern Africa.[3], [5], [9], [10]

In South Africa, *R. rubiginosa* is a declared Category 1 invader in terms of Section 29 of the Conservation of Agricultural Resources Act (Act no. 43 of 1983) and may it therefore not be planted, harvested, nor traded.[11], [12] However, in the Lesotho mountains in Southern Africa, isolated rural communities are allowed to harvest *R. rubiginosa* rosehips by hand and sell them to production companies. Harvesting of the *R. rubiginosa* rosehips and plant material, from a conservation point of view, has a positive impact on the ecosystem through the management and control of invader plants, whilst positively contributing towards the sustainability of rural communities' economies.[13], [14], [15] According to the literature, valuable cosmetic constituents are found in rosehip seed oil. This vegetable oil contains vitamin C, tocopherols, phytosterols, β-carotene, various bio-flavonoids, all-*trans*-retinoic acid (tretinoin) and relatively high concentrations of saturated and unsaturated fatty acids.[4], [5], [16] It has been established that *R. rubiginosa* rosehips contain high levels of poly-unsaturated fatty acids (PUFAs) of approximately 62.91 ± 3.64%. The PUFA contents reportedly consists of 37.25 ± 2.40% linoleic acid (C18:2) and 25.65 ± 1.92% α-linolenic acid (C18:3). Another relatively high fatty acid content being identified in the oil, was 11.12 ± 0.83% of oleic acid (C18:1).[3] Its anti-oxidant activity, established through the determination of the contents of the phenolic

compounds (natural anti-oxidants) in the seed lipids, has been documented as values of up to 140.5 mg/100 g of dry matter.[16] Furthermore, a phytochemical analysis demonstrated that whole rosehips from the *R. rubiginosa* plant material, contained an average flavonoid content (expressed as quercetin equivalent) of 72.0 mg/100 g of dry mass.[17]

In light of all of the bio-active constituents present in this oil and the structure of the skin, its possible positive impact on topical skin care product formulations seems obvious. The ceramides, for instance, which contain fatty acids, are significant components of the skin's cornified lipid envelope system, responsible for covalently binding the corneocytes. The free fatty acids that are located in the stratum corneum are predominantly long chain, saturated fatty acids. Two unsaturated fatty acids that are also present in rosehip oil, namely oleic- and linoleic acid, are also present (unbound) of the stratum corneum.[18], [19]

Important functions of the skin's lipid matrix system include the prevention of trans-epidermal water and salt loss, the obstruction of water soluble substances from penetrating the epidermal layers and its contribution towards the tight cohesion among the corneocytes. Deficiencies in the stratum corneum lipid composition may result in skin barrier function abnormalities, which in turn would affect skin barrier permeability.[18], [19], [20], [21] Altered epidermal lipid composition (either due to dermatological diseases, or nutritional fatty acid deficiencies) may cause a disruption of the healthy skin barrier function and an increase in inter-cellular molecule permeation.[22]

The topical application of fatty acids, such as linoleic acid, may therefore stabilise the skin’s fatty acid balance by preventing a skin lipid ceramide deficiency, or an impaired production of ceramides and lipids in the skin, which would prevent excessive drying of the skin and its inability to facilitate the regeneration of the skin barrier function.[18] The topical application of fatty acids bypasses metabolic conversion in the liver, causing linoleic acid to be more readily available for use in the skin, than when acquired through the diet. According to the literature also, topically applied linoleic acid has proven to decrease both trans-epidermal water loss (TEWL) and wrinkle formation.[18], [23] Label claims with regards to cosmetic formulations that contain *R. rubiginosa* seed oil generally include those ranging from the improvement of skin damage (such as ultraviolet (UV) irradiation and skin deterioration from overexposure to the sun), scars from surgery, stretch marks, eczema, psoriasis, wrinkles and premature skin ageing, hyper-pigmentation, dermatitis, burns (including those from radiation and sunburn), as well as age spots. Rosehip seed oil is considered a safe, inexpensive and effective ingredient for use in "skin repair" formulations.[24], [25], [26] The lack of validated scientific information enables skin care product owners to push the boundaries of formulation innovation, whilst utilising unsubstantiated label claims as a marketing tool. Contributing towards the complexity of this issue, currently very few "naturally" derived components are standardised and scientifically

monographed ingredients.[14] To date, no information is available regarding any clinical studies being performed on *R. Rubiginosa* rosehip seed oil that is sourced from Lesotho. Since geographical distribution and different extraction methods may contribute towards a variation in the plant material contents and because of the mere extent of unsubstantiated label claims found in the literature, an investigation of the clinical efficacy of this specific rosehip seed oil was deemed necessary. For the purpose of this study, *in vivo* clinical efficacy studies of *R. rubiginosa* seed oil, harvested from the Lesotho mountains in Southern Africa, were conducted.

The objectives of the clinical studies during this project were to substantiate any label claims, related to changes in skin hydration, -elasticity and -topography. A basic cosmetic oil-in-water (o/w) emulsion, stabilised in a dispersion gel system (emulgel), was formulated.[27] This basic topical formulation excluded any non-essential excipients, hence any ingredients that would not contribute towards the stability of the formulation, in order to minimise any possible clinical skin effects by excipients.[28] Because emulsions generally are thermo-dynamically unstable systems, emulsifiers, such as surfactants and various viscosity enhancers, were incorporated into the formulation. Modification of the dispersion media, by forming an emulsion gel structure (emulgel), was employed to increase the stability character of this unstable emulsion system.[29], [30]

MATERIALS AND METHODS

Test materials

The active ingredient, *R. rubiginosa* seed oil, was purchased from The Rosehip Company (Mohale's Hoek, Lesotho). The excipients that were used in the investigational test formulation included methylparaben, propylparaben and Tween® 80 (PEG-20 sorbitan mono-oleate), acquired from Merck Laboratory Supplies (Midrand, South Africa). Carbopol® Ultrez 21 was acquired from Lubrizol Advanced Materials (Brussels, Belgium). Butylated hydroxytoluene (BHT) was obtained from Sigma-Aldrich Corporation (Steinheim, Germany). Deionised water, throughout the duration of this study, was prepared with a Milli-Q® water purification system, equipped with a Millipak® 40 Millipore (0.22 µm) water filter system (Millipore, Milford, USA). Sodium hydroxide (NaOH) pearls (97% purity) were acquired from Merck Laboratory Supplies (Midrand, South Africa) for use in pH adjustments.

Preparation of the newly formulated emulgel

A newly formulated emulgel, containing 20% of rosehip seed oil, was prepared during this study.

To prepare the emulgel, the respective ingredients were weighed and added into two separated glass containers, i.e. one container for the preparation of the dispersed oil phase and another for the continuous water phase, as summarised in Table I. Both glass containers were

separately placed on Labcon® MSH10 magnetic stirrer plates for 10 min to ensure proper wetting and mixing of the ingredients. A cold processed method was followed to limit degradation of the unstable tretinoin and no external heat was applied during the preparation process.

Table I: Formulation ingredients and their functionalities, used in the preparation of a 20% (w/w) rosehip seed oil emulgel.

Following the wetting and dissolving of the ingredients, the oil phase and water phase mixtures were homogenised at 13,500 rpm for 5 min, utilising a Heidolph Diax 600 homogeniser (Labotec, Halfway House, South Africa). Thereafter, the continuous phase mixture was kept in the homogeniser, while the oil phase was added sparingly. Following the complete addition of the dispersion oil phase to the continuous water phase, the mixture was homogenised for another 10 min at 13,500 rpm. Finally, the pH was adjusted to approximately 5.5, with a 2 M NaOH (8% m/v) solution.

In vivo clinical studies

Mono-centric, open label, non-randomised, comparative *in vivo* clinical efficacy studies were performed on the prepared emulgel formulation. All clinical studies were conducted in accordance with the World Medical Association (WMA) Declaration of Helsinki's ethical principles of medical research, involving human participants and consistent with the principles of the International Conference on Harmonisation (ICH) Good Clinical Practice (GCP).[31] These studies were approved beforehand by the institutional ethical review board, i.e. the North-West University Research Ethics Regulatory Committee (NWU RERC), under the umbrella application that is titled "(*In vivo*) cosmetic efficacy studies" (approval number: NWU-00097-10-A5). Participant enrolment followed after written informed consent had been obtained from each participant. All participants were allowed to retract their informed consents at any stage during the studies.

Equipment used during the *in vivo* clinical studies

All measurements were performed with non-invasive, bio-engineering instruments to quantify the clinical effects. Measurements were carried out following an acclimatisation period of at least 30 min in environmentally controlled conditions of 20 - 25°C and 40 - 60% relative humidity (RH).

Skin capacitance tests were performed to measure skin moisture, using a Corneometer® CM 825 (Courage & Khazaka, Cologne, Germany). The Corneometer® measures the water content of the stratum corneum (skin hydration) by means of the capacitance method. These measurements are then converted into arbitrary Corneometer® units, ranging from 0 to 120.[32], [33], [34] Since water has the highest di-electrical constant in the skin, an increase in the capacitance values would be indicative of an increase in the water content of the skin (skin hydration).

Changes in dermal connective tissue, as a result of ageing, are investigated by measuring the mechanical properties of the skin. As the skin ages, its elasticity and ability to recover from deformation decrease. These changes in mechanical properties are assessed by using various non-invasive, bio-engineering instruments.[35], [36] To evaluate skin firmness and tone, the Cutometer® (Courage & Khazaka, Cologne, Germany) was utilised to measure the visco-elastic properties of the epidermis (upper skin layer).[35], [36], [37]

The Cutometer® MPA 580 (Courage & Khazaka, Cologne, Germany) deforms the upper layer of the skin mechanically, by applying a negative pressure of 200 - 400 mbar to the skin, which pulls the upper skin layer into a 2 mm probe aperture for 2 sec. A non-contact optical measuring system calculates the penetration depth of the skin inside the probe.[37] The Cutometer® MPA 580 software analyses the measurements of the deformation-relaxation profile of the skin to generate a computerised skin deformation curve, from which the R-parameters are calculated. Skin visco-elastic properties are characterised and quantified by the following R-parameters: skin distensibility (R0), gross elasticity (R2), stretch capacity (R6), elastic recovery (R7) and total recovery change (R8).[37], [38]

Skin surface analyses are used to evaluate skin condition and any changes thereof. Various methods have been developed through which skin surface properties can be investigated, by using non-invasive, digital imaging, the analyses of skin replicas and counter-replicas of skin surface, or additionally, through examination of the skin surface's scaliness by employing adhesive tape stripping methods.[39], [40] For the purpose of this study, the Visioscan® VC 98 (Courage & Khazaka, Cologne, Germany) was used to evaluate changes in skin topography through digital imaging. The Visioscan® VC 98 emits an ultraviolet A (UVA A) light and records high resolution (256 grey pixel level) skin surface images. Light or bright pixels, visible on the images, reveal skin scaliness, whereas dark areas depict wrinkles. The skin, as represented by these images, are analysed by computer software, utilising the surface evaluation of living skin (SELS) method to generate SELS parameters, which are useful for quantifying any changes in skin topography.[41] The following SELS parameters were used to evaluate changes in skin topography: skin scaliness (SEsc), skin roughness (SEr), skin smoothness (SEsm) and skin wrinkles (SEw).

Study design: short-term (4 h) single application and long-term (84 days) multiple application studies

A short-term (4 h), single application and a longer term (84 days), multiple application study were performed. The two investigational cosmetic products (ICPs) that were assessed during this study included 100% (w/w) *Rosa rubiginosa* seed oil and a topical emulgel formulation (as described above), containing 20% (w/w) of rosehip seed oil.

During the short-term study, baseline (T0) skin measurements were performed and were the ICPs applied directly thereafter. Measurements were performed at 60 (T1), 120 (T2) and 240 (T3) min, following application. The total duration of the short-term study was approximately 5 h (including acclimatisation of 30 min and the time needed to perform the baseline measurements).

The longer term multiple application study was completed over a period of 84 days and were measurements performed on days 28 (T1), 56 (T2) and 84 (T3). The ICPs were applied twice daily, as per the test protocol and the application schedule. The treatment areas (20 x 40 mm) were demarcated on the volar forearms of the participants by means of a stencil and a non-toxic skin marker. This consistent demarcation of areas with a stencil facilitated the application procedure of the ICPs and ensured that corresponding areas on all participants were subjected to treatment and evaluation. The stencil also ensured repeatable evaluation of the same treated skin area at each subsequent measurement interval.

The active ingredient dose was pre-calculated and ranged between 1 and 3 mg/cm^2, when applied to the skin treatment areas.

Any changes in skin condition, relative to the initial (T0) values, were determined for each participant. The short-term study comprised of a skin hydration assessment, whereas skin hydration, topography and visco-elastic properties were assessed during the longer term study.

Study participants

To minimise inter-participant variations, pre-determined inclusion and exclusion criteria, relating to gender, age, ethnicity or skin type, and health conditions, were included in the trial protocol and strictly adhered to during the recruitment and screening processes.[42]

Healthy Caucasian (Fitzpatrick skin type I-III) females, between the ages of 20 - 55 years, neither pregnant nor breast feeding, not suffering from known chronic diseases, such as diabetes or thyroid conditions, with no known dermatological diseases, qualified for taking part in the study. Participants had to refrain from consuming any caffeine, or alcohol on those days that measurements were taken. A list of prohibited medication, which could cause cutaneous

micro-vascular vaso-dilatation or –constriction, or that could possibly contribute towards variations in the measurements, was given to each participant. Participants had to inform the investigator of any changes in their state of health, or of any pharmacological treatments taken during the study, as well as whether they had experienced any adverse effects, related, or non-related to the treatment with the ICPs. All participants received a pH balanced soap for use the whole week prior to each study (wash-out period) and were they instructed not to use any other skin care products on the treatment areas, until final measurements were performed.

Table II: Participant disposition table for each clinical trial.

Statistical analysis

Raw data, representing the various skin parameters, were subjected to statistical analysis. To gain a better understanding of the significance of the changes in skin parameters as a function of the duration of each treatment, the data was converted by means of Equation 1 to express the change in a parameter value as a percentage, relative to the baseline value (T0) of that particular parameter.

$$\text{Percentage (\%) Change} = ((Tn-T0)/T0) \times 100 \qquad \textbf{Equation 1}$$

Where:

T0 = baseline value.

Tn = Measurement value at given time with n = 1 (28 days), n = 2 (56 days) and n = 3 (84 days) for the long-term study and n = 1 (60 min), n = 2 (120 min) and n = 3 (240 min) for the short-term study.

The percentage (%) change data for the various skin parameters was also subjected to statistical analysis. Interaction analyses were performed by employing a mixed analysis model, with fixed repeated measures concerning the treatment and an analytical review (AR(1)) covariance structure. Statistical significance was determined at a 95% confidence interval and a p-value of < 0.05. Restricted maximum likelihood value analyses (Type III tests of fixed effects) were used to determine any statistically significant differences among treatments, exposure times and interactions with regards to time (duration) and treatment. It was assumed that the random participant samples being used during this study were equivalent and representative of a population that would normally apply such treatments.

Pairwise comparisons between treatments were performed, based upon an estimated marginal means (mean value of measurements obtained from T0 - T3), using Sidak adjustments for multiple comparisons at a 95% confidence interval for differences ($p < 0.05$), to determine statistically significant differences.

Univariate F-tests, based upon the linearly independent pairwise comparisons between the estimated means of each treatment at each measurement interval, were performed to determine the treatment effect size. In addition, Cohen's d-value was used to evaluate the practical significances of observed differences. The extent of any effect (effect size) (d) ≈ 0.20 would be indicative of a small effect, or practically non-significant difference, d ≈ 0.50 would point towards a medium effect, or practically visible difference, whereas d ≈ 0.80 would signify a large effect, or practically significant difference.[43]

RESULTS AND DISCUSSION

The effect of a single ICP application on skin hydration

Pairwise comparisons, based upon estimated marginal means, using Sidak adjustments for multiple comparisons, revealed statistically significant differences ($p < 0.05$) for the ICPs, i.e. for the emulgel ($p < 0.0001$) and for the rosehip seed oil ($p = 0.007$), when compared to their respective untreated skin areas.

Both ICPs had increased skin hydration, relative to the baseline (T0) measurements, with a maximum mean percentage hydration increase being observed after 60 min. Rosehip seed oil had improved skin hydration (mean ± SD) with 34.56 ± 15.82% after 60 min, followed by 20.46 ± 11.04% and 20.26 ± 17.14% at 120 min and 240 min, respectively (determined using Equation 1). The emulgel had improved skin hydration by 20.29 ± 10.86% at T1 (60 min), 11.26 ± 8.27% at T2 (120 min) and 10.96 ± 9.41% at T3 (240 min) (Fig.1).

Figure 1: Mean percentage changes in skin hydration levels relative to T0 for the short-term study.

Although both ICPs had improved skin hydration over time, pairwise comparisons, based upon estimated marginal means, using Sidak adjustments for multiple comparisons, revealed a statistically significant difference between the extent of hydration being achieved by the emulgel, compared to the rosehip seed oil ($p = 0.003$). This statistically significant difference was also supported by the results that were obtained when performing univariate F-tests. Univariate F-tests, based upon linearly independent, pairwise comparisons of the estimated means of the

effect of each treatment over time, were performed to determine the magnitude of the effect (Cohen's d-value). Cohen's d-values were indicative of the extent to which skin hydration had been increased at each time interval and revealed that significantly larger effects, as summarised in Table III, had been achieved by the rosehip seed oil.

Table III: Extent of the effects of each treatment relative to the initial skin hydration level (T0).

Topically applied products may increase skin hydration levels, by either supplying water directly to the skin surface, or indirectly through occlusion. Occlusion prevents TEWL from the stratum corneum into the atmosphere, by trapping water in the stratum corneum, to subsequently increase skin hydration levels. It is therefore possible that, in the case of rosehip oil, the higher increase in skin hydration after a single application could have been attributed to the occlusion properties of the rosehip seed oil.[44] The initial improvement in skin hydration that had been achieved with the emulgel, may have been due to the emulgel-water portion that had been applied directly to the skin surface. The relatively smaller oil component, compared to the rosehip oil, may have contributed towards the fact that a similar occlusive effect had not been achieved with the emulgel. Subsequent evaporation of the water being applied to the skin was hence not hindered, due to insufficient occlusive properties that had been revealed by the emulgel. Consequently, the TEWL had not been reduced to a similar extent, as observed with the rosehip seed oil and as a result, no longer, sustained skin hydration effect had been achieved.[21], [22], [45], [46], [47]

The effect of multiple, longer term ICP applications on skin hydration

Following the long-term multiple applications, a maximum increase in skin hydration was observed for rosehip oil after 84 days and for the emulgel after 28 days (Fig. 2).

Figure 2: Mean percentage changes in skin hydration levels relative to T0 for the long-term study.

The effect of the rosehip seed oil treatment, as indicated by the mean percentage changes in skin hydration (Equation 1), revealed that the skin hydration levels (mean ± SD) had increased by 15.78 ± 16.90% after 28 days and by 21.36 ± 26.33% after 84 days.

With regards to the emulgel, the maximum hydration effect had been achieved after 28 days of twice daily applications and had the baseline skin hydration level been increased by 18.52 ± 24.78%. After 56 days, the analysis of the emulgel data revealed a 12.31 ± 25.48% improvement in skin hydration. This hydration effect seemed to have decreased over time, with no practically significant differences in skin hydration being observed from baseline up to day 84 (Fig. 2 and Table IV).

The information in Fig. 2 was further elucidated by the univariate F-tests, based upon linearly independent, pairwise comparisons of the estimated means of the effect of each treatment over time, during which the magnitude of the effect, Cohen's d-value, was determined. Practically significant differences, indicative of a hydration effect, were observed for both the rosehip seed oil and emulgel ICPs (Table IV). Thus, similar to the clinical effect, as depicted in Fig. 2, the maximum extent of the skin hydration effect, relative to the baseline value for the emulgel, had been achieved after 28 days, whereas the maximum increase in hydration by the rosehip seed oil had been achieved after 84 days.

Table IV: Extent of the effects of each treatment relative to the initial skin condition (T0).

Pairwise comparisons, based upon the estimated marginal means, using Sidak adjustments for multiple comparisons, were performed and were statistically significant differences ($p < 0.05$) found with regards to the interaction of the emulgel and the skin, relative to untreated skin ($p = 0.001$), and between rosehip seed oil and the skin, compared to untreated skin ($p = 0.012$), but not amongst the effects being achieved by the emulgel and the rosehip seed oil ICPs ($p = 0.902$). These outcomes suggested that interactions between the emulgel and rosehip oil had demonstrated similar clinical effects with regards to interactions over time, which had significantly differed from the untreated skin area, providing evidence of the beneficial results by both ICPs.

Skin topography analyses

Visioscan® VC 98 (Courage & Khazaka, Cologne, Germany) measurements were performed to investigate any possible skin topography changes as a result of the application of the two ICPs. The following SELS parameters were investigated: SEsc, SEsm, SEr and SEw. The SEsm values are determined from the average width of the wrinkles, and would a positive change (higher SEsm values) be indicative of an increase in skin smoothness and would it also suggest an increase in the skin moisturising effects.[41] In addition, a negative change in the SEsc, SEr and SEw parameters would also be indicative of an improvement in the skin's condition, because of a reduction in the scaliness, roughness and wrinkle appearances of the skin.

Pairwise comparisons, based upon estimated marginal means over time, using Sidak adjustments for multiple comparisons, by analysing the skin smoothness parameter, SEsm, revealed statistically significant differences ($p < 0.05$) between the effects of the emulgel and rosehip seed oil on the skin ($p < 0.0001$), as well as between the emulgel ($p < 0.0001$) and rosehip oil ($p < 0.0001$), compared to their respective control areas. This comparison indicated that statistically significant differences had existed between the measured clinical effects of the emulgel and the rosehip oil over time, as well as among the applied ICPs individually, compared to untreated skin. An unfavourable negative change was observed for SEsm, which indicated that skin smoothness had decreased with the use of both ICPs.

These results did not mirror the long-term skin hydration/moisturising results, as were obtained with the Corneometer®. This outcome is important to R&D formulators for consideration when formulating a functional moisturiser. Additional vehicle emollients should be incorporated into the formulation, to purposely fill the spaces in between the upper layer corneocytes, thus to smooth out the stratum corneum skin surface, by providing cohesion and a flattening of the desquamating corneocyte edges.[44]

SEw parameter values were obtained, following the computerised calculation of the average number and average width of the wrinkles. Decreasing SEw values would be indicative of reduced wrinkle appearances over time.[41] Practically significant differences were investigated by employing univariate F-tests, based upon linearly independent, pairwise comparisons of the estimated means of the effects of each treatment over time. The extent of the achieved effects (Cohen's d-value) was calculated (Table V). It is important to note that practically significant differences for SEw were observed, for both the emulgel and rosehip oil, from between T0 to T2 (56 days). Wrinkle appearances had hence been practically significantly reduced, following multiple applications of both the emulgel and the rosehip seed oil, until day 56 (Table V).

Table V: Extent of the effects of treatments over time relative to the initial skin condition (T0).

Coinciding with the results in Table V, the statistical analyses of SEw through pairwise comparisons, based upon the estimated marginal means over time, using Sidak adjustments for multiple comparisons, revealed statistically significant differences ($p < 0.05$) for each ICP, compared to untreated control skin areas. However, no statistically significant differences had existed between the effects of the emulgel and rosehip oil on the skin. The calculated percentage changes illustrated that multiple ICP applications had decreased the skin's wrinkle appearance (mean percentage (%) ± SD) by 7.65% (± 17.61) and 6.25% (± 12.04) for the emulgel and rosehip seed oil, respectively, measured after 56 days (Fig. 3).

Figure 3: Mean percentage changes in skin wrinkle appearances relative to T0 for the long-term study.

Pairwise comparisons, based upon estimated marginal means over time, using Sidak adjustments for multiple comparisons, by analysing the SEsc and SEr, showed no statistically significant differences ($p < 0.05$) between the hydration effects of the emulgel and rosehip seed oil, nor between the emulgel and rosehip oil, compared to their respective control areas.

Statistically significant difference among SEsc and SEr treatments, following the hierarchical linear models to test the effects of the treatment, the time and the treatment-time interactions, were computed. Results indicated statistically significant differences ($p < 0.05$) with regards to the measured areas over time. Together with the results from the preceding pairwise comparisons, a possible time dependent effect for both SEsc and SEr was suggested (Table VI).

Table VI: Pairwise comparisons between treatments and between each treatment and the control.

Skin visco-elastic property assessments

Bio-mechanical parameters, based upon the deformation character of the skin, were evaluated following the long-term, multiple ICP applications. Cutometer® MPA 580 software was used to analyse the measured deformation-relaxation properties of the skin. Calculated R-parameters were obtained from the software generated, deformation curve, for the objective assessment of skin firmness and elasticity.[37], [38]

Figure 4: Typical skin deformation curve, generated from measurements with a Cutometer® MPA 580.

Where:

R0 = Uf, R1 = Uf-Ua, R2 = Ua/Uf, R3 = last maximum amplitude, R4 = last minimum amplitude, R5 = Ur/Ue, R6 = Uv/Ue, R7 = Ur/Uf, R8 = Ua, R9 = R3-R0 (adapted from [37]).

R0, or skin distensibility, represents skin firmness and the skin's passive resistance when negative pressure is applied. R0 is dependent upon skin thickness, where a positive change is indicative of an improvement in skin firming and an increase in stratum corneum thickness.[37] The mean percentage change over time, relative to the baseline measurements, were determined and further analysed. During this study, rosehip seed oil had improved skin firmness by 12.12 ± 18.77% (after 28 days), 5.24 ± 21.83% (after 56 days) and by 32.81 ± 28.54% (after 84 days), relative to T0 (Fig. 5). Treatment with the emulgel, however, revealed that the emulgel and the untreated skin area had followed a similar clinical effect trend, which was indicative thereof that the emulgel application had possibly not influenced skin distensibility, nor improved skin thickness.

Figure 5: Percentage skin firmness changes (R0 parameter) relative to T0.

Figure 6: Percentage gross elasticity changes (R2 parameter) relative to T0.

The R2 parameter is a relatively elastic parameter, representing the gross, or overall elasticity of the skin. Where R2 is closer to 1 (100%), higher skin elasticity properties are illustrated.[37] Positive changes are therefore indicative of an improvement in skin elasticity. Both ICPs had exhibited similar clinical trends by increasing skin elasticity, according to the measurements on

days 28 and 56. Again, this same trend had also been observed for the untreated skin areas, indicating that the R2 parameter changes had possibly not resulted from the ICP application (Fig. 6).

The R6 parameter reflects the stretch capacity of the skin and would a negative change be indicative of an improvement in the skin's condition.[37] Both ICPs exhibited a similar trend with regards to improving the skin's stretch capability, with a maximum improvement relative to T0 being achieved after 84 days (Fig. 7).

Figure 7: Percentage stretch capacity changes (R6 parameter) relative to T0.

The R7 parameter represents the degree of elastic recovery after total deformation, where the "immediate retraction" equals the "final" distension ratio and is a portion of elasticity hence compared to the complete deformation curve. Values closer to 1 (100%) reflect proportionally higher elasticity properties.[37] Positive changes demonstrate an improvement in the skin's elasticity properties. Positive changes were revealed for both the ICPs, after 28 and 56 days, relative to T0. Again, a similar clinical trend was also measured for the untreated skin areas (Fig. 8).

Figure 8: Percentage elastic recovery changes (R7 parameter) relative to T0.

The R8 parameter represents the skin's total recovery and the closer that R8 is to 0, depicted by a negative change, the better the skin's ability to return to its original condition.[37] A similar clinical trend was followed by both ICPs, but neither of the applied ICPs had demonstrated any improvement in the skin's elasticity (Fig. 9).

Figure 9: Percentage total recovery changes (R8 parameter) relative to T0.

CONCLUSION

During this study, beneficial clinical efficacy results for rosehip seed oil were demonstrated. A statistically significant ($p < 0.05$) improvement in skin hydration had been achieved, following

both the single and multiple applications of 100% rosehip seed oil and the 20% rosehip seed oil emulgel.

The results being obtained from the short-term study indicated that statistically significant increases in skin hydration had been achieved from both the emulgel and the rosehip seed oil, following a single application, compared to the untreated skin areas. A maximum hydration increase had been achieved by both ICPs, 60 min after a single application. Noteworthy higher hydration levels were measured for the rosehip oil, compared to the emulsion, which were supported by a statistically significant difference ($p = 0.003$) that had been observed between the two ICPs, and the much larger extent of the hydration effect (Cohens-D-values) by the rosehip oil. Considerably better skin hydration results had been achieved by the rosehip seed oil at 60, 120 and 240 min after single application, than by the emulgel.

The long-term study results indicated that although both ICPs had demonstrated beneficial skin hydration effects, the rosehip emulgel and rosehip seed oil had not followed exact similar clinical trends. The rosehip emulgel had achieved maximum hydration after 28 days, after which all subsequent measurements had constantly decreased. The rosehip seed oil had, however, demonstrated an opposite clinical trend, with the increase in skin hydration having reached a maximum after 84 days of treatment. Statistically significant increases in skin hydration were shown for both the rosehip emulgel and rosehip oil, when compared to the untreated skin areas, as a function of treatment time, thereby providing supportive evidence of the beneficial results for both ICPs.

Since untreated control skin areas followed a similar clinical trend, a possible time dependant effect was suggested for both the SEsc and SEr assessments. Both the rosehip seed oil and rosehip emulgel had decreased skin wrinkle formation, thus improving skin condition, following the long-term, multiple ICP applications.

Contrary to the beneficial results being obtained for skin wrinkle formation and skin hydration assessments, the evaluation of SEsm yielded an unfavourable clinical effect. Results showed that both the rosehip emulgel and rosehip oil had decreased SEsm at all measurement intervals, relative to the baseline values.

The assessment of Cutometer® MPA 580 R-parameters, R0, R2, R6, R7 and R8, initially suggested potential visco-elastic changes, following the long-term, multiple applications. Following the statistical analysis of the R0-parameter, the results demonstrated skin firming and increased stratum corneum thickness properties by the rosehip oil, whilst a similar clinical effect had not been achieved by the emulgel. Further assessment of both ICPs' treatment effects, compared to untreated skin areas, relating to the R2, R6, R7 and R8-parameters, did not indicate any significant differences, nor any clinical effect changes for both ICPs.

Rosehip seed oil had improved wrinkle appearance and skin firmness. These results of a decrease in the skin smoothness, emphasised the importance of employing careful deliberation when choosing the vehicle ingredients. Multi-functional formulation vehicle ingredients should be incorporated to achieve a favourable clinical effect, complementary to the clinical efficacy objective that the formulator wishes to achieve.

To conclude, *R. rubiginosa* seed oil can be considered a valuable and functional ingredient for the improvement of skin hydration, wrinkle appearance and skin firmness. *R. rubiginosa* seed oil contains biologically significant substances, which may be synergistically incorporated into cosmetic formulations.

ACKNOWLEDGEMENTS

This work was carried out with the financial support of the National Research Foundation of South Africa (NRF) (Grants no. IFRR81178 and CPRR13091742482) and the Centre of Excellence for Pharmaceutical Sciences (Pharmacen) of the North-West University, Potchefstroom Campus, South Africa. Statistical analysis was performed by the Statistical Consultation Services of the Potchefstroom Campus of the North-West University.

DISCLAIMER

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

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FIGURE LEGENDS

Figure 1: Mean percentage changes in skin hydration levels relative to T0 for the short-term study.

Figure 2: Mean percentage changes in skin hydration levels relative to T0 for the long-term study.

Figure 3: Mean percentage changes in skin wrinkle appearances relative to T0 for the long-term study.

Figure 4: Typical skin deformation curve, generated from measurements with a Cutometer® MPA 580.

Figure 5: Percentage skin firmness changes (R0 parameter) relative to T0.

Figure 6: Percentage gross elasticity changes (R2 parameter) relative to T0.

Figure 7: Percentage stretch capacity changes (R6 parameter) relative to T0.

Figure 8: Percentage elastic recovery changes (R7 parameter) relative to T0.

Figure 9: Percentage total recovery changes (R8 parameter) relative to T0.

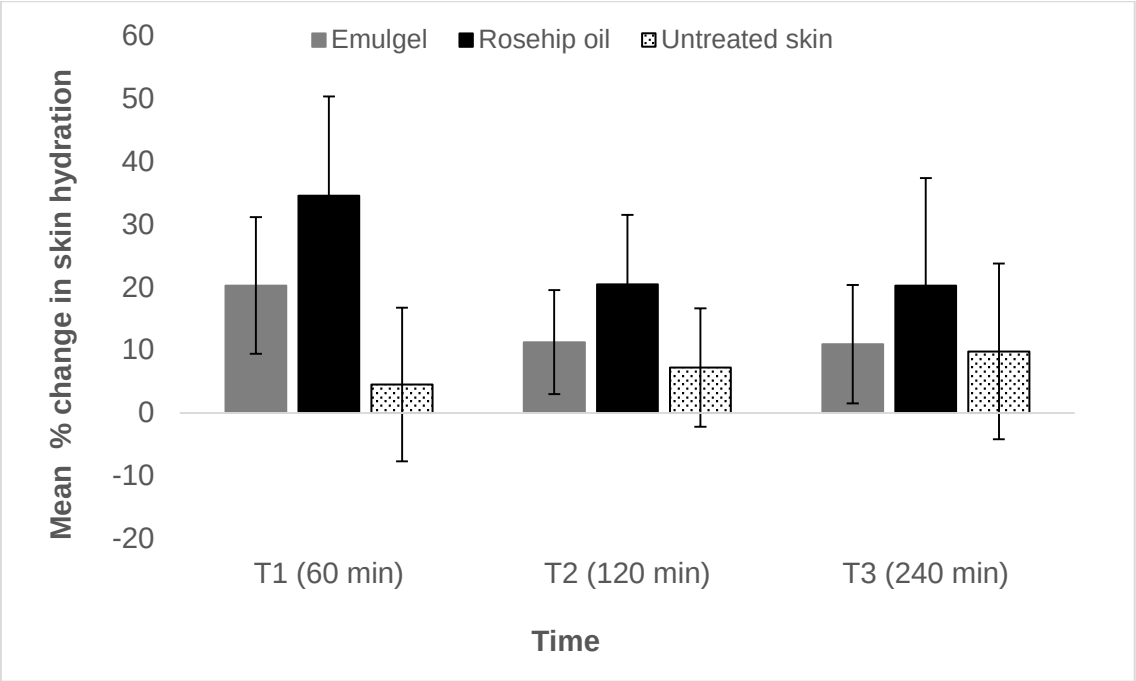

Figure 1: Mean percentage changes in skin hydration levels relative to T0 for the short-term study.

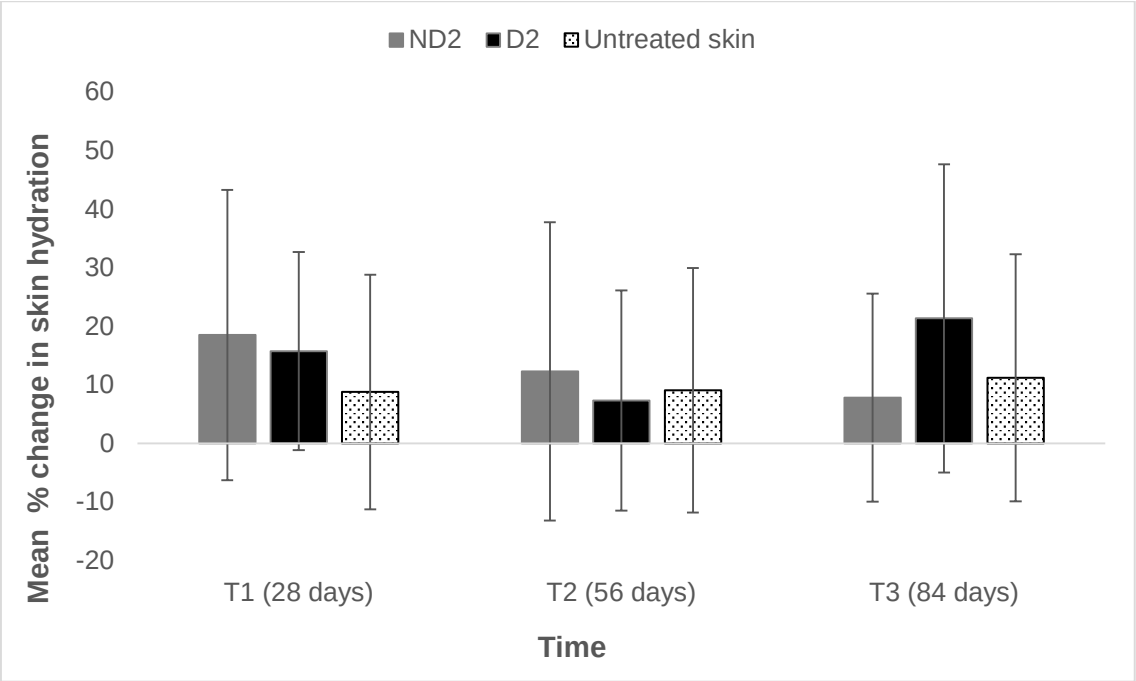

Figure 2: Mean percentage changes in skin hydration levels relative to T0 for the long-term study.

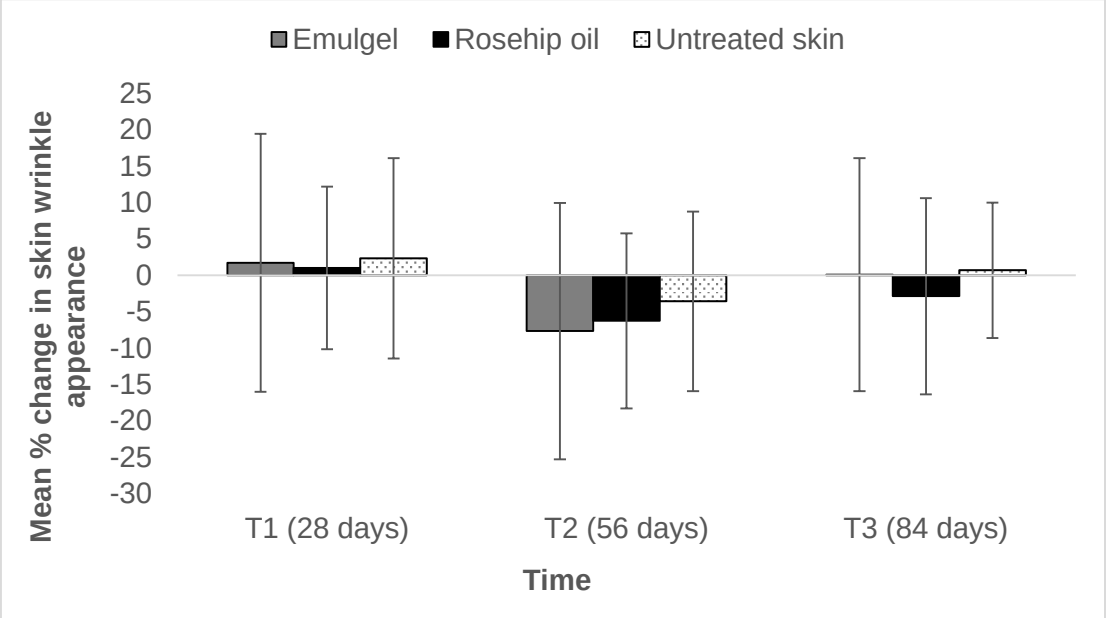

Figure 3: Mean percentage changes in skin wrinkle appearances relative to T0 for the long-term study.

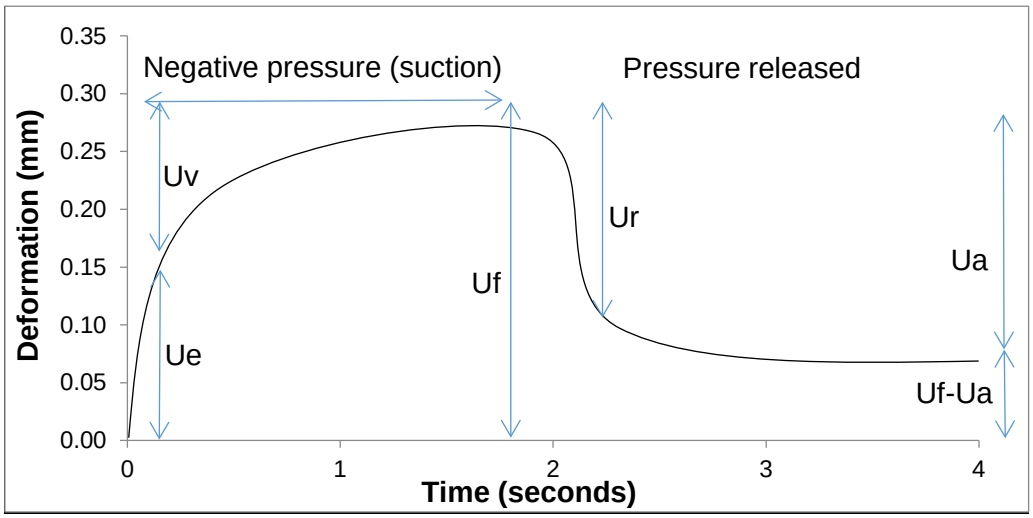

Figure 4: Typical skin deformation curve, generated from measurements with a Cutometer® MPA 580.

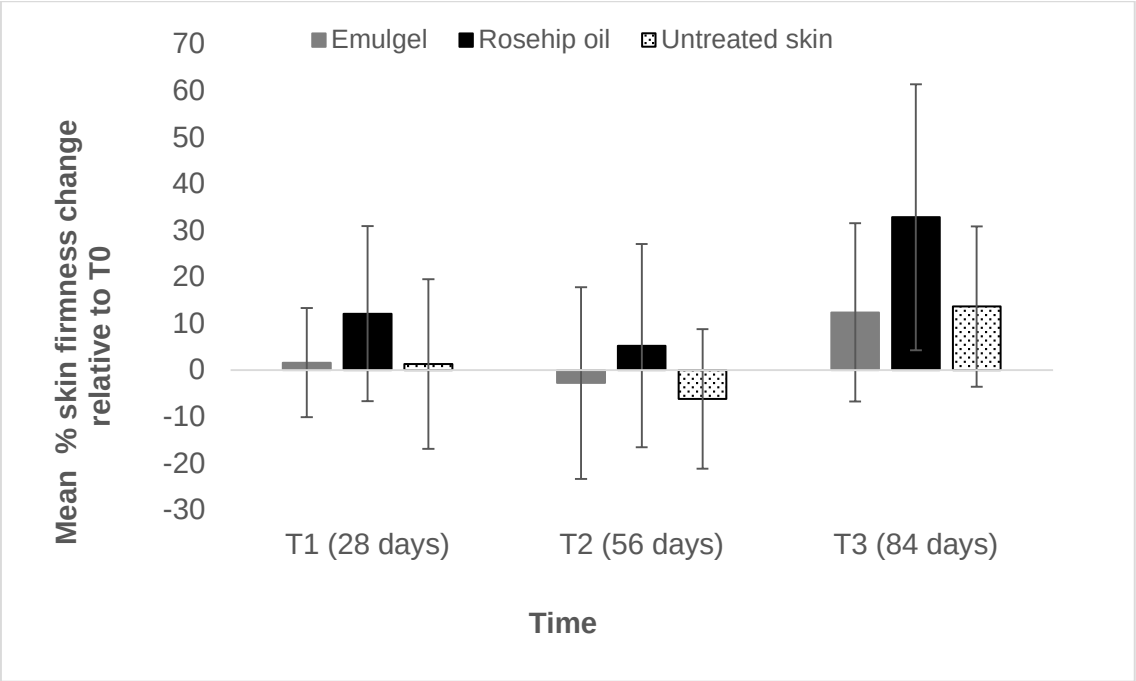

Figure 5: Percentage skin firmness changes (R0 parameter) relative to T0.

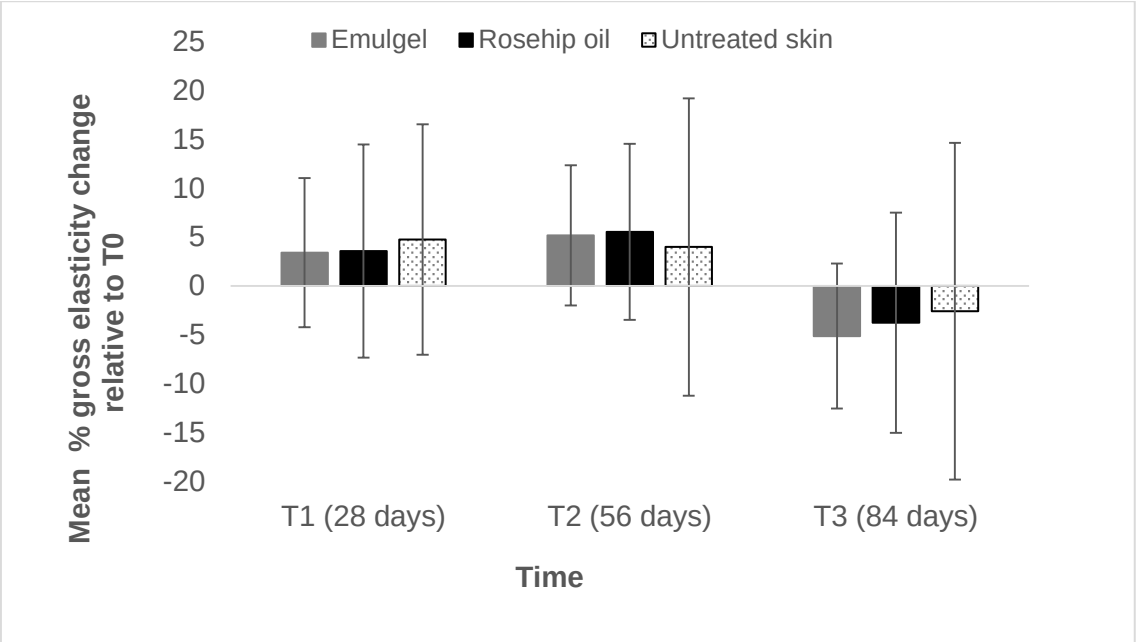

Figure 6: Percentage gross elasticity changes (R2 parameter) relative to T0.

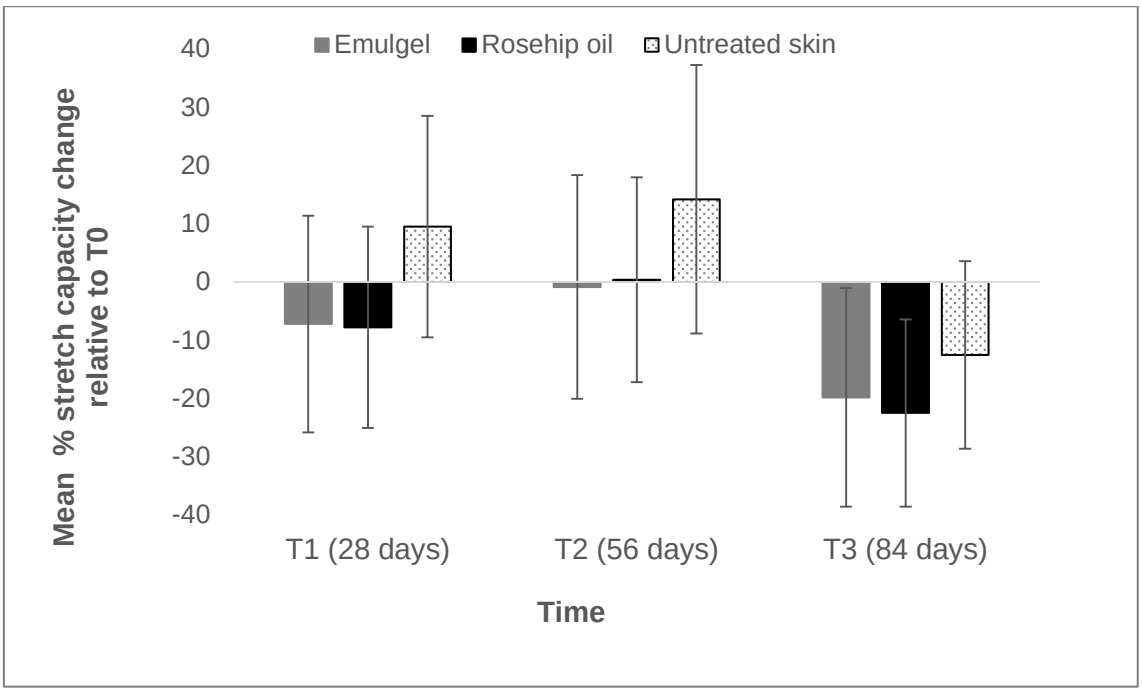

Figure 7: Percentage stretch capacity changes (R6 parameter) relative to T0.

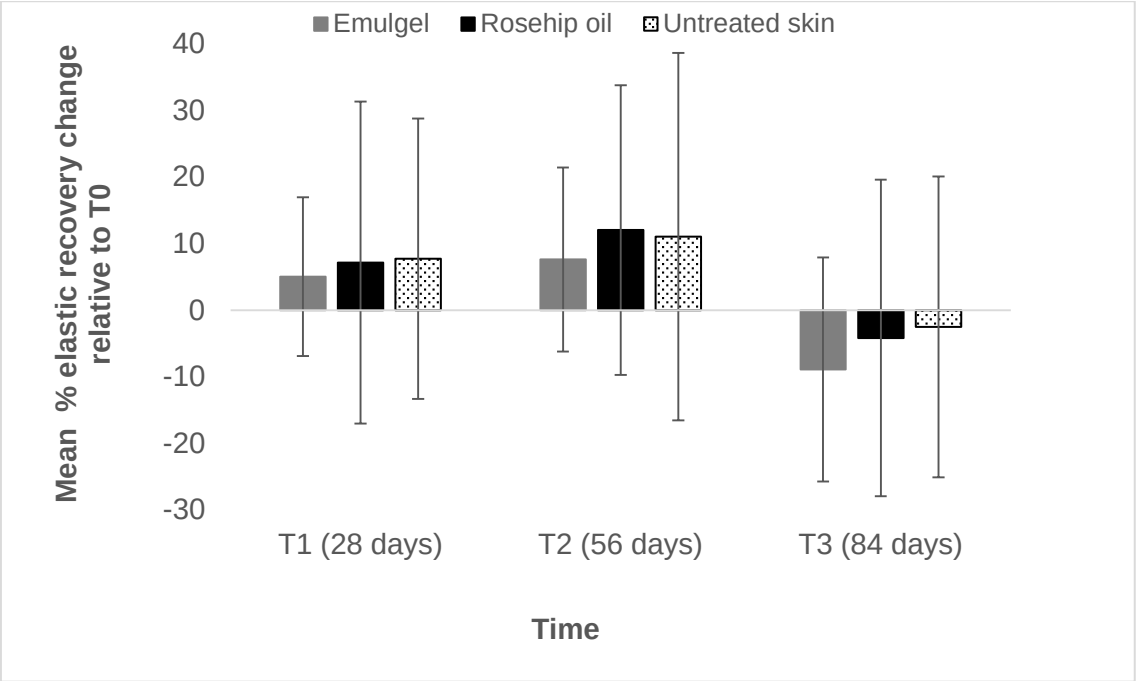

Figure 8: Percentage elastic recovery changes (R7 parameter) relative to T0.

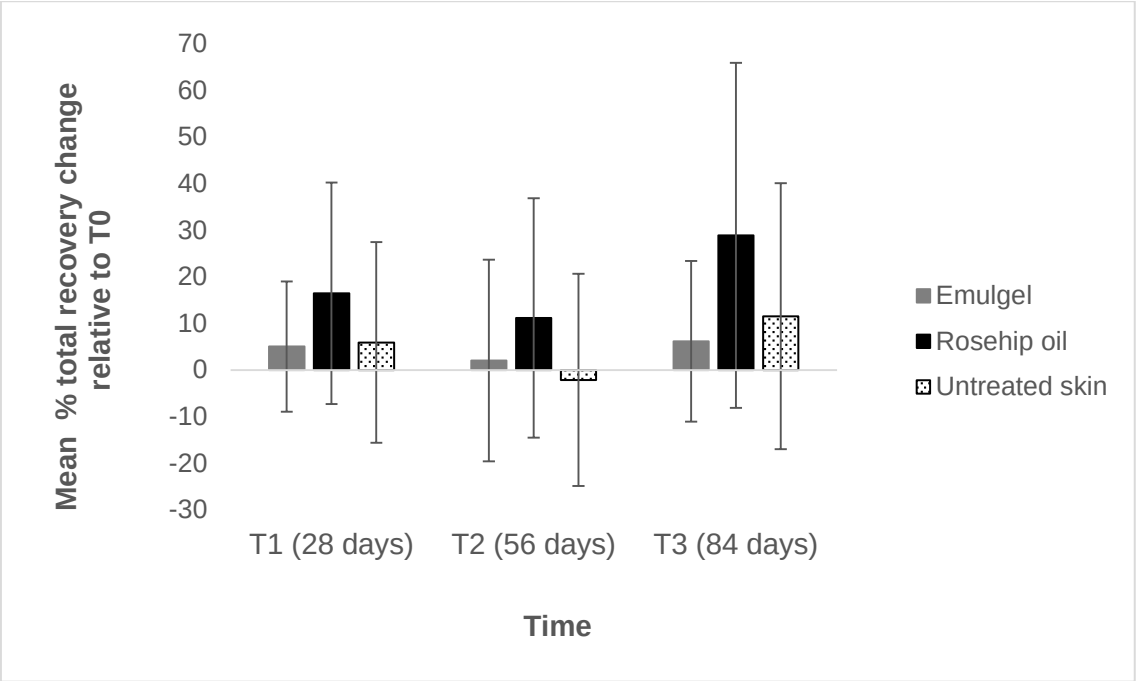

Figure 9: Percentage total recovery changes (R8 parameter) relative to T0.

TABLE LEGENDS

Table I: Formulation ingredients and their functionalities, used in the preparation of a 20% (w/w) rosehip seed oil emulgel.

Table II: Participant disposition table for each clinical trial.

Table III: Extent of the effects of each treatment relative to the initial skin hydration level (T0).

Table IV: Extent of the effects of each treatment relative to the initial skin condition (T0).

Table V: Extent of the effects of treatments over time relative to the initial skin condition (T0).

Table VI: Pairwise comparisons between treatments and between each treatment and the control.

Table I: Formulation ingredients and their functionalities, used in the preparation of a 20% (w/w) rosehip seed oil emulgel.

Ingredient	Percentage	Functionality
Dispersed oil phase		
Rosa rubiginosa seed oil	20.00	Oil (bio-active component)
Labrafac™ WL 1349	6.15	Emulsifier
Butylated hydroxytoluene (BHT)	0.50	Anti-oxidant
Propylparaben	0.04	Preservative
Continuous water phase		
Tween® 80	3.85	Surfactant
Carbopol® Ultrez 21	0.60	Thickening/gelling agent
Methylparaben	0.20	Preservative
Deionised water	68.66	Solvent

Table II: Participant disposition table for each clinical trial

	Short-term (4 h) study	**Longer term (84 days) study**
Number of participants screened	19	23
Number of participants eligible for each study	18	18
Number of participants enrolled	17	16
Participants age group	23-43	35-53
Number of participants that completed the study	17	14*
Comments		
* Due to personal reasons, participants were unable to complete the study.		

Table III: Extent of the effects of each treatment relative to the initial skin hydration level (T0)

Time	Extent of the effect (Cohen's d-value) relative to T0		
	Emulgel	**Rosehip seed oil**	**Untreated skin**
T1	1.33*	2.25*	0.28
T2	0.77*	1.31*	0.49
T3	0.72	1.29*	0.70

*d ≈ 0.8 signifies a large effect, or a practical significant difference

Table IV: Extent of the effects of each treatment relative to the initial skin condition (T0)

Time	Extent of the effect (Cohen's d-value) relative to T0		
	Emulgel	**Rosehip seed oil**	**Untreated skin**
T1	1.10*	0.86*	0.44
T2	0.70*	0.45	0.49
T3	0.41	1.15*	0.56

*d ≈ 0.8 signifies a large effect, or a practical significant difference

Table V: Effect sizes of treatments over time relative to initial skin condition (T0)

	Time	Extent of the effect (Cohen's d-value) relative to T0		
		Emulgel	**Rosehip seed oil**	**Untreated skin**
SEw	T1	0.04	0.05	0.16
	T2	1.00*	0.72*	0.43
	T3	0.12	0.26	0.00

*d ≈ 0.8 signifies a large effect, or a practical significant difference

Table VI: Pairwise comparisons between treatments and between each treatment and the control

Pairwise comparisons between treated skin and the control		**Statistically significant difference (p-value)**			
		SEsm	**SEsc**	**SEw**	**SEr**
Emulgel	Rosehip seed oil	< 0.0001*	0.877	0.813	0.769
Untreated skin	Emulgel	< 0.0001*	0.695	< 0.0001*	0.861
	Rosehip seed oil	< 0.0001*	0.996	0.001*	0.987

* Statistical significant difference $p < 0.05$

Chapter 7

Final conclusion and Future prospects

7.1 Introduction

The aim of this study generally was to examine the research and development (R&D) process, from concept to *in vivo* clinical efficacy, of a newly formulated investigational cosmetic product (ICP), containing commercially acquired *Rosa rubiginosa* seed oil, sourced from South African soil. During the R&D process, it was also important to review and assess the bio-active ingredients that were present in the oil, and to evaluate the API's penetration through the stratum corneum into the target deposition area, as well as to perform ICP stability assessments, before any clinical trials would commence (Millikan, 2001:371).

To achieve these aims, the following objectives (as per Chapter 1) were set:

1. Develop and validate a high performance liquid chromatography (HPLC) method to quantitatively determine the concentrations of: a) tretinoin in the *R. rubiginosa* seed oil and b) the API and excipients in an investigational cosmetic product (ICP) to assess their concentration changes, when subjected to accelerated storage conditions (stability studies).

2. Formulate an oil in water (o/w) emulsion, utilising commercially acquired rosehip seed oil.

3. Perform stability tests on the ICP, when stored at long-term (25°C / 60% RH), intermediate (30°C / 60% RH) and accelerated (40°C / 75% RH) storage conditions. The following evaluations were performed at 0, 1, 2, 3 and 6 months: assays of the concentrations of the API and excipients, pH, viscosity, conductivity, particle size, visual appearance and creaming index.

4. Perform membrane release studies, utilising vertical Franz cell methods to determine the API release from the ICP.

5. Perform transdermal diffusion studies by employing *in vitro* vertical Franz cell methods, followed by tape stripping to determine and compare transdermal and topical delivery of the API from the ICP, respectively.

6. *In vivo* clinical efficacy trials, evaluating *R. rubiginosa* seed oil (100% oil) alone and in the formulated ICP (20% rosehip seed oil in o/w emulsion). The following clinical efficacy trials were performed:

- o A short-term study (over 4 h): to measure skin hydration and the improvement thereof by the ICPs.
- o A long-term study (over 84 days): to evaluate skin hydration and the anti-ageing effects of the ICPs.
- o An erythema study: to investigate the anti-inflammatory properties of *R. rubiginosa* seed oil.

The set aims and objectives are discussed in this chapter and possible recommendations for future projects are made in the sections below.

7.2 Development and validation of quantitative HPLC assay methods

7.2.1 Determination of the tretinoin content in *R. rubiginosa* seed oil

The validated HPLC method, as described in Appendix A, was employed to perform the tretinoin assays. This assay method was developed and validated for the purpose of investigating the tretinoin concentrations, present in commercially available *R. rubiginosa* seed oil and to determine its concentrations in test samples, following the membrane and transdermal diffusion studies (Chapter 5). This method was found suitable for the analysis of the tretinoin content.

According to the literature, the assay outcomes of *R. rubiginosa* seed oil had indicated that different extraction methods would influence the concentration of tretinoin, with reported tretinoin concentrations ranging between 0.051 and 0.324 mg/L (Concha *et al.*, 2006:772). Tretinoin is a naturally occurring derivative of vitamin A and one of the main retinoids being used in cosmetic applications (Brand *et al.*, 2011:142). Due to its clinical significance, the tretinoin content of rosehip seed oil was investigated during this study.

The investigation of the tretinoin concentration, present in commercially available *R. rubiginosa* seed oil, resulted in an interesting finding (Appendix E). Three different batches of rosehip seed oil from the same rose species, *R. rubiginosa*, commercially sourced from three different eco-regions, namely Lesotho, Spain and Chile, were analysed. Unfortunately, no detectable amounts of tretinoin were found in any of these commercially available oils. It was hypothesised that commercially available *R. rubiginosa* seed oil may have contained tretinoin during the extraction processes, such as optimised cold press conditions that are employed to minimise tretinoin breakdown. However, following extraction, rosehip oil had been exposed to a chain of diverse handling and storage conditions, such as variations in temperature and light. Tretinoin is highly susceptible to degradation from heat, light and oxidising agents, and is it also sensitive to air (BP, 2015:1; Lai *et al.*, 2013:104). Exposure of the oil to such factors during transport and

storage, following its extraction, until acquisition by the manufacturer and consumer, may have resulted in the degradation of tretinoin to undetectable levels.

For a future research project, rosehip seed oil extraction may be performed in-house, under environmentally controlled conditions, to determine the possible tretinoin loss being inflicted following extraction, as a result of exposure during transport and storage and during the manufacturing process. It is also recommended that the HPLC assay method should be adjusted and re-validated, so that assays can be performed at more sensitive conditions, which would accommodate lower detection ranges.

Furthermore, a more complete rosehip seed oil assay, by possibly including a gas chromatography/mass spectrometry (GC/MS) oil analysis for the determination of its fatty acid profile, may also be beneficial and worthwhile.

Future research may further focus on identifying and evaluating any other phyto-actives that may be present in rosehip seed oil. A qualitative bio-active composition assay, or phytochemical characterisation of the plant source may confirm that supplementary ingredients that are present in the oil could be effectively utilised in the personal care industry.

7.2.2 Determination of the concentrations of the API and excipients in the new 20% cosmeceutical formulation

A single HPLC method, as described in Appendix B, was developed for the combined analyses of methyl paraben, propyl paraben, butylated hydroxytoluene (BHT) and tretinoin. This method was validated in accordance with the ICH Guidelines, for the purpose of employing it during the 6 months stability testing of the new rosehip seed oil formulation that had been developed and prepared during this study (ICH-Q2 (R1), 2006). The stability study assays of the emulsion, containing 20% of rosehip seed oil, aimed at assessing any concentration changes in the API and excipients, when subjected to accelerated storage conditions.

From an overall evaluation of the method validation outcomes, it was concluded that the developed HPLC method was reliable and adequately sensitive for the multi-component determination of the concentrations of methyl- and propyl paraben, BHT and of tretinoin, present in the formulated ICP. This method was therefore regarded as suitable for: 1) the analysis of the four analytes during the stability testing of the ICP, 2) for quality control and 3) for batch release purposes.

7.3 Formulation and preparation of the new cosmeceutical formulation

Emulsions are of the most commonly used formulation vehicles in topical skin care formulations (Buchmann, 2001:149) and was it the selected vehicle system for this project. Emulsions have the ability to incorporate both hydrophilic and lipophilic actives in the same product, to generate different textures and viscosities and to facilitate the permeation of the APIs through the skin (Attwood, 2013:87; Eccleston, 2013:442; Izquierdo, 2008:172; Otto *et al.*, 2009:5).

One of the challenges during this study was that emulsions are thermo-dynamically unstable systems (Otto *et al.*, 2009:6). Therefore, to increase the formulation's stability, emulsifiers and a viscosity enhancer were utilised. The hydrophilic-lipophilic balance (HLB) system was employed to calculate the surfactant ratio, required to stabilise the emulsion's water-oil phase. To prepare a mathematical model that would predict the characteristics of an optimised cosmetic formulation is difficult, when formulating with natural ingredients, since not all of the data that is required for these models is known. In this case, the pre-formulation studies were performed to narrow down the preparation trials during the R&D process (Guesnet *et al.,* 1994:65; Mitsui, 1997:321).

The ICP was formulated to only contain "functional" vehicle ingredients and the rosehip seed oil. Only constituents that would assist with the vehicle functioning and stability were utilised and were neither any fragrance, nor colouring ingredients incorporated into the formulation. All ingredients were chosen in accordance with the Cosmetic ingredient review board's permitted ingredient list and were utilised in acceptable cosmetic application concentrations (CIR, 2016). Finally, an oil-in-water (o/w) emulsion, incorporated within a stabilising gel structure, was formulated and prepared, as discussed in Chapter 3.

To prevent any possible API degradation, a cold process method was followed and no external heat was added at any stage during the ICP formulation's preparation.

The emulsion had a silky feel when applied to the skin, and had a smooth and uniform texture, with a light yellow colour and with no distinct smell.

7.4 Stability testing

Accelerated stability testing procedures were performed to assess any possible formulation character changes, which may affect the final product's appeal and efficacy (Barry, 2009:595; York, 2009:5), when stored at different storage conditions over a period of 6 months. The following evaluations, as described in Chapter 4, were performed at 0, 1, 2, 3 and 6 months: assays to determine the concentrations of the API and excipients, pH, viscosity, conductivity, particle size, visual appearance and creaming index. Acceptance criteria were set in

accordance with the ICH Guidelines Q1A (R2) and were the allowed changes from the baseline values over time limited to 5% (ICH, 2003:10).

The outcomes of the HPLC concentration assays demonstrated that only methylparaben, stored at 40 ± 2°C /7 5 ± 5% RH, had completely remained within the acceptable 5% variation limit. Of all of the ingredients being evaluated, tretinoin had displayed the highest concentration change, for all test samples and at all time intervals. These outcomes again emphasised the importance of employing extreme caution when handling tretinoin APIs and formulations that contain tretinoin, especially by limiting UV light exposure (Ioele *et al.,* 2005:256).

The pH value assessment revealed no significant changes for all of the test samples at all storage conditions, when analysing the mean percentage changes, relative to the baseline measurements (ICH, 2003:10). The average pH values of all of the test samples were found to have remained within the pre-determined range.

The calculated mean percentage viscosity changes, relative to the baseline measurements, revealed that significant changes in the viscosity of all of the formulations investigated, had occurred over time and at all time intervals. In addition, fluctuation in conductivity measurements had also occurred over time, for all of the samples tested. It was suggested that both the measured viscosity and conductivity changes may have been attributed to the possible breakage of the hydrogen bonds, causing free water to be released from the gel system structure, as observed on the inside of the screw caps of the sample containers (Behera *et al.*, 2015:259; Korać *et al.*, 2014:272; Ngawhirunpat *et al.*, 2013:800).

The mean D(0.5) droplet diameter of freshly prepared emulsions was 3.331 µm and had it remained within the acceptable limit of 5% for all of the emulsions and at all storage conditions. It was therefore assumed that no droplet coalescence, nor Ostwald ripening had occurred during any stage of sample storage.

No visible phase separation, creaming, nor sedimentation was revealed at any of the test intervals and for any of the tested formulation samples. Analysis of the test samples, by applying polarisation microscopy methods, did not reveal substantial information regarding the gel structure, nor about any o/w emulsion changes over time. Visual inspection revealed no visible phase separation, nor texture changes throughout the duration (6 months) of the study.

The stability assessments being performed revealed unacceptably significant changes, higher than 5%, following the API and excipient assays, and viscosity and conductivity measurements (ICH, 2003:10).

Although no observed changes had altered the general visual, or sensorial acceptance criteria of the test formulations at any of the time intervals, the mere fact that the viscosity, assay and

conductivity measurements had not remained within the 5% acceptance criteria, this formulation could be considered neither stable, nor suitable for manufacturing purposes.

Also, as discussed in Chapter 4, the following shortcoming was identified during this study. All samples were stored in 50 ml glass containers with tightly closed screw caps, containing polyvinylidene chloride (PVDC) liners, impermeable to water. Since water evaporation into the atmosphere was therefore not possible, mass changes and water loss could not be assessed. Future studies of such test samples should hence be stored in semi-permeable containers, allowing for possible water evaporation, to determine the effect of this parameter and to determine the effects of increased temperature conditions on the test formulations more extensively.

7.5 Membrane release studies

Membrane release studies are performed to give an indication of the extent to which a drug is released from the formulation vehicle. During skin diffusion studies, however, the ability of a vehicle to release the drug at the local site is limited by numerous factors, including drug-skin and vehicle-skin interactions, and not only the drug-vehicle factors, as are examined during membrane release studies. During membrane release studies, no biological diffusion processes are suggested by the synthetic membrane being utilised, which may lead to an overestimation of dermal absorption values (Shin *et al.*, 2005:67).

The release of the API from the ICP was confirmed during the membrane release studies. The average cumulative concentration of 28.060 µg/cm^2, also expressed as an average percentage of 4.97% of tretinoin, was released from the formulations through the membranes after 6 h. The average flux that was obtained by the slope of the straight line between 2 and 6 h for tretinoin, was 9.0586 µg/cm^2.h.

A significant obstacle of using tretinoin is its unstable, light sensitive chemical structure, responsible for its rapid degradation that leads to newly formed isomers, such as 13-*cis* (isotretinoin) and 9-*cis* (alitretinoin) (Ioele *et al.*, 2005:256). The degradation of tretinoin in the ICP being tested during this study resulted in a similar reaction. One additional peak had eluted and was visible on the HPLC chromatograms and it was identified as the tretinoin isomer, isotretinoin (Bagatin *et al.,* 2015:87; Lehman & Malany, 1989: 597; Manconi *et al.,* 2006; Ourique *et al.*, 2011:97).

For the purpose of the membrane release study, hydrophilic polyvinylidene fluoride (PVDF) membrane filters (FP Vericel, 0.45 µm, 25 mm, Pall®) were utilised. Different flux values may be obtained from using different types of synthetic membranes (Ng *et al.*, 2010:219). For a future study, therefore, it may be interesting to assess the influence of the hydrophilic and/or lipophilic

nature of different membranes, of membrane thickness and pore size, and of the type of materials and coatings of various synthetic membranes, while employing the vertical Franz cell method.

7.6 Transdermal diffusion studies and tape stripping

Skin diffusion studies, utilising the Franz cell diffusion method, as described in Chapter 5, were performed to determine the concentration of tretinoin in the formulation that had permeated into and through the skin.

For cosmetic applications, it is required that a final product should remain in the upper skin layer, or at least not penetrate deep into the viable epidermis. Hence, for cosmetic applications, minimal permeation is required to cause an accumulation of the APIs in the top skin layer, or target deposition areas (Izquierdo, 2008:174; WHO, 2006:1; Wiechers, 2008:10). According to the literature, the highest amounts of tretinoin are usually retained in the stratum corneum, regardless of its concentration in certain cream formulations (Bagatin *et al.,* 2015:87). The preferred target deposition area for this study was that the API should remain within the stratum corneum.

The concentration of tretinoin that had permeated the dermatomed skin and reached the receptor compartment was measured as an average of 0.362 µg/ml, and had an average 0.071% of tretinoin in the applied dose hence diffused from the formulations and through the skin after 12 h. It was also established that 0.049 µg/ml (0.0095%) of the isotretinoin had been retained in the receptor fluid after 12 h.

The skin fractionation procedure, using the tape stripping method, had generated unexpected results. The average concentration of tretinoin being retained within the stratum corneum-epidermis (SCE) was 0.020 µg/ml, whereas a slightly higher concentration of 0.027 µg/ml was located within the epidermis-dermis (ED). Because of the lipophilic nature of the SCE, together with the lipophilic character of tretinoin, it had been predicted that considerably higher amounts of tretinoin would be retained within the lipophilic stratum corneum, than what would permeate through the skin. This was, however, not the findings during this investigation (Bagatin *et al.,* 2015:87). The preferred target deposition area had not been preserved and had the API not remained within the lipophilic stratum corneum.

It was concluded that lipid solubilisation, the penetration enhancer effect being demonstrated by surfactants and fatty acids, the disruption of the skin barrier function and the mechanism thereof, would require investigation in future studies as this may have yielded the results obtained (Franco *et al.,* 2005:443; OECD, 2011:42; Sigma-Aldrich, 2015).

7.7 *In vivo* clinical efficacy trial studies

Since the label claim, *natural*, of a topical formulation does not necessarily indicate that the ingredients of such products are safe, nor effective, the clinical efficacy of active ingredients being incorporated into cosmetic formulations and their final products require evaluation (Brandt *et al.*, 2011:141; Gagliardi *et al.*, 2007:45; Nohynek *et al.*, 2010:240, 252).

Non-invasive *in vivo* methods, by utilising bio-engineering instruments, have been developed to assess various skin conditions. These applied methods are capable of detecting sub-clinical skin effects that are not observable by the naked eye, and are they used to quantify and substantiate cosmetic claims in an objective and unbiased way (Berardesca, 2011:89).

The challenge remains the difficulty of separating the performance of the finished product from those of its individual ingredients. *In vivo* clinical studies were therefore performed on human volunteers to evaluate the clinical efficacy of both the 100% rosehip seed oil and of the 20% rosehip seed oil o/w emulsion, as described in Chapter 6 and Appendix C. The following label claims with regards to the two rosehip seed oil ICPs were investigated:

- Short- and long-term skin moisturising effects.
- Anti-ageing properties, such as:
    - Improvement of skin elasticity of the upper skin layers.
    - Influence on skin topography through surface evaluations of the living skin (SELS) parameters.
    - Anti-wrinkle properties.
- Skin barrier repair (assessment of transepidermal water loss (TEWL) changes).
- Anti-inflammatory properties.

Statistically significant ($p < 0.05$) improvement of skin hydration was achieved, following single and multiple applications of 100% rosehip seed oil and the 20% rosehip seed oil emulsion. A maximum increase in skin hydration (± 35%) had been achieved, 60 min after a single application of the 100% rosehip seed oil test sample.

In the longer term study, a statistically significant ($p < 0.05$) skin moisturising effect was observed for both test products. The maximum moisturising effect by the rosehip emulgel (± 19%) was recorded at 28 days, whereas the maximum increase by the rosehip seed oil (± 21%) was obtained after 84 days. The long-term study results hence indicated that both test products had achieved beneficial skin hydration effects.

The changes in skin wrinkles and smoothness were evaluated by means of the wrinkle parameter (SEw) and skin smoothness parameter (SEsm), respectively, and generated through the SELS method. The changes in the SEw showed statistically significant differences ($p < 0.05$) for each ICP, compared to untreated skin areas. The maximum decrease in skin wrinkles of approximately 7% was recorded after 56 days of multiple applications. Both the rosehip seed oil and the 20% rosehip emulsion had thus decreased skin wrinkles and had consequently improved the skin's condition, following long-term, multiple ICP applications.

Contrary to the beneficial results obtained with regards to skin wrinkle improvement and skin hydration assessments, the evaluation of skin smoothness had yielded an unfavourable clinical effect. The results showed that both the rosehip emulsion and rosehip oil had decreased skin smoothness at all test intervals, relative to the baseline values. The changes in the SEsm were statistically significantly different ($p < 0.05$) for each test product, compared to untreated skin, indicative thereof that the clinical changes, as reflected by this parameter, had not supported a complimentary clinical outcome.

Following the statistical analysis of the skin's elasticity, R-parameters were evaluated. The results demonstrated that skin firming and increased stratum corneum thickness properties had occurred with the application of a 100% rosehip oil, but had a similar clinical effect not been achieved from the emulsion. Visco-elastic property assessments revealed improvements of the skin's stretch capability and had a maximum clinical effect been achieved after 84 days.

Although rosehip seed oil had improved both wrinkle appearance and skin firmness, a decrease in skin smoothness had been observed. Consequently, during the formulation R&D process, it would be important to take this into consideration, when choosing appropriate formulation vehicle ingredients.

As for the anti-erythema study that had been performed to investigate the anti-inflammatory and skin barrier repair properties of the ICPs, unfortunately no better clinical effect, nor significant changes with regards to reducing TEWL and erythema, compared to the positive controls, were revealed for both the ICPs tested. It was hence concluded that neither of the test products (100% rosehip seed oil, 20% rosehip seed oil emulsion) had shown superior clinical anti-inflammatory, nor TEWL reduction effects, relative to the 1% hydrocortisone acetate (w/w) water dispersible cream (control), utilised during this study.

No adverse events were reported during, or after the study for all of the clinical studies performed. Both ICPs were well tolerated and accepted by all participants, although no skin irritation, sensitisation, phototoxicity and immediate-type allergy studies were performed.

Poly-unsaturated fatty acids (PUFAs), as found in rosehip seed oil, offer skin protection against dehydration, which partially contribute towards the occlusive properties that resultantly decrease TEWL that cause an increase in the skin's moisturise content (Sharafzadeh, 2013:234). PUFAs and essential fatty acids (EFAs) complement the structures of the inter-cellular lipid matrix of the stratum corneum and play an important role in the biological functioning of the skin. In the case of a deficiency, it is possible to provide compounds, rich in PUFAs, for use on the skin through topical supplementation, in the form of cosmetic emulsions to illustrate their beneficial role (Krasodomska & Jungnickel, 2015:469). Topical applications bypass metabolic conversion in the liver and are EFAs more readily available for use in the skin, than when taken through the diet (Regiert, 2008:357). For instance, the topical application of linoleic acid, also found in rosehip seed oil, stabilises the skin's fatty acid balance that prevents skin lipid ceramide deficiency and assists with the regeneration of the skin's function. Topically applied linoleic acid can decrease TEWL and assist with the repair of the skin barrier function (Regiert, 2008:357).

It is therefore suggested that future research should further investigate the clinical applications of rosehip seed oil as sources of PUFAs and EFAs, in topically applied formulations.

To conclude, beneficial clinical efficacy results were demonstrated during this study. *R. rubiginosa* rosehip seed oil can be considered as a value adding cosmetic ingredient for the improvement of skin hydration, wrinkle appearance and skin firmness. *R. rubiginosa* seed oil contains biologically significant substances, which may be synergistically incorporated into cosmetic and topical personal care vehicle systems. However, multifunctional formulation vehicle ingredients should be incorporated to achieve a desired biological effect, complementary to the clinical efficacy objectives that the formulator wishes to achieve.

A definite challenge remains the meeting of consumers' demands for natural personal care products, whilst ensuring that, when developing such botanically derived cosmetic products, to simultaneously focus on the sustainability of use, as well as the preservation of the natural resources, which was during this study found to be quite possible, with regards to *R. rubiginosa*.

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Appendix A

Method validation of tretinoin (all-*trans*-retinoic acid) HPLC assay

A.1 Introduction

The purpose of the validation process is to confirm that the analytical method, used for determining the amount of active pharmaceutical ingredient (API) in test samples is sensitive, reliable and suitable for its intended purpose. The API used during this study was tretinoin (all-*trans*-retinoic-acid). A high performance liquid chromatographic (HPLC) method for the analysis of tretinoin was developed and validated at the Analytical Technology Laboratory (ATL) at the North-West University, Potchefstroom Campus. The method validation was performed in accordance with the International Conference on Harmonisation (ICH-Q2 (R1), 2005) guidelines.

This assay method was developed and validated for the purpose of investigating the tretinoin concentrations present in commercially available *Rosa rubiginosa* (rosehip) seed oil and to determine its concentrations in test samples, following membrane and transdermal diffusion studies. The summarised validation results in Table A.1 indicate that this method was found suitable for the analysis of tretinoin content.

Table A.1: Summary of the validation outcomes of the tretinoin HPLC assay method

Test	Result
Specificity	Complies
Concentration range	0.04 – 97.70 µg/ml
Linearity	R^2 = 0.9998
Accuracy	98.94%
Precision	RSD = 2.13%

A.2 Chromatographic conditions

The chromatic conditions of the developed tretinoin assay method were as follows:

Analytical instrument: HP1100 series HPLC, equipped with a pump, auto-sampler, UV-detector and Chemstation Rev. A.10.03 data acquisition and analysis software, or equivalent (Agilent Technologies, Palo Alto, CA).

Column: A Luna $C_{18(2)}$, 150 x 4.6 mm, 5 µm, 100 Å pores, 17.8% carbon load, end-capped (Phenomenex, Torrance, CA), and a Venusil XBP $C_{18(2)}$, 150 x 4.6 mm, 5 µm (Agela Technologies, Newark, DE) columns were validated.

Mobile phase: Acetonitrile, containing 0.5% of glacial acetic acid (v/v) and filtered through Whatman™ GF/F glass microfiber filters (0.7 µm pore size).

Flow rate: 1.0 ml/min

Injection volume: 10 µl

Detection: UV at 349 nm

Retention time: ± 5.2 min

Stop time: 7 min

Solvents: Acetonitrile, tetrahydrofuran (THF) and phosphate buffer solution (PBS) pH 7.4.

A.3 Sample preparation

Two separate sample sets were analysed by utilising this HPLC method and were they prepared as follows:

- The samples for the *Rosa rubiginosa* seed oil assays were prepared by weighing 1 g of rosehip seed oil and by filling each up to a volume of 25 ml with THF. These samples were transferred into amber HPLC auto-sampler vials and analysed.
- The samples, collected from the Franz diffusion cells, following the membrane release and skin diffusion studies, were filtered and transferred into amber HPLC auto-sampler vials and analysed without any further processing.

A.4 Standard samples preparation

Four standard solutions were prepared as follows:

1. Approximately 5 mg of tretinoin was accurately weighed into a 100 ml amber volumetric flask (stock solution).

2. The tretinoin stock solution was dissolved in approximately 10 ml of acetonitrile, sonicated and filled to volume with acetonitrile (Std 1).

3. 10 ml of the Std 1 solution was diluted to 50 ml with acetonitrile (Std 2).

4. 10 ml of the Std 2 solution was diluted to 50 ml with acetonitrile (Std 3).

5. 10 ml of the Std 3 solution was further diluted to 50 ml with acetonitrile (Std 4).

6. These dilutions yielded standard solutions containing 0.4, 2, 20 and 50 µg/ml of tretinoin.

7. These four standard solutions (Std 1 - 4) were each transferred into amber auto-sampler vials and analysed on HPLC.

A.5 Calculations

The four standard solutions were used to determine the linearity of the tretinoin API, by performing a linear regression analysis on the measured HPLC peak areas *versus* their standard sample concentrations (µg/ml). A standard curve was calculated and the resultant slope and y-intercept were used to calculate the concentrations of tretinoin in the test samples from their HPLC generated peak areas.

A.6 Validation test procedures and acceptance criteria

A.6.1 Specificity

To determine specificity, the following method was followed:

1. Two placebos were prepared in auto-sampler vials, by filling the one with THF and the other with PBS (pH 7.4).

2. The placebos were injected into the HPLC in duplicate.

3. Four forced degradation solutions were prepared by adding 100 µl of water, 2.0 M of hydrochloric acid, 2.0 M of sodium hydroxide and 10% hydrogen peroxide respectively to four separate 1 ml samples of the Std 3 standard solution (prepared as described in Section A.4).

4. These solutions were stored for 3 h in closed test tubes at room temperature, protected from ultraviolet light conditions, to allow for them to degrade.

5. Another standard solution (Std 3) were prepared and transferred into a volumetric flask which was left to stand in an area subjected to unprotected light conditions for 3 h.

6. All of these samples were injected into the HPLC and analysed with a run time of 7 min.

7. The chromatographs were examined to determine whether any additional peaks were detected by HPLC analysis.

A.6.1.1 Acceptance criteria

The degraded samples should not generate any peaks on HPLC that would interfere with the determination of tretinoin. Similarly, no peaks should be generated by any of the ingredients in the placebos that would interfere with the tretinoin analyses (ICH-Q2(R1), 2005:7).

A.6.2 Linearity

The linearity of the method was validated by employing the following method:

1. Four standard solutions were prepared, as described in Section A.4.

2. 1.0, 2.5, 5.0, 7.5 and 10 µl of each standard solution (Std 1 to 4) were injected into the HPLC in duplicate and analysed.

A.6.2.1 Acceptance criteria

Linear regression analysis should yield a regression coefficient (R^2) of ≥ 0.99. The concentration range is determined as the lowest and highest concentrations between which the response remains linear, and/or where acceptable precision is obtained (ICH-Q2(R1), 2005:8).

A.6.3 Accuracy

According to the ICH-Q2(R1) guidelines, the accuracy of an analytical procedure demonstrates the closeness of agreement (degree of scatter) between the value that is accepted as either a conventional true value, or an accepted reference value and the value found (ICH-Q2(R1), 2005:4).

The HPLC validation method did not involve any test sample preparations. Accuracy and precision were validated by preparing a set of standard solutions that was analysed against another set of standard solutions as follows:

1. Approximately 10 mg of tretinoin was weighed into a 100 ml volumetric flask. The tretinoin was dissolved in approximately 10 ml of acetonitrile and filled to volume with THF (stock solution).

2. 10 ml of this solution was transferred into a 50 ml volumetric flask and filled to volume with THF.

3. Another 2 ml of the stock solution was transferred into a 100 ml volumetric flask and filled to volume with THF.

4. These three dilutions contained concentrations of about 2, 20 and 100 µg/ml of tretinoin.

5. These three standard solutions were transferred into amber HPLC auto-sampler vials and analysed against the set of four standard solutions that were prepared, as described in Section A.4, to determine the accuracy of the HPLC method.

A.6.3.1 Acceptance criteria

According to the FDA (2001:5), the mean value being determined for accuracy should be within 15% of the true value. For the purpose of this study, a limit of between 95 - 105% was set.

A.6.4 Precision

According to the ICH-Q2(R1) guidelines, the precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements, obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. During this study, precision was considered at repeatability, intermediate precision and reproducibility levels (ICH-Q2(R1), 2005:4).

A.6.4.1 Intra-day precision (repeatability)

Intra-day precision was validated, using the following method:

1. Three standard solutions were prepared, one each with low, medium and high concentrations (n = 9).

2. A set of standards solutions was prepared, as described under Section A.4.

3. These solutions were injected into the HPLC and analysed in duplicate.

A.6.4.2 Inter-day precision

Three samples of the middle standard solution was analysed, as described above for intra-day precision determinations, on two more days to determine the between-day variability of the method. If possible, a different analyst should perform the analysis, preferably using different equipment, as was done during this study.

A.6.4.3 Acceptance criteria

The limit being set for precision of bio-analytical methods is 15% of the coefficient of variation, except for the lower limit of quantification (LLOQ), where it should not exceed 20% (FDA, 2001:5).

For the purposes of our study, the limits were set as follows:

- Intra-day repeatability had to be better than 5% (n = 9).
- Inter-day precision had to be better than 10% (n = 9).

A.6.5 Ruggedness

A.6.5.1 Stability of sample solutions

The stability of the samples was validated by using the following method:

1. A standard solution (Std 1) was prepared, as described under Section A.4.
2. The standard sample was injected into the HPLC and analysed in duplicate.
3. The standard sample was left in the auto-sampler and re-analysed at hourly intervals for up to 24 h to determine the stability of the sample over the period.
4. The HPLC pump was programmed to reduce the mobile phase flow rate to 0.1 ml/min after elution of the peak after 7 min, and to reset the flow rate to 1 ml/min 5 min before injecting the next sample.

A.6.5.1.1 Acceptance criteria

Sample solutions should not be used for a period longer than it would take to degrade with 2%. During this study, special precautions had to be followed to compensate for degradation.

A.6.5.2 System repeatability

A sample, or standard solution was injected six times consecutively in order to test the repeatability of the peak area, as well as the repeatability of the retention time.

A.6.5.2.1 Acceptance criteria

For the purpose of this study the peak area and retention times should have a write out (RSD) of 2% or less (USP 37, 2014: 5).

A.6.6 Robustness

According to the ICH-Q2(R1) guidelines, the robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and is it hence indicative of the method's reliability during normal usage (ICH-Q2(R1), 2006:5). Deliberate changes to the flow rate, injection volume, wavelength and mobile phase composition were therefore made and the standard solutions analysed accordingly to establish the influence of these changes on the chromatographic results.

A.6.7 System suitability

System suitability is a measure of the system and method performance characteristics. An extended performance report on the standard solution is generated by taking care that only the relevant peaks are integrated.

A.6.7.1 Acceptance criteria

The performance results obtained are examined by setting realistic performance characteristics that must be complied with in order to perform the analysis successfully.

A.6.8 Uncertainty of measurement

The sources of error and the extent thereof must be identified to determine the degree of uncertainty of measurement of the developed analytical method. The calculated uncertainty of measurement during this study was established by combining the uncertainties of each step in the analysis process by expressing each as a contribution factor. For reporting purposes, the set value for uncertainty of measurement should be included in the method's validation outcomes (Taverniers *et al*., 2004:487).

A.7 Validation outcomes

A.7.1 Specificity

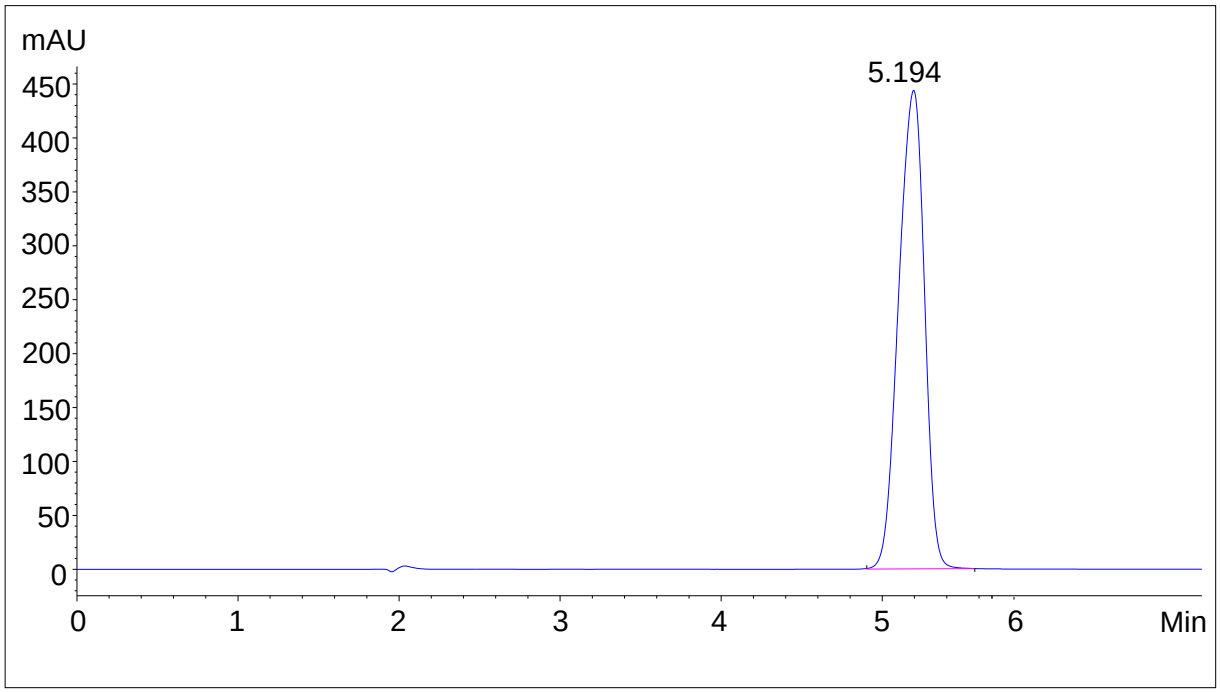

Figure A.1: HPLC chromatogram of a tretinoin standard solution (2 ug/ml).

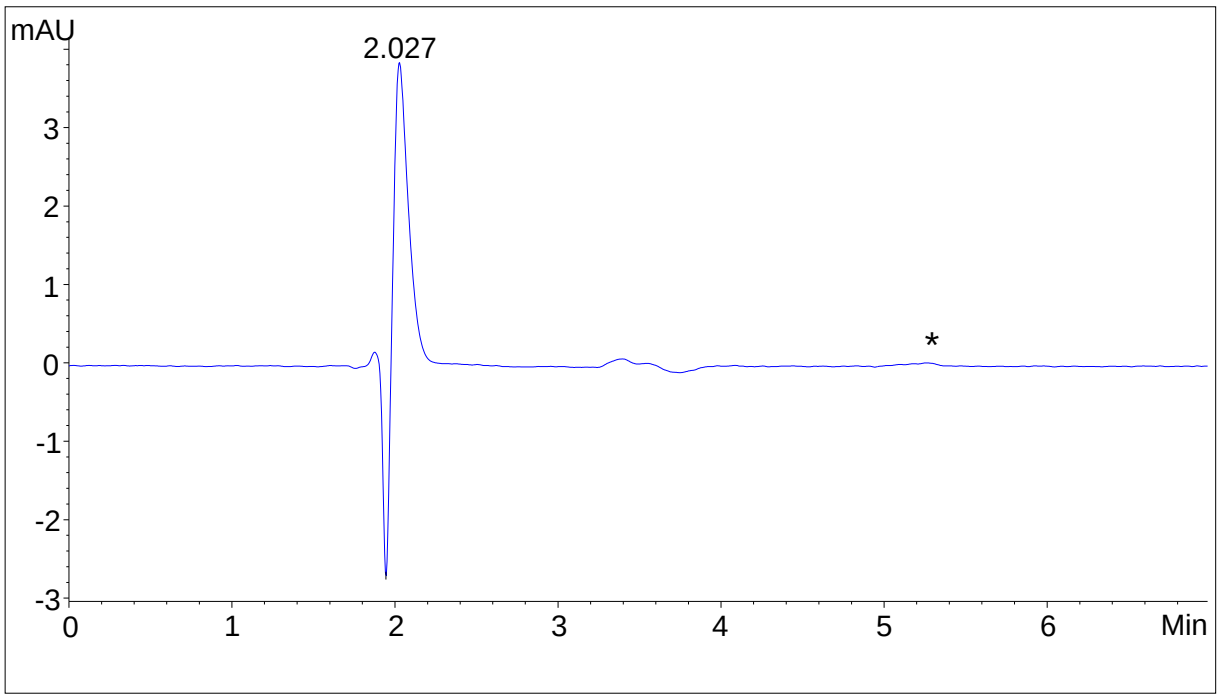

Figure A.2: HPLC chromatogram of the THF placebo, with the * indicating the elution of tretinoin.

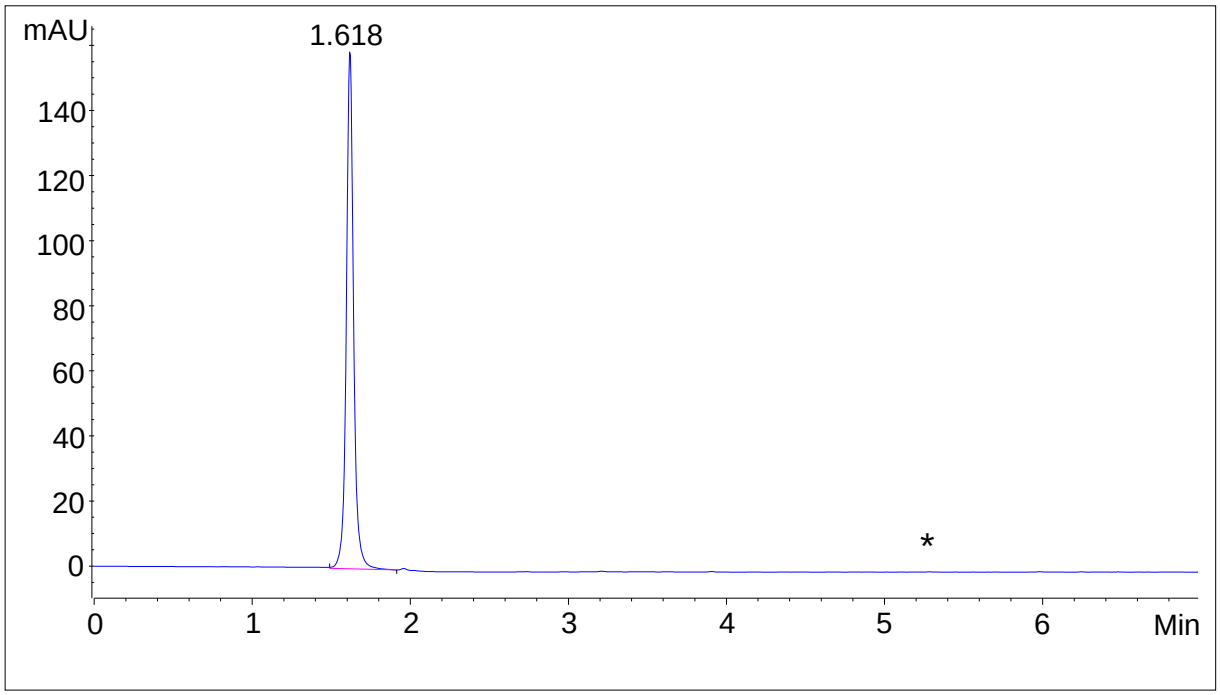

Figure A.3: HPLC chromatogram of the PBS placebo (pH 7.4) solution, with the * indicating the elution of tretinoin.

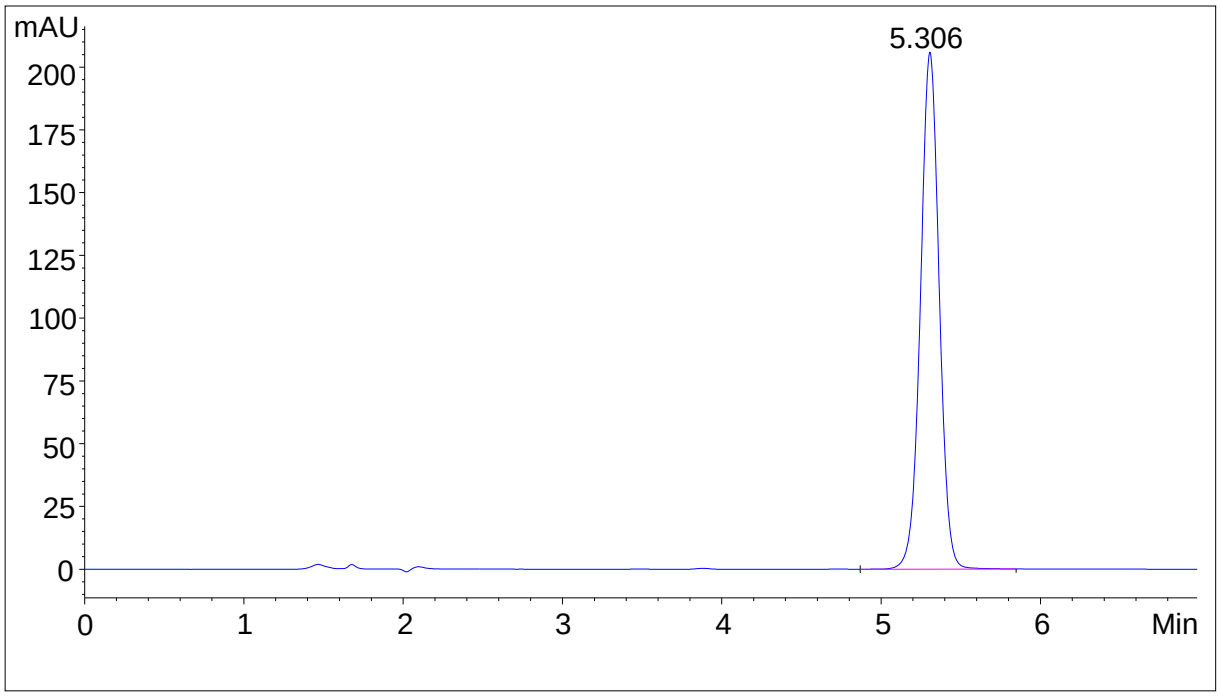

Figure A.4: HPLC chromatogram of a 2 ug/ml standard solution diluted in water.

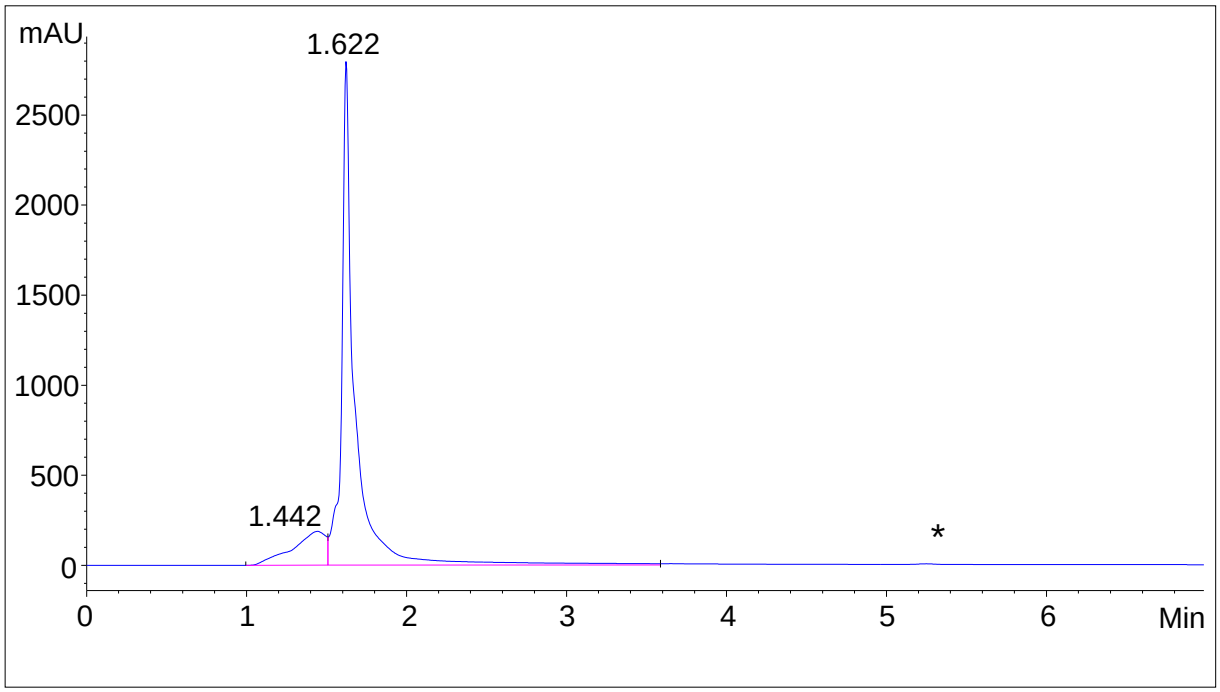

Figure A.5: HPLC chromatogram of a 2 ug/ml standard solution diluted in 0.1 M HCl, with the * indicating the elution of tretinoin.

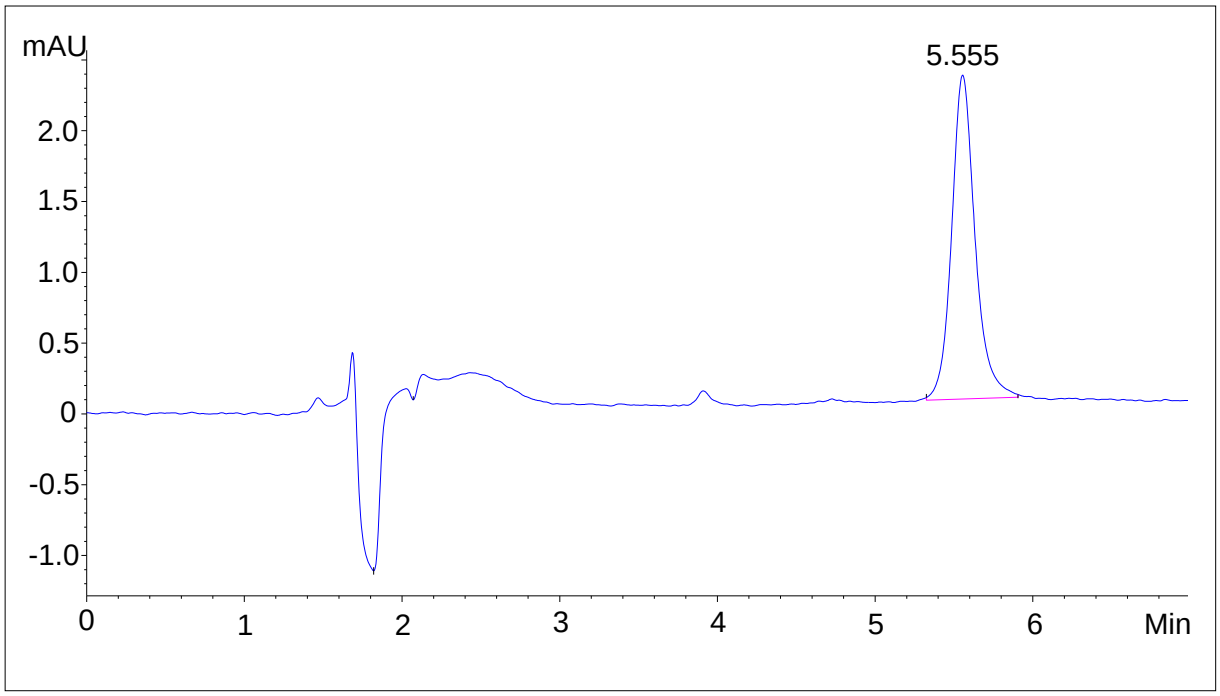

Figure A.6: HPLC chromatogram of a 2 ug/ml standard solution diluted in 0.1 M NaOH.

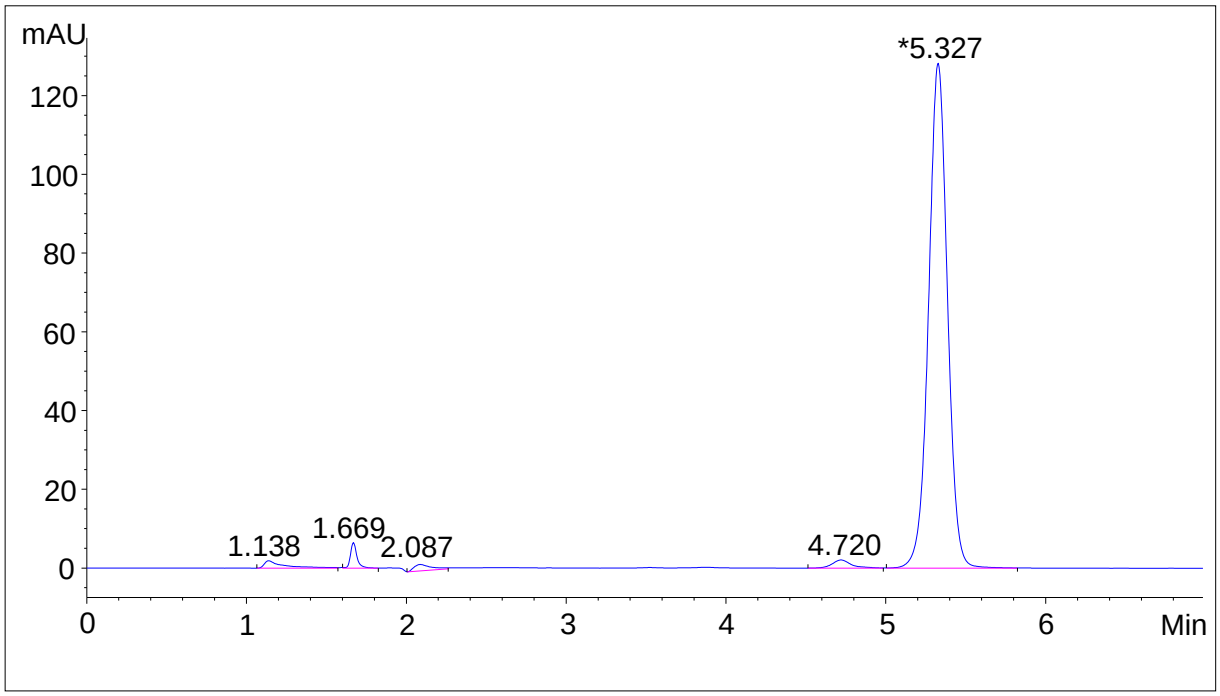

Figure A.7: HPLC chromatogram of a 2 ug/ml standard solution diluted in 10% H_2O_2, with the * indicating the elution of tretinoin.

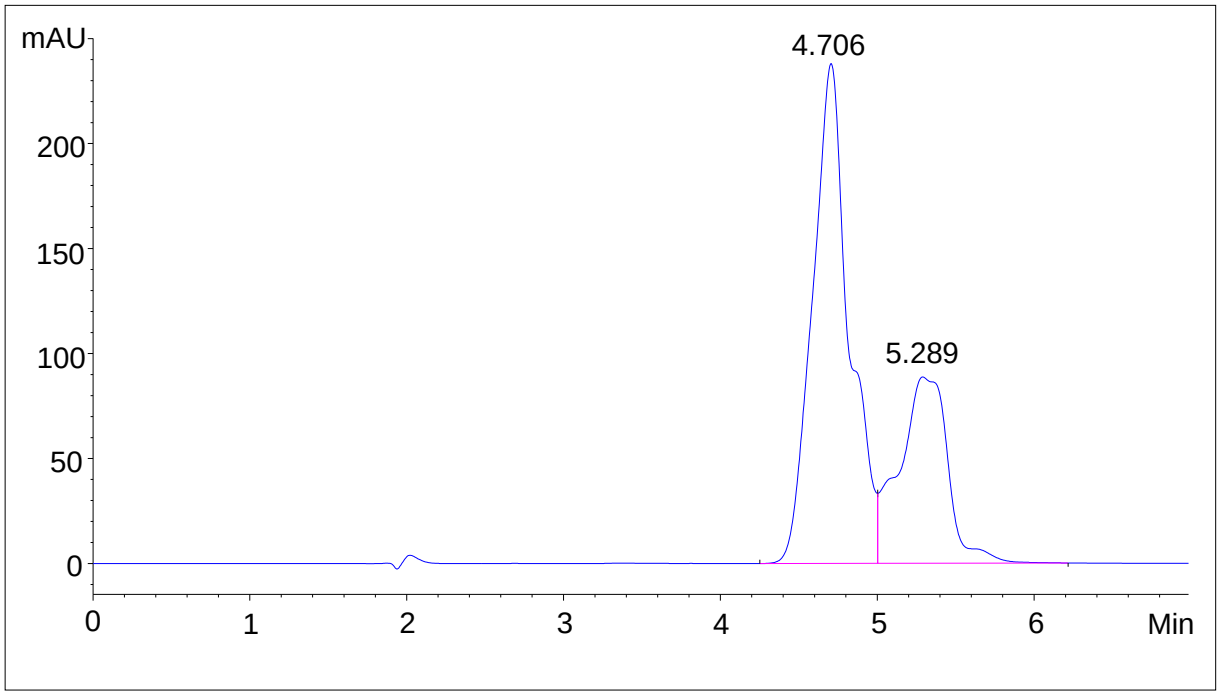

Figure A.8: HPLC chromatogram of a 2 ug/ml standard solution after exposure to uncontrolled light conditions.

Following the specificity validation method, as described in Section A.6.1, it was found that both of the analysed placebo solutions, THF and PBS (pH 7.4), did not interfere with the elution of the tretinoin peak. Additional degradation peaks were formed during the forced degradation processes by diluting the standard sample solutions in different stressor solutions.

According to the literature study outcomes, the dissolving of tretinoin in ethanol and its exposure to UV-light irradiation lead to its rapid and complete degradation to form 13-*cis* retinoic acid (isotretinoin) and 9-*cis* retinoic acid (alitretinoin), with a typical HPLC peak shift from 349 nm to 346 nm and 340 nm, respectively for each isomer formed. Also, a subsequent slower degradation process, as reported for the newly formed 13-*cis* and 9-*cis* isomers, is responsible for the formation of several additional isomers (Ioele *et al*., 2005:256; Lehman & Malany, 1989: 597).

During the validation of this developed HPLC method, UV-light degradation has proven to have significantly altered the results obtained. As can be observed on the chromatograms of the standard samples after being subjected to the various stress conditions, peak shifting, tretinoin degradation and degradation peak formation had occurred frequently. These outcomes re-emphasised the importance that tretinoin should be handled with extreme caution during experiments, by limiting laboratory UV-light exposure and by employing preventative measures, such as the use of amber glassware (BP, 2015:1).

The British Pharmacopoeia (BP) states that tretinoin is susceptible to degradation, due to heat, light, oxidising agents and that it is sensitive to air. Tretinoin should be stored at temperatures below 25°C and in containers filled with an inert gas. All assays on samples containing tretinoin should be carried out without delay to avoid exposure to actinic light and as revealed, other conditions that may cause tretinoin peak degradation (BP, 2015:1; Lai *et al*., 2013:104).

A.7.2 Linearity and range

Table A.2: Tretinoin linearity results

Concentration (µg/ml)	Peak area 1	Peak area 2	Mean peak area
0.04	4.34	3.97	4.16
0.10	9.19	8.54	8.87
0.20	16.83	17.51	17.16
0.29	32.87	32.15	32.51
0.39	47.26	47.38	47.32
1.95	175.61	180.22	177.92
4.89	439.34	439.89	439.62
9.77	878.14	876.51	877.33
14.66	1301.74	1301.32	1301.53
19.54	1732.75	1735.15	1733.95
24.43	2215.68	2208.67	2212.18
48.85	4366.82	4367.41	4367.11
48.85	4498.38	4488.43	4493.40
73.28	6508.16	6512.73	6510.44
97.70	8795.80	8796.89	8796.35
		R^2	0.9998
	Regression statistics	**Intercept**	9.2227
		Slope	89.242

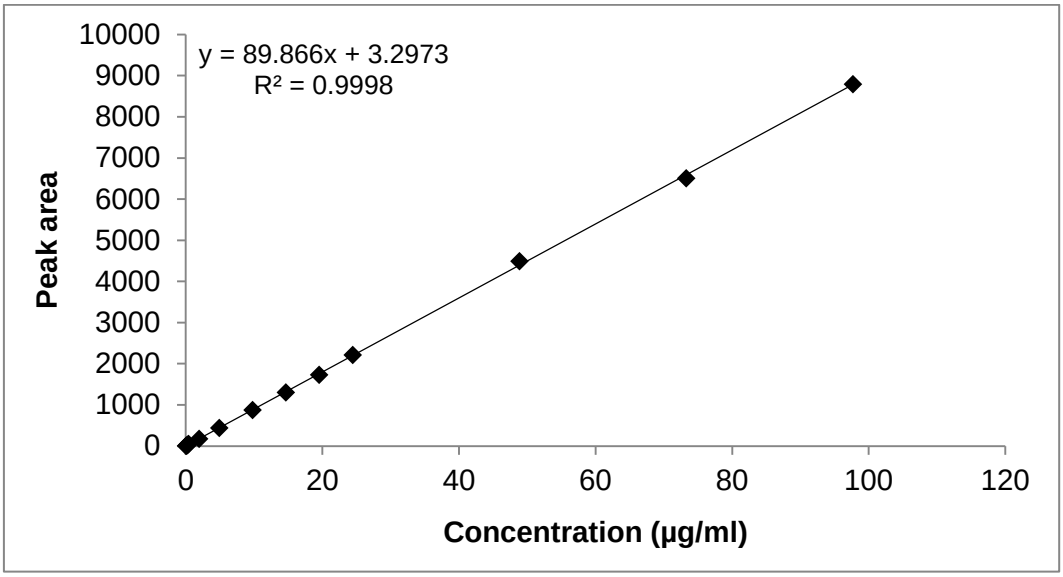

Figure A.10: Linear regression curve for tretinoin.

The developed HPLC method was found linear for tretinoin over the concentration range 0.04 - 97.70 µg/ml. The resultant regression value (R^2 = 0.9998) was within the acceptance criteria.

A.7.3 Accuracy

Table A.3: Accuracy parameters of tretinoin

Concentration (µg/ml)	Peak area 1	Peak area 2	Mean peak area	Recovery	
				µg/ml	%
1.80	159.21	149.37	154.29	1.73	95.89
1.94	172.40	185.50	178.95	2.00	103.31
1.85	162.99	162.15	162.57	1.82	98.53
18.04	1584.24	1591.51	1587.88	17.77	98.48
19.38	1743.74	1738.63	1741.18	19.48	100.52
18.46	1650.75	1656.46	1653.61	18.50	100.23
90.20	7791.72	7820.03	7805.88	87.34	96.83
96.90	8586.08	8609.42	8597.75	96.20	99.28
92.30	8040.97	8031.11	8036.04	89.91	97.41
				Mean	98.94
				SD	2.11
				% RSD	2.13

Over the tretinoin concentration range of 1.8 – 96.9 µg/ml, the HPLC assay method yielded a mean recovery of 98.94%. Precision was satisfactory with an RSD of 2.13%.

A.7.4 Precision

A.7.4.1 Intra-day precision (reproducibility)

Table A.4: Intra-day precision parameters of tretinoin

Concentration (µg/ml)	Peak area 1	Peak peak area 2	Mean	Recovery	
				µg/ml	%
10.14	769.49	771.58	770.54	10.55	104.00
11.26	863.84	866.19	865.01	11.70	103.92
9.93	758.84	758.22	758.53	10.40	104.72
50.70	3870.91	3880.62	3875.76	48.55	95.75
56.30	4309.22	4312.95	4311.08	53.87	95.69
49.65	3788.19	3786.11	3787.15	47.46	95.59
101.40	8287.45	8302.04	8294.75	102.62	101.20
112.60	9232.09	9305.59	9268.84	114.54	101.72
99.30	8392.42	8394.11	8393.26	103.83	104.56
				Mean	100.79
				SD	3.79
				% RSD	3.76

A.7.4.2 Inter-day precision (reproducibility)

Table A.5: Inter-day precision parameters of tretinoin

Area	Day 1	Day 2	Day 3	Inter-day results
1	8625.10	8649.28	8713.48	
2	8631.13	8645.90	8703.88	
Mean	8628.11	8647.59	8708.68	8661.46
SD	3.01	1.69	4.80	34.32
% RSD	0.035	0.020	0.055	0.04

The repeatability was found to be within acceptable limits, with an intra-day variance of 3.76% and an inter-day variance of 0.04%. This assay was hence expected to perform well, even when executed by other personnel and/in a different laboratory.

A.7.5 Ruggedness

A.7.5.1 Stability of sample solutions

A sample was left in the HPLC auto-sampler and was it re-analysed at hourly intervals over the duration of 24 h to determine the stability of the sample.

Table A.6: Sample stability parameters of tretinoin

Time interval (h)	Peak area	Remaining tretinoin %
0	8278.02	101.99
1	8291.47	102.16
2	8294.28	102.19
3	8275.14	101.96
4	8281.96	102.04
5	8279.41	102.01
6	8276.47	101.98
7	8291.83	102.16
8	8296.61	102.22
9	8294.19	102.19
10	8295.82	102.21
11	8289.93	102.14
12	8087.77	99.66
13	8039.67	99.07
14	8292.03	102.17
15	8323.68	102.55
16	8189.90	100.91
17	8332.18	102.66
18	8263.38	101.82
19	8283.77	102.07
20	8252.30	101.68
21	8321.66	102.53
22	8293.58	102.19
23	8307.23	102.35
24	8272.83	101.93
Mean	102.89	101.87
SD	0.80	0.79
% RSD	0.78	0.78

The tretinoin sample solution was found stable over the 24 h period, with a mere 0.78% variation in concentration.

A.7.5.2 System repeatability

A standard tretinoin sample was injected six times in order to test the repeatability of the peak area, as well as its retention time.

Table A.7: System repeatability of the peak area and retention time of tretinoin

	Peak area	Retention time (min)
	1743.74	5.150
	1738.63	5.079
	1741.87	5.133
	1741.11	5.115
	1778.33	5.150
	1741.16	5.136
Mean	1747.47	5.127
SD	13.88	0.025
% RSD	0.79	0.479

System performance proved well within the acceptable range, with RSD values of 0.79% with regards to the peak areas and 0.479% for the retention times.

A.7.6 Robustness

The following changes in the HPLC chromatographic operating parameters were found acceptable:

Column: Both a Luna $C_{18(2)}$ column, 150 x 4.6 mm, 5 µm particle size, 100 Å pores, 17.8% carbon load, end-capped (Phenomenex, Torrance, CA), and a Venusil XBP $C_{18(2)}$ column, 150 x 4.6 mm, 5 µm particle size, 100 Å pores, 19% carbon load, end-capped, were found suitable.

Mobile phase: Acetonitrile (CH_3CN) containing 0.4 - 0.6 % glacial acetic acid (CH_3)COOH (v/v), filtered and degassed, was found suitable.

Flow rate: 0.8 - 1.2 ml/min was found suitable, although the tretinoin peak area decreased significantly at low flow rates. Flow rates below 1 ml/min were hence not recommended.

Wavelength: The wavelength could be altered by ± 3 nm without any adverse effect.

Environment: The analysis was performed by two different analysts on days 2 and 3 of the inter-day precision experiment and on two different HPLC systems (Agilent 1100 series with diode array detection and Agilent 1200 series with variable wavelength UV-detection). The intra-day variation was established as 3.76%.

The method was found to be capable of tolerating small changes in the chromatographic conditions and should it perform well under normal use.

A.8 Chromatographic performance parameters

System suitability tests, as defined in the USP 38, 2015:431, are as follows:

Retention time (min): ± 5.213 min

Number of theoretical plates (N) plates/column (tangent method): 11136

USP Tailing factor (T): 1.050

Capacity factor (k'): 2.075

A.8.1 System suitability parameters

The uncertainty of measurement was determined by utilising both empirically calculated and validation data. The calculated uncertainty of measurement was obtained by combining the uncertainties of each step in the analysis process, expressed as a contribution factor. The value for uncertainty of measurement was determined as follows:

1. A standard solution was injected into the HPLC in triplicate.
2. The relative standard deviation of the peak areas obtained was calculated.
3. The number of theoretical plates for the tretinoin peak was calculated.
4. The tangent method was used to calculate the parameters.

The system was found suitable to perform the analyses, if the following criteria were met:

- The RSD of three injections should not exceed 2%.
- The column must have more than 8352 theoretical plates for tretinoin (75% of the validated value).

A.8.2 Uncertainty measurements

Table A.8: Uncertainty calculations

Empirical calculation	Calculation	% Uncertainty
Weighing of standard	0.010 mg/25 mg x 100	0.04%
100 ml volumetric flask	0.080 ml/100 ml x 100	0.08%
5 ml pipette	0.015 ml/5 ml x 100 (3 x diluted)	0.90%
50 ml volumetric flask	0.050 ml/50 ml x 100 (3 x diluted)	0.30%
	Injection inaccuracy (repeatability)	**1.04%**
	Total uncertainty	**2.36%**

Validation outcomes	Calculation	% Uncertainty
Recovery	99.6%, thus 100 - 99.6%	0.40%
Intra-day and inter-day precision	(3.76 + 0.04%)/2	1.90%
	Total uncertainty	**2.30%**

A.9 Conclusion

This tretinoin HPLC assay method was found to perform well and was it expected to be suitable for the analysis of the tretinoin content in the diffusion samples, as well as in those samples generated during the stability testing, quality control and for batch release purposes. Measurement uncertainty was within the limits for assays in biological matrices.

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ICH ***see*** International Conference on Harmonisation of technical requirements for registration of pharmaceuticals for human use.

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Appendix B

Validation of a single HPLC method for the combined analyses of methyl paraben, propyl paraben, butylated hydroxytoluene and tretinoin

B.1 Introduction

A single HPLC method was developed for the combined analyses of methyl paraben, propyl paraben, butylated hydroxytoluene (BHT) and tretinoin. This method was validated in accordance with the ICH Guidelines, with the purpose of using it for the stability testing of the new rosehip seed oil formulation during this study (ICH-Q2 (R1), 2005). The objective of validating this new HPLC assay method was to ensure that it would be responsive, sensitive, specific and consistent in its determination of the concentrations of each of the four active ingredients, present in a 20% water-in-water (w/w) *Rosa rubiginosa* (rosehip) seed oil, oil-in-water (o/w) emulsion.

As discussed in Appendix A, an HPLC method for the single analysis of tretinoin had also been developed and validated at the Analytical Technology Laboratory at the North-West University, Potchefstroom Campus. The single tretinoin analysis was utilised following the diffusion studies whereas the multiple component analytical method was required during the stability testing formulation assay.

Based upon the successful validation outcomes of this multi-component analytical method, it was indeed used during this study for the combined analyses of the four active ingredients, present in the new cosmeceutical formulation. The preparation of the test samples from the new formulation and the results are discussed in Chapter 3. Tretinoin spiked rosehip oil (Chapter 3, Section 3.4.3.1) was utilised in the preparation of the o/w emulsion, which was then subjected to accelerated stability conditions for a period of 6 months, during which the samples were analysed on HPLC at monthly intervals, as discussed in Chapter 4.

Table B.1: Summary of the successful validation outcomes of a single HPLC method for the analysis of four active ingredients

	Validation results of multiple active ingredients			
Test	**Methyl paraben**	**Propyl paraben**	**BHT**	**Tretinoin**
Range (µg/ml)	15.88 - 476.52	3.33 - 99.84	100.36 - 301.08	2.76 - 82.68
Linearity (R^2)	0.999	0.999	0.998	0.999
Accuracy (%)	100.91	103.25	102.08	99.44
Precision (%RSD*)	2.22	2.22	2.13	2.20

*Relative Standard Deviation

B.2 Chromatographic conditions

The chromatic conditions of the developed HPLC assay method were as follows:

Analytical instrument: HP1200 series HPLC, equipped with a pump, auto-sampler, UV-detector and Chemstation Rev. A.10.03 data acquisition and analysis software, or equivalent (Agilent Technologies, Palo Alto, CA).

Column: A Luna $C_{18(2)}$ column, 150 x 4.6 mm, 5 µm, 100 Å pores, 17.8% carbon load, end-capped (Phenomenex, Torrance, CA), and a Venusil XBP $C_{18(2)}$, 150 x 4.6 mm, 5 µm (Agela Technologies, Newark, DE) column were validated.

Mobile phase A: HPLC water and 0.5% of glacial acetic acid, filtered through Whatman™ GF/F glass micro-fiber filters (0.07 µm).

Mobile phase B: Acetonitrile, containing 0.5% of glacial acetic acid (v/v), filtered through Whatman™ GF/F glass micro-fiber filters (0.07 µm).

Gradient table: The gradient elution method was employed, starting at a 55% acetonitrile/acetic acid solution (Mobile phase B) and a 45% HPLC water/acetic acid solution (Mobile phase A) for the first 0.5 min, followed by a linear increase to a 100% acetonitrile/acetic acid solution (Mobile phase B) at 3.0 min. The mobile phase composition was kept at a 100% acetonitrile/acetic acid solution until 7.5 min has elapsed, whereafter a gradient decrease to a 55% acetonitrile/acetic acid solution (Mobile phase B) was set to be reached at 7.9 min. Thereafter, once the mobile phase composition was maintained, the system was re-equilibrated at the starting conditions for the next analysis.

Flow rate: 1.0 ml/min

Injection volume: 10 µl

Detection: UV was set at 354 nm until 6.0 min, changed to 210 nm until 7.9 min, whereafter it was set at 349 nm until 12.0 min.

Retention time: Methyl paraben (± 3.4 min), propyl paraben (± 4.6 min), BHT (± 7.8 min), tretinoin (± 9.2 min).

Stop time: 12 min

Solvent: Acetonitrile and tetrahydrofuran (THF).

B.3 Standard and Sample preparations

B.3.1 Standard preparation

Standard solutions were prepared as follows:

1. The following quantities of each of the active ingredients were weighed into a single 100 ml volumetric flask and dissolved by filling the flask to volume with acetonitrile: 40 mg of methyl paraben, 8 mg of propyl paraben, 25 mg of BHT and 7 mg of tretinoin (Std 1) (concentrations: 400 µg/ml (methyl paraben), 80 µg/ml (propyl paraben), 250 µg/ml (BHT), 70 µg/ml (tretinoin).

2. 5 ml of the Std 1 solution was transferred into a 50 ml volumetric flask and filled to volume with acetonitrile (Std 2) (concentrations: 40 µg/ml (methyl paraben), 8 µg/ml (propyl paraben), 25 µg/ml (BHT), 7 µg/ml (tretinoin).

3. Each standard solution was prepared in duplicate.

4. The Std 1 and 2 standard solutions were each transferred into amber HPLC auto-sampler vials and analysed in duplicate.

B.3.2 Sample preparation

Sample solutions were prepared as follows:

1. Approximately 1 g of rosehip o/w emulsion was weighed into a 50 ml volumetric flask, dissolved in approximately 20 ml of THF and filled to volume with THF.

2. Three samples were prepared.

3. Each sample was transferred into an amber HPLC auto-sampler vial and analysed in duplicate.

B.4 Calculations

The concentrations of the standard solutions and the generated HPLC peak areas of the standard and sample solutions were captured onto an Excel spreadsheet. The linearity of the active ingredients was determined by performing linear regression analysis on the standard sample peak areas *versus* their concentrations (µg/ml). A standard curve was generated and the slope and y-intercept were used to calculate the concentrations of the samples from their obtained peak areas.

B.5 Validation test procedures and Acceptance criteria

B.5.1 Linearity

The linearity of the HPLC method was validated by employing the following method:

1. Two standard solutions, as described in Section B.3.1 (Standard preparation), were utilised.

2. 4, 6, 8, 10, and 12 µl of each standard solution (Std 1 and Std 2) were injected in duplicate into the HPLC and analysed.

B.5.1.1 Acceptance criteria

The HPLC standard solution assay results were examined and their useable concentration range determined. The useable concentration range was plotted and linear regression analysis performed. Linear regression analysis should yield a regression coefficient (R^2) of ≥ 0.99.

B.5.2 Accuracy

According to the ICH-Q2(R1) guidelines, the accuracy of an analytical procedure demonstrates the closeness of agreement (degree of scatter) between the value that is accepted either as a conventional true value, or an accepted reference value, and the value found (ICH-Q2(R1), 2005:4). The following method was used for the validation of the accuracy of the HPLC method:

1. Std 1 standard solutions were prepared in triplicate, as described above in Section B.3.1 (Standard preparation).

2. 8, 10, and 12 µl of each of the three Std 1 solutions were injected in duplicate into the HPLC and analysed.

B.5.2.1 Acceptance criteria

Percentage recovery is an indication of the accuracy of the analytical system. According to the acceptance criteria, the percentage recovery of the HPLC assay system should be between 98% and 102% (ICH, 2005:9).

B.5.3 Precision

According to the ICH-Q2(R1) guidelines, the precision of an analytical procedure expresses the closeness of agreement (degree of scatter) among a series of measurements, obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision was considered at repeatability, intermediate precision and reproducibility levels (ICH-Q2(R1), 2005:4). The precision was validated, by using the following intra-day and inter-day precision methods:

B.5.3.1 Intra-day precision (repeatability)

The purpose of this test was to establish the variability of multiple equivalent test samples, prepared and analysed together on the same day.

The intra-day precision was validated by employing the following method:

1. Nine samples of approximately 1.0 g each of the rosehip o/w emulsion was each weighed into a 50 ml volumetric flask (9 flasks) and filled to volume with THF.

2. A single standard solution, at 100% of the expected sample concentration (Std 2), as described in the Standard preparation method (Section B.3.1), was prepared.

3. The sample and standard solutions were injected into the HPLC and analysed in duplicate.

B.5.3.2 Inter-day precision

To determine the between-day variability of the same batch of the prepared emulsion formulation that had been utilised during the intra-day precision testing (Section B.5.3.1), each sample was kept in the HPLC auto-sampler tray at controlled laboratory conditions protected from ambient light and re-analysed in triplicate on two more days, by using the same method as for the intra-day precision analyses (at 100% of the sample concentration). Where possible, a different analyst performed the analyses, using different equipment.

B.5.3.3 Acceptance criteria

For the purpose of this study, the limits were set for the intra-day repeatability to be more than 5% (n = 9), whereas the inter-day precision had to be higher than 10% (n = 9).

B.5.4 Ruggedness

B.5.4.1 Stability of sample solutions

The purpose of this test was to establish the time that it would take for a test sample to degrade by 2%, starting immediately following sample preparation, by using the same controlled laboratory conditions than what would prevail during the sample analyses.

The stability of the test samples was validated by employing the following method:

1. One sample solution was prepared, as described in Section B.3.2.

2. The sample solution was injected in duplicate into the HPLC for analysis.

3. Following the first set of HPLC injections, the samples were kept in the auto-sampler and re-analysed at hourly intervals in duplicate to determine their stability.

4. The HPLC pump was programmed to reduce the mobile phase flow rate to 0.1 ml/min for most of the time in between the hourly analyses, from after the elution of the tretinoin peak at 12 min, and to reset the flow rate back to 1.0 ml/min at 5 min prior to repeating the next set of hourly analyses.

B.5.4.1.1 Acceptance criteria

Sample solutions should be analysed within that established time period within which they have not yet degraded by more than 2%.

B.5.4.2 System repeatability

The system repeatability was validated by injecting a sample and/or a standard solution for six consecutive times and were the repeatability of the peak areas, as well as their retention times determined.

B.5.4.2.1 Acceptance criteria

The peak area and retention times should each have a relative standard deviation (RSD) of 2%, or less.

B.5.5 Robustness

According to the ICH-Q2(R1) guidelines, the robustness of an analytical procedure is a measurement of its capacity to remain unaffected by small, but deliberate variations in the method parameters and is it indicative of the reliability of the method during normal usage (ICH-Q2(R1), 2005:5).

Deliberate changes to the flow rate, injection volume, wavelength and mobile phase composition were therefore made and were the standard solutions analysed to establish the impact of such changes on the chromatographic results.

B.5.6 System suitability (system and method performance characteristics)

System suitability was validated by generating an extended performance report on the standard solution, taking care that only the relevant peaks were integrated.

B.5.6.1 Acceptance criteria

The performance results obtained were examined and were realistic performance characteristics established that had to be complied with in order to successfully perform the analyses.

B.5.7 Uncertainty of measurement

Identification of the sources of error and the extent thereof are required to determine the degree of uncertainty of measurement during the utilisation of a quantitative method. The calculated uncertainty of measurement was established by combining the uncertainties of measurement of

each step in the analytical process and to express each as a contribution factor. For reporting purposes, the set value of the uncertainty of measurement should be included (Taverniers *et al.*, 2004:487).

B.6 Validation results

B.6.1 Linearity and Range

B.6.1.1 Methyl paraben

Table B.2: Linearity test results of methyl paraben

Concentration µg/ml	Peak area 1	Peak area 2	Mean peak area
15.88	214.06	221.97	218.01
23.83	336.36	320.64	328.50
31.77	478.19	435.65	456.92
39.71	569.32	549.76	559.54
47.65	643.66	655.67	649.67
158.84	2509.06	2554.14	2531.60
238.26	3703.72	3692.33	3698.03
317.68	4848.93	4843.93	4846.43
397.10	5978.16	5985.38	5981.77
476.52	7036.87	7048.19	7042.53
Regression statistics		**R^2**	0.999
		Intercept	0.98
		Slope	15.05

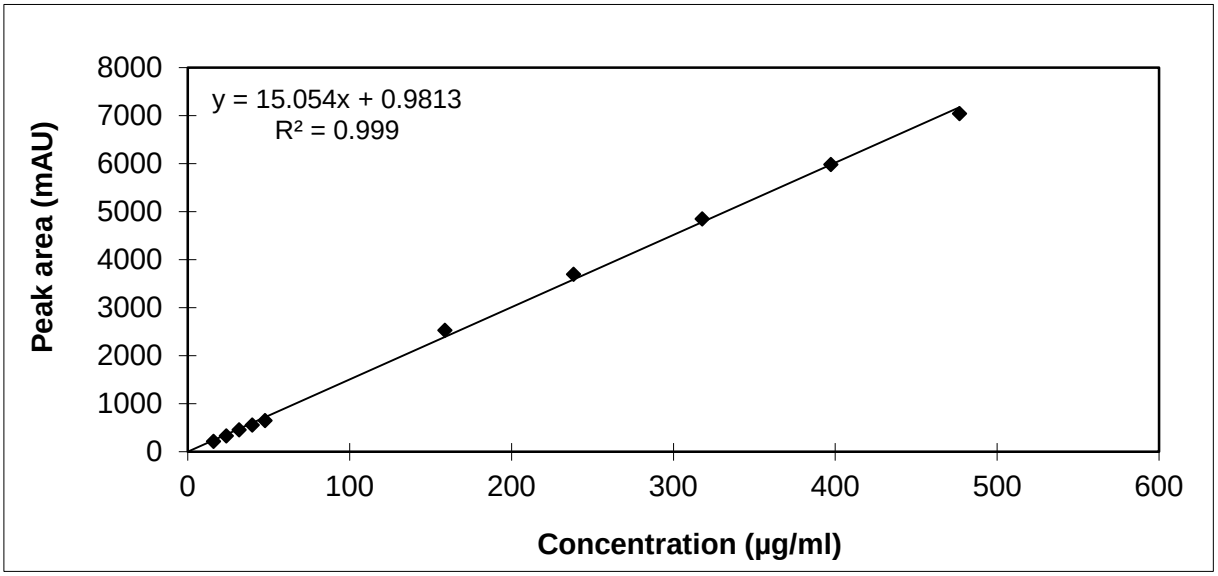

Figure B.1: Linear regression curve of the methyl paraben standard solutions.

The method was linear for methyl paraben over the concentration range of 15.9 - 476.5 µg/ml. The established regression value (R^2 = 0.999) was within the set acceptance criteria limits.

B.6.1.2 Propyl paraben

Table B.3: Linearity test results of propyl paraben

Concentration µg/ml	Peak area 1	Peak area 2	Mean peak area
3.33	43.95	43.74	43.84
4.99	71.36	73.75	72.56
6.66	102.17	99.17	100.67
8.32	121.60	107.29	114.45
9.98	135.72	150.11	142.92
33.28	467.92	446.49	457.21
49.92	719.55	691.39	705.47
66.56	935.44	945.59	940.51
83.20	1144.61	1105.31	1124.96
99.84	1371.96	1384.88	1378.42
Regression statistics		**R^2**	0.999
		Intercept	4.75
		Slope	13.75

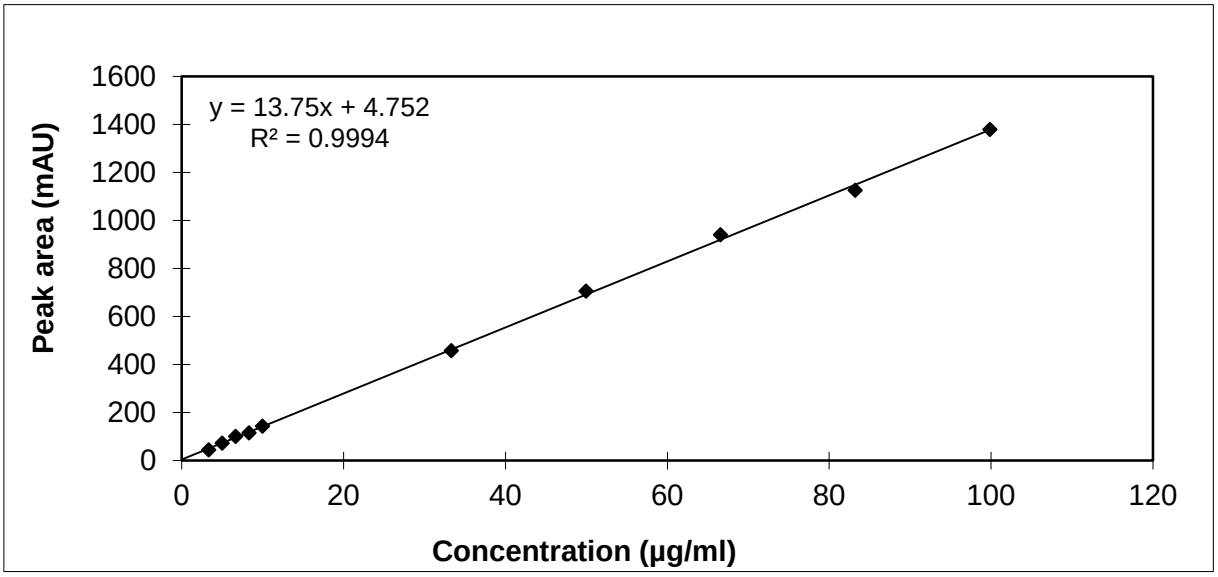

Figure B.2: Linear regression curve of the propyl paraben standard solutions.

The method was linear for propyl paraben over the concentration range of 3.3 - 99.8 µg/ml. The established regression value (R^2 = 0.999) was within the set acceptance criteria limits.

B.6.1.3 BHT

Table B.4: Linearity test results of BHT

Concentration µg/ml	Peak area 1	Peak area 2	Mean peak area
100.36	1664.05	1606.14	1635.10
125.45	1988.24	1981.40	1984.82
150.54	2353.19	2334.63	2343.91
175.63	2711.48	2697.03	2704.26
200.72	3038.97	3022.72	3030.85
225.81	3321.49	3276.62	3299.06
250.90	3600.21	3599.46	3599.84
275.99	3887.81	3933.14	3910.48
301.08	4196.65	4221.10	4208.88
Regression statistics		**R^2**	0.998
		Intercept	411.41
		Slope	12.74

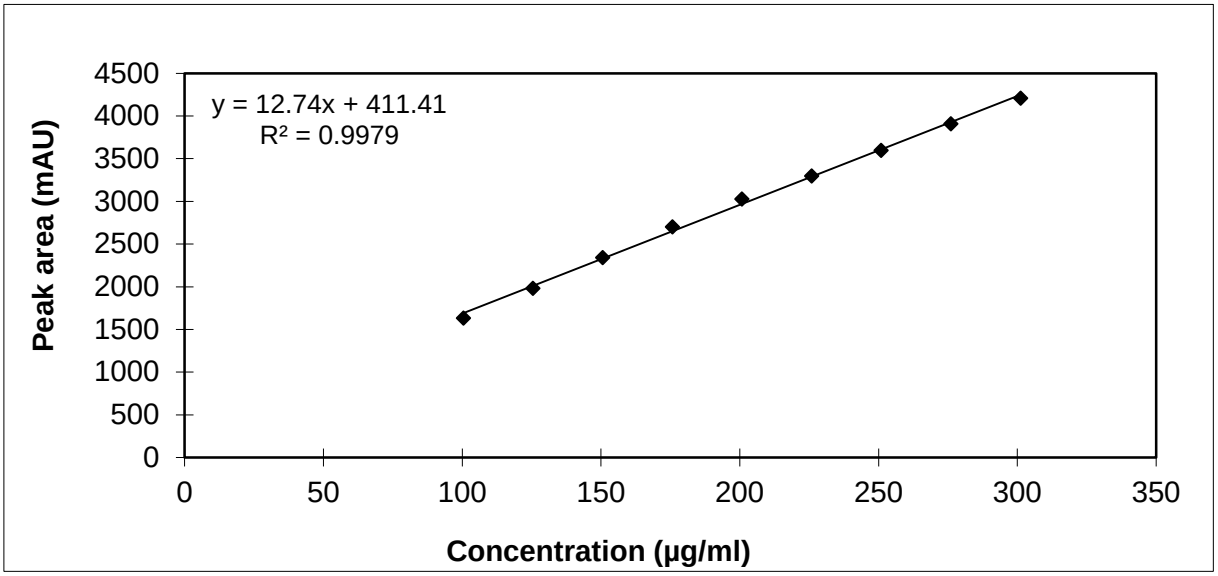

Figure B.3: Linear regression curve of the BHT standard solutions.

The method was linear for BHT over the concentration range of 100.4 - 300.1 µg/ml. The established regression value (R^2 = 0.999) was within the set acceptance criteria limits.

B.6.1.4 Tretinoin

Table B.5: Linearity test results of tretinoin

Concentration µg/ml	Peak area 1	Peak area 2	Mean peak area
2.76	254.91	236.51	245.71
4.13	391.02	390.02	390.52
5.51	496.88	499.43	498.16
6.89	627.29	642.98	635.14
8.27	765.76	749.48	757.62
27.56	2570.50	2547.58	2559.04
41.34	3739.56	3775.75	3757.66
55.12	5008.19	5011.33	5009.76
68.90	6222.56	6223.09	6222.83
82.68	7419.79	7423.82	7421.81
Regression statistics		**R^2**	0.999
		Intercept	20.96
		Slope	90.01

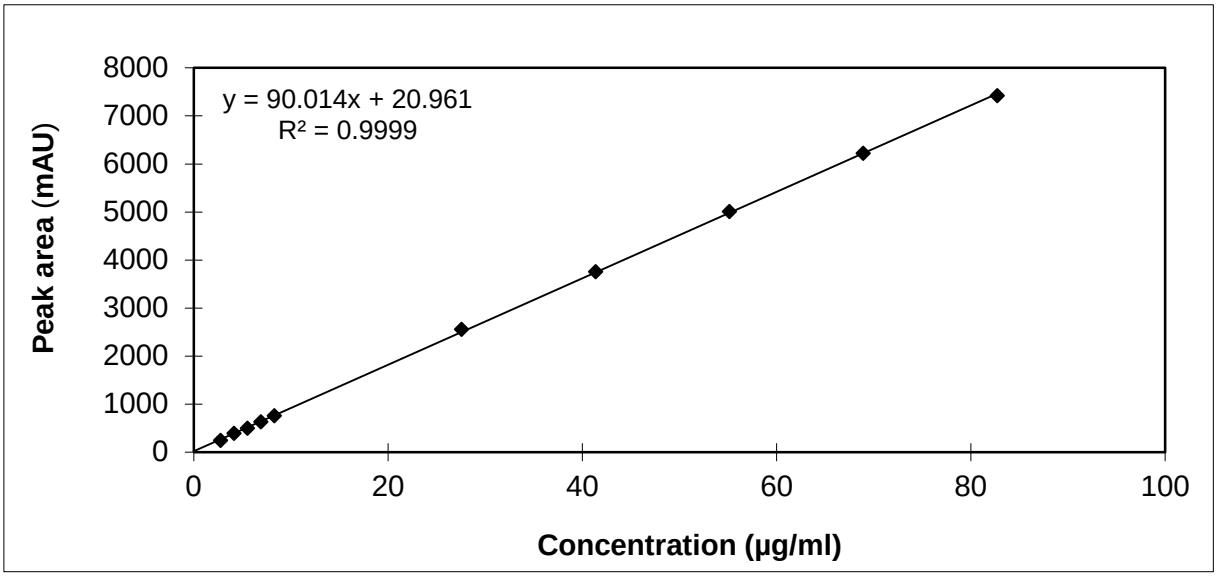

Figure B.4: Linear regression curve of the tretinoin standard solutions.

The method was linear for tretinoin over the concentration range of 2.8 - 82.7 µg/ml. The established regression value (R^2 = 0.999) was within the set acceptance criteria limits.

B.6.2 Accuracy

B.6.2.1 Methyl paraben

Table B.6: Accuracy test parameters of methyl paraben

Concentration (µg/ml)	Peak area 1	Peak area 2	Mean peak area	Recovery	
				(µg/ml)	(%)
327.60	5013.99	4971.32	4992.66	336.17	102.62
327.92	5020.69	4954.58	4987.64	335.83	102.41
322.24	4902.69	4926.05	4914.37	330.90	102.69
409.50	6140.46	6133.77	6137.12	413.23	100.91
409.90	6143.78	6146.95	6145.37	413.79	100.95
402.80	6044.26	5996.20	6020.23	405.36	100.64
491.40	7254.84	7240.38	7247.61	488.00	99.31
491.88	7284.65	7259.99	7272.32	489.67	99.55
483.36	7123.07	7103.87	7113.47	478.97	99.09
				Mean	100.91
				SD	1.34
				%RSD	1.33

Over the 80 - 120% standard concentration range, the method yielded an average methyl paraben recovery of 100.91%, which was within the set accepted criteria limits of between 98 - 102% (ICH, 2005:9).

B.6.2.2 Propyl paraben

Table B.7: Accuracy test parameters of propyl paraben

Concentration (µg/ml)	Peak area 1	Peak area 2	Mean peak area	Recovery	
				(µg/ml)	(%)
66.56	947.69	948.03	947.86	69.73	104.76
70.00	1011.03	1001.35	1006.19	74.02	105.74
68.56	936.22	963.32	949.77	69.87	101.91
83.20	1175.94	1152.42	1164.18	85.64	102.93
87.50	1197.64	1246.28	1221.96	89.89	102.73
85.70	1224.58	1171.31	1197.95	88.13	102.83
99.84	1394.94	1366.18	1380.56	101.56	101.72
105.00	1466.99	1471.27	1469.13	108.07	102.93
102.84	1448.15	1450.29	1449.22	106.61	103.67
				Mean	103.25
				SD	1.22
				%RSD	1.18

Over the 80 - 120% standard concentration range, the method yielded an average propyl paraben recovery of 103.25%, which was slightly higher than the set accepted criteria limits of 98 - 102%, but was it found acceptable for biological matrices (ICH, 2005:9).

B.6.2.3 BHT

Over the 80 - 120% standard concentration range, the method yielded an average BHT recovery of 102.08%, which was within the set accepted criteria limits of between 98 - 102% (ICH, 2005:9).

Table B.8: Accuracy test parameters of BHT

Concentration (µg/ml)	Peak area 1	Peak area 2	Mean peak area	Recovery	
				(µg/ml)	(%)
160.06	2497.01	2468.53	2482.77	162.59	101.58
162.56	2535.11	2530.42	2532.77	166.51	102.43
165.06	2553.90	2592.86	2573.38	169.70	102.81
198.82	3027.40	3028.43	3027.92	205.38	103.30
200.07	3033.63	3020.62	3027.13	205.32	102.62
202.57	3033.03	3095.18	3064.11	208.22	102.79
238.59	3472.88	3496.25	3484.57	241.22	101.10
239.09	3490.93	3474.94	3482.94	241.09	100.84
240.09	3507.44	3506.68	3507.06	242.99	101.21
				Mean	102.08
				SD	0.85
				%RSD	0.83

B.6.2.4 Tretinoin

Over the 80 - 120% standard concentration range, the method yielded an average tretinoin recovery of 99.44%, which was within the set accepted criteria limits of between 98 - 102% (ICH, 2005:9).

Table B.9: Accuracy test parameters of tretinoin

Concentration (µg/ml)	Peak area 1	Peak area 2	Mean peak area	Recovery	
				(µg/ml)	(%)
54.56	5186.93	5213.53	5200.23	55.92	102.48
55.04	5168.16	5142.06	5155.11	55.43	100.71
55.76	5115.54	5113.92	5114.73	55.00	98.63
68.20	6398.43	6419.35	6408.89	68.91	101.04
68.80	6306.27	6337.09	6321.68	67.97	98.80
69.70	6319.27	6355.81	6337.54	68.14	97.77
81.84	7621.74	7627.45	7624.60	81.98	100.18
82.56	7548.07	7466.94	7507.51	80.72	97.78
83.64	7604.45	7570.76	7587.61	81.59	97.54
				Mean	99.44
				SD	1.64
				%RSD	1.65

B.6.3 Precision

B.6.3.1 Intra-day precision (repeatability)

B.6.3.1.1 Methyl paraben

Table B.10: Intra-day precision test parameters of methyl paraben

Mass (g)	Peak area 1	Peak area 2	Mean peak area	Recovery	
				(µg/ml)	(%)
1.08	834.54	837.76	836.15	59.64	105.93
1.03	850.10	877.09	863.60	58.91	101.31
1.06	838.07	864.06	851.06	59.64	104.07
1.02	831.86	867.15	849.50	57.34	100.25
1.00	851.69	826.50	839.09	55.80	98.77
1.06	857.15	883.44	870.29	60.86	103.85
1.01	846.04	851.06	848.55	56.71	99.26
1.02	839.76	846.88	843.32	57.25	100.82
1.03	851.44	941.33	896.39	61.12	101.26
				Mean	101.72
				SD	2.26
				%RSD	2.22

Precision was satisfactory with an RSD of 2.22% (acceptance criteria %RSD lower than 10%) and a recovery of 101.72%.

B.6.3.1.2 Propyl paraben

Precision was satisfactory with an RSD of 2.22% (acceptance criteria %RSD lower than 5%) and a recovery of 100.32%.

Table B.11: Intra-day precision test parameters of propyl paraben

Mass (g)	Peak area 1	Peak area 2	Mean peak area	Recovery	
				(µg/ml)	(%)
1.08	170.46	183.47	176.96	13.60	104.46
1.03	169.31	154.95	162.13	11.92	99.91
1.06	179.37	172.05	175.71	13.27	102.63
1.02	151.99	161.29	156.64	11.39	98.87
1.00	138.23	143.70	140.96	10.10	97.40
1.06	161.22	167.36	164.29	12.38	102.42
1.01	161.92	173.11	167.52	12.06	97.89
1.02	147.88	152.61	150.24	10.99	99.43
1.03	175.70	167.28	171.49	12.60	99.86
				Mean	100.32
				SD	2.23
				%RSD	2.22

Precision was satisfactory with an RSD of 2.22% (acceptance criteria %RSD lower than 10%) and a recovery of 100.32%.

B.6.3.1.3 BHT

Table B.12: Intra-day precision test parameters of BHT

Mass (g)	Peak area 1	Peak area 2	Mean peak area	Recovery	
				(µg/ml)	(%)
1.08	2695.61	2727.39	2711.50	180.54	94.66
1.03	2657.67	2669.04	2663.36	176.76	99.01
1.06	2708.71	2662.84	2685.78	178.52	97.58
1.02	2683.00	2705.08	2694.04	179.17	100.07
1.00	2664.07	2682.29	2673.18	177.53	101.51
1.06	2696.13	2698.98	2697.56	179.45	96.97
1.01	2679.65	2676.32	2677.99	177.91	101.44
1.02	2687.74	2682.42	2685.08	178.47	99.95
1.03	2723.70	2720.41	2722.06	181.37	99.72
				Mean	98.99
				SD	2.10
				%RSD	2.13

Precision was satisfactory with an RSD of 2.13% (acceptance criteria %RSD lower than 5%) and a recovery of 98.99%.

B.6.3.1.4 Tretinoin

Table B.13: Intra-day precision test parameters of tretinoin

Mass (g)	Peak area 1	Peak area 2	Mean peak area	Recovery	
				(µg/ml)	(%)
1.08	587.65	610.52	599.08	6.42	100.32
1.03	596.02	583.74	589.88	6.05	104.89
1.06	581.46	557.92	569.69	6.00	102.11
1.02	570.01	557.07	563.54	5.72	106.00
1.00	616.53	569.91	593.22	5.93	107.59
1.06	576.58	578.92	577.75	6.07	102.32
1.01	509.58	518.38	513.98	5.16	107.06
1.02	506.88	525.58	516.23	5.27	105.40
1.03	557.40	553.78	555.59	5.69	104.94
				Mean	104.51
				SD	2.30
				%RSD	2.20

Precision was satisfactory with an RSD of 2.20% (acceptance criteria %RSD lower than 5%) and a recovery of 104.51%.

B.6.3.2 Inter-day precision

B.6.3.2.1 Methyl paraben

Table B.14: Inter-day precision test parameters of methyl paraben

	Day 1	Day 2	Day 3	Inter-day results
	776.49	775.17	775.8	
	794.76	791.86	793.3	
	772.22	766.89	769.6	
Mean	781.16	777.97	779.57	779.57
SD	9.78	10.39	10.05	10.16
%RSD	1.25	1.34	1.29	1.30

The inter-day precision was acceptable at 1.30% (acceptance criteria %RSD lower than 10%). Repeatability was within acceptable limits, and should the assay method perform well, even when performed by other personnel in different laboratories.

B.6.3.2.2 Propyl paraben

Table B.15: Inter-day precision test parameters of propyl paraben

	Day 1	Day 2	Day 3	Inter-day results
	137.62	141.06	139.34	
	144.68	143.53	144.10	
	138.52	139.00	138.76	
Mean	140.27	141.20	140.73	140.73
SD	3.14	1.85	2.40	2.55
%RSD	2.24	1.31	1.70	1.81

The inter-day precision was acceptable at 1.81% (acceptance criteria %RSD lower than 10%). Repeatability was within acceptable limits, and should the assay method perform well, even when performed by other personnel in different laboratories.

B.6.3.2.3 BHT

Table B.16: Inter-day precision test parameters of BHT

	Day 1	Day 2	Day 3	Inter-day results
	2733.96	2747.91	2740.94	
	2757.90	2748.07	2752.99	
	2769.14	2765.78	2767.46	
Mean	2753.67	2753.92	2753.79	2753.79
SD	14.67	8.39	10.84	11.59
%RSD	0.53	0.30	0.39	0.42

The inter-day precision was acceptable at 0.42% (acceptance criteria %RSD lower than 10%). Repeatability was within acceptable limits, and should the assay method perform well, even when performed by other personnel in different laboratories.

B.6.3.2.4 Tretinoin

The inter-day precision was acceptable at 4.89% (acceptance criteria %RSD lower than 10%). Repeatability was within acceptable limits, and should the assay method perform well, even when performed by other personnel in different laboratories.

Table B.17: Inter-day precision test parameters of tretinoin

	Day 1	Day 2	Day 3	Inter-day results
	593.80	616.05	604.93	
	551.38	557.83	554.60	
	532.98	553.96	543.47	
Mean	559.39	575.95	567.67	567.67
SD	25.47	28.40	26.73	27.73
%RSD	4.55	4.93	4.71	4.89

B.6.4 Ruggedness

B.6.4.1 Stability of sample solutions

A freshly prepared sample solution was transferred into two amber HPLC vials and injected in duplicate each for initial analysis. These samples were left in the auto-sampler and was care taken to limit any exposure to UV-light. The samples were re-analysed over several time intervals to determine their stability.

The propyl paraben, BHT and tretinoin were found stable over a period of 12 h. The methyl paraben parameters, however, as indicated in red in Table B.18, did not remain within acceptable limits, indicative thereof that all samples had to be analysed without any delay, immediately following preparation in controlled laboratory conditions.

Sample preparation was performed under controlled laboratory conditions (temperature, 23 ± 2 °C and light protection). Precautions were taken to limit sample exposure to direct sunlight and artificial light. During sample preparation the laboratory lights were switched off, doors and windows were closed. Amber laboratory glassware was used and together with immediately transferring prepared samples into amber HPLC vials, promptly placing the filled vials into the auto-sampler tray and by keeping the auto-sampler door closed.

Table B.18: Sample stability test parameters of methyl paraben, propyl paraben, BHT and tretinoin

Time (h)	Methyl paraben		Propyl paraben		BHT		Tretinoin	
	Peak area	%RSD	Peak area	%RSD	Peak area	%RSD	Peak area	%RSD
0	542.1	100.0	120.2	100.0	2646.2	100.0	739.3	100.0
1	554.2	102.2	119.2	99.2	2642.9	99.9	740.3	100.1
2	545.0	100.5	120.6	100.3	2647.7	100.1	741.1	100.2
3	551.7	101.8	120.3	100.2	2656.0	100.4	742.5	100.4
4	511.9	94.4	120.5	100.3	2661.2	100.6	744.8	100.7
5	554.3	102.3	120.9	100.6	2671.1	100.9	748.8	101.3
6	560.3	103.3	122.3	101.8	2676.6	101.1	748.5	101.2
7	554.3	102.2	122.4	101.8	2686.7	101.5	754.5	102.1
8	529.3	97.6	122.7	102.2	2696.4	101.9	757.4	102.4
9	572.3	105.6	122.2	101.7	2710.8	102.4	758.8	102.6
10	563.1	103.9	122.6	102.1	2714.3	102.6	761.9	103.1
11	531.1	98.0	124.1	103.3	2719.4	102.8	764.1	103.4
12	548.5	101.2	125.1	104.1	2736.5	103.4	767.4	103.8
13	546.2	100.7	124.6	103.7	2734.1	103.3	769.6	104.1
14	579.2	106.8	126.0	104.8	2752.6	104.0	775.3	104.9
15	579.3	106.8	126.8	105.5	2763.4	104.4	776.8	105.1
Mean	551.4	101.7	122.5	102.0	2694.7	101.8	755.7	102.2
SD*	17.4	3.2	2.2	1.8	38.3	1.4	12.2	1.7
%RSD	**3.2**	**3.2**	1.8	1.8	1.4	1.4	1.6	1.6

*Standard Deviation

B.6.4.2 System repeatability

Samples were injected six times consecutively in order to test the repeatability of the generated peak areas and retention times.

B.6.4.2.1 Methyl paraben

Table B.19: System repeatability of the peak area and retention time of methyl paraben

	Peak area	Retention time (min)
	833.45	3.39
	810.54	3.39
	817.24	3.40
	809.99	3.40
	815.35	3.40
	796.60	3.41
Mean	813.86	3.40
SD	10.96	0.01
%RSD	1.35	0.20

System performance proved well within the acceptable range (an RSD of 2%, or less), with RSD values of 1.35% for the peak area and 0.20% for the retention time.

B.6.4.2.2 Propyl paraben

Table B.20: System repeatability of the peak area and retention time of propyl paraben

	Peak area	Retention time (min)
	144.93	4.62
	141.48	4.62
	143.69	4.64
	142.90	4.64
	143.72	4.64
	142.90	4.65
Mean	143.27	4.63
SD	1.05	0.01
%RSD	0.73	0.25

System performance proved well within the acceptable range (an RSD of 2%, or less), with RSD values of 0.73% for the peak area and 0.25% for the retention time.

B.6.4.2.3 BHT

Table B.21: System repeatability of the peak area and retention time of BHT

	Peak area	Retention time (min)
	2758.79	7.78
	2756.60	7.79
	2762.55	7.80
	2752.41	7.78
	2746.41	7.80
	2746.77	7.82
Mean	2753.92	7.80
SD	5.99	0.01
%RSD	0.22	0.18

System performance proved well within the acceptable range (an RSD of 2%, or less), with RSD values of 0.22% for the peak area and 0.18% for the retention time.

B.6.4.2.4 Tretinoin

Table B.22: System repeatability of the peak area and retention time of tretinoin

	Peak area	Retention time (min)
	565.28	9.23
	563.51	9.24
	566.02	9.25
	561.38	9.22
	565.05	9.28
	573.10	9.23
Mean	565.72	9.24
SD	3.63	0.02
%RSD	0.64	0.20

System performance proved well within the acceptable range (an RSD of 2%, or less), with RSD values of 0.64% for the peak area and 0.20% for the retention time.

B.6.5 Robustness

The following changes in the chromatographic operating parameters of this HPLC assay were found to be acceptable:

Column: A (1) Luna $C_{18(2)}$ column, 150 x 4.6 mm, 5 µm, 100 Å pores, 17.8% carbon load, end-capped (Phenomenex, Torrance, CA), and a (2) Lichrospher 100-5 RP-18ec cartridge column, 125 x 4.0 mm, 5 µm, 100 Å pores, 21.5% carbon load, end-capped (Machery-Nagel, Düren, Germany) were found suitable for this multi-component analytical method. The latter column was found to be operated at a flow rate of 0.7 - 1.0 ml/min.

Mobile phase: Concentrations of 27 - 33% methanol was still suitable in spite of different retention times of the active ingredients. The stop time of 12 min was, however, still adequate for complete elution of all actives.

Flow rate: 0.8 - 1.2 ml/min (0.7 - 1.0 ml/min for the Lichrospher 100-5 RP-18ec cartridge column).

Wavelength: The wavelengths could be altered by ± 3 nm, without any undesirable impact on the test outcomes.

The validated analytical method was capable of tolerating small changes in the chromatographic conditions and should it perform well under conditions of normal use.

B.6.6 Chromatographic performance parameters

Table B.23: Chromatographic performance parameters, as described in the USP 38 (2015a)

Parameter	Methyl paraben	Propyl paraben	BHT	Tretinoin
Retention time (min)	± 3.406 min	± 4.634 min	± 7.799 min	± 9.311 min
Number of theoretical plates (N) plates/column (tangent method)	7662	26634	35975	34747
USP Tailing factor (T)	1.351	1.092	1.040	0.970
Capacity factor (k')	2.75	3.71	6.21	7.43

B.6.6.1 System suitability parameters

The uncertainty of measurement of this HPLC method was determined by utilising empirically calculated data, as well as the validation data being generated during this study. The calculated uncertainty of measurement was established by combining the uncertainties of each step in the analytical process, which was each expressed as a contribution factor. The value for uncertainty of measurement was determined as follows:

1. A standard solution was analysed in triplicate on the HPLC.
2. The relative standard deviation of the generated peak areas was calculated.
3. The number of theoretical plates for each of the methyl paraben, propyl paraben, BHT and tretinoin peaks was calculated.
4. The tangent method to calculate the parameters was applied.

The validated HPLC analytical system was found suitable to perform the analyses, provided the following criteria were met:

1. If the %RSD of 3 injections was ≤ 2%.
2. The column should have more than:
    - 5,746 theoretical plates for methyl paraben (75% of the validation value).
    - 19,975 theoretical plates for propyl paraben (75% of the validation value).
    - 26,981 theoretical plates for BHT (75% of the validation value).
    - 26,060 theoretical plates for tretinoin (75% of the validation value).

B.6.6.2 Uncertainty measurements

Table B.24: Uncertainty calculations

Empirical calculation	Calculation	% Uncertainty
Weighing of standard	0.01 mg/25 mg x 100	0.04%
100 ml flask	0.08 ml/100 ml x 100	0.08%
5 ml pipette	0.015 ml/5 ml x 100 (3 x diluted)	0.90%
50 ml volumetric flask	0.05 ml/50 ml x 100 (3 x diluted)	0.30%
Injection inaccuracy (repeatability)		1.04%

Total uncertainty		**2.36%**
Methyl paraben validation data	**Calculation**	**% Uncertainty**
Recovery	100.9%, thus (100 - 100.9%)	0.90%
Intra-day and inter-day precision	[(2.2% + 1.30%)/2]	1.75%
Total uncertainty		**2.65%**

B.7 Conclusion

The performance of the validated HPLC method was found satisfactory and suitable for the combined analyses of the four active ingredients, i.e. methyl paraben, propyl paraben, butylated hydroxytoluene (BHT) and tretinoin, present in the emulsionformulation. This method was validated with the purpose of using it for the stability testing of the new rosehip seed oil formulation during this study (Chapter 4). The measurement of uncertainty was found to be within the limits set for assays of biological matrices, i.e. between 98% and 102%.

From an overall evaluation of the method validation outcomes, it was concluded that this developed HPLC method was reliable and adequately sensitive for the multi-component determination of the concentrations of methyl- and propyl paraben, BHT and of tretinoin, present in the new semi-solid emulsion formulation. This method was hence regarded as suitable for the analysis of the four analytes during stability testing of the new rosehip seed oil containing formulations for quality control and batch release purposes.

References

ICH ***see*** International Conference on Harmonisation of technical requirements for registration of pharmaceuticals for human use.

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Appendix C

Clinical efficacy of topical formulations containing *Rosa rubiginosa* seed oil

C.1 Introduction

In vivo clinical efficacy studies are performed to verify the final product efficacy label claims, as presented by manufacturers, or final product owners. Product owner claims for cosmetic formulations containing *Rosa rubiginosa* (rosehip) oil, generally include statements about the reversal of skin damage, improvement of skin condition or skin repair. Label claims typically include: reducing the appearance of stretch marks, scarring resulting from surgery or burns (including those from radiation and sunburn), improvement of eczema, psoriasis, dermatitis, wrinkle formation and visible signs of premature skin ageing such as hyper-pigmentation or age spots. Rosehip seed oil is considered a safe, inexpensive and effective ingredient that is used to aid in skin repair and even to prevent skin damage (Concha *et al*., 2006:771; Ioele *et al*., 2005:251; Mountain Rose Herbs.com, 2011).

Rosehip powder contains fatty acid constituents which has exhibited clinically significant anti-inflammatory action when taken orally, or following topical application (Cohen, 2012:496; Evangelista *et al*., 2014:106). Due to the potential alleviation of inflammation when applied topically, the change in erythema values, measured through *in vivo* irritated skin models, was therefore investigated during this study.

It is known that the skin's lipid matrix assists in optimising the skin barrier function, by reducing trans-epidermal water loss (TEWL) and by deterring water soluble substances from penetrating the epidermal layers. Deficiencies in stratum corneum lipid composition would therefore result in insufficient skin barrier protection (Baroni *et al.,* 2012:259; Menon *et al.,* 2012:6; WHO, 2006:14). A previous study, aimed at assessing the reinforcement of the skin barrier function resulting from the application of externally applied lipids, had indicated the possibility of accelerated skin barrier repair and a reduction in TEWL (Coderch *et al.,* 2002:144). Contradictory research information states that free fatty acids, which are the building blocks of triglycerides and lipids found in vegetable plant oils, such as rosehip seed oil, are known to disrupt the stratum corneum lipid structural order, which is often associated with increased TEWL values and a resultant deficient skin barrier function (Mack Correa *et al*., 2014:39). It

was therefore deemed necessary to investigate this clinical effect, as well as the label claims associated with skin barrier repair properties.

In this Appendix, *in vivo* clinical studies, performed on human subjects to evaluate the clinical efficacy of 100% rosehip seed oil and 20% rosehip seed oil o/w (oil in water) emulsion, are discussed. The following label claims with regards to these two rosehip seed oil products were investigated:

- Short- and long-term skin moisturising effects.
- Anti-ageing properties, such as:
    - Improvement of skin elasticity of the upper skin layers.
    - Influence on skin topography through surface evaluations of the living skin (SELS) parameters.
    - Anti-wrinkle properties.
- Skin barrier repair (assessment of TEWL change).
- Anti-inflammatory properties.

C.2 Materials and methods

C.2.1 Formulations and test products

Commercially available *Rosa rubiginosa* (rosehip) seed oil (The Rosehip Company (Pty) Ltd, Lesotho) was acquired and utilised as a test product. Oil in water (o/w) emulsions, containing 20% rosehip seed oil (w/w) and vehicle components, as summarised in Chapter 3 (Table 3.1.), were prepared prior to each clinical study and utilised as the test product. The o/w emulsion vehicle components included Labrafac® WL 1349 (Gattefossé, Cedex, France), Tween® 80 (Merck (Pty) Ltd, Modderfontein, South Africa), Carbopol® Ultrez 21 (Lubrizol Advanced Materials, Brussels, Belgium), butylated hydroxytoluene (BHT) (Merck KGaA, Darmstadt, Germany), methyl paraben (Merck (Pty) Ltd, Modderfontein, South Africa), propyl paraben (Merck (Pty) Ltd, Midrand, South Africa) and deionized water. For utilisation during the irritation patch studies, commercially available 1% hydrocortisone acetate (w/w) water dispersible cream (Mylocort® cream) was used as positive control on 1% sodium lauryl sulphate (SLS) (w/v) irritated skin areas.

C.2.2 Non-invasive measurements

Bio-engineering instruments were used to perform *in vivo*, non-invasive measurements. Instrument check calibration was performed prior to every study to eliminate possible variations in measurements, resulting from inaccurate instrument standardisation (Berardesca *et al*., 2002:449).

C.2.2.1 Hydration level of the stratum corneum

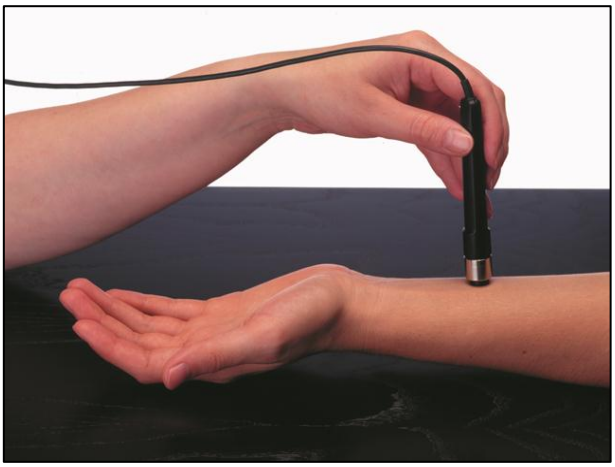

Figure C.1: Corneometer® CM 825 (Adapted from Courage & Khazaka electronic GmbH, 2015a).

The Corneometer® CM 825 (Courage & Khazaka, Cologne, Germany) calculates skin surface water content by measuring the skin's electrical capacitance value (di-electric constant) at a skin depth ranging within 10 - 20 µm, thus ensuring that deeper skin layers do not influence the measurements. Skin conductance values are determined by measuring the resistance to an electrical current when it flows through the skin. Skin capacitance and conduction increase with an increase in skin moisture. The highly sensitive Corneometer® CM 825 probe, designed to measure small capacitance changes resulting from variations in water content, measures changes in the stratum corneum water content by means of a precision measuring capacitor inside the probe. Changes are quantified and converted into arbitrary units (AU) (Berardesca, 1997:127; Darlenski & Fluhr, 2011:128; Li *et al*., 2001:24).

C.2.2.2 Visco-elastic properties of the skin

The Cutometer® MPA 580 (Courage & Khazaka, Cologne, Germany) deforms the upper layer of the skin mechanically by applying a negative pressure of 400 mbar for 2 sec that pulls the upper skin layer into the 2 mm probe aperture (Figure C.2).

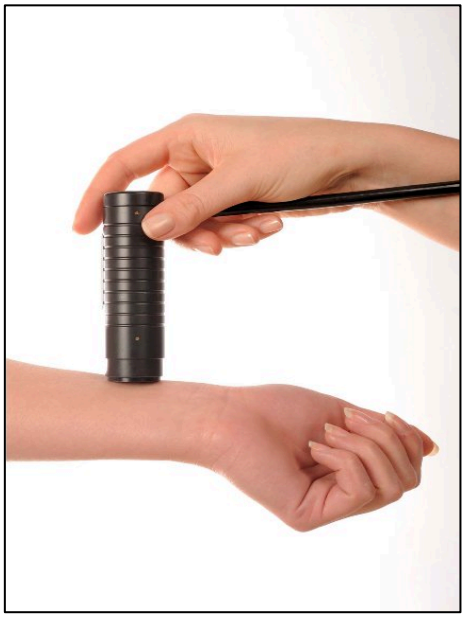

Figure C.2: Cutometer® MPA 580 (Adapted from Courage & Khazaka electronic GmbH, 2015b).

Skin resistance (firmness), when sucked into the probe opening, and the skin's ability to recover from such deformation (elasticity), are thus measured. These values are calculated by a non-contact optical measuring system and are represented by generating a deformation curve. The negative pressure deforms the skin mechanically when the skin is drawn into the probe aperture, which represents the elastic properties of skin. Skin suction is discontinued after a pre-determined time and do the measured values reflect the visco-elastic characteristics of the skin. The deformation curve (Figure C.3) is described where: Ue = immediate deformation, Uv = delayed distension, Uf = final deformation (skin distensibility), Ur = immediate retraction, Ua = total recovery, R = residual deformation at the end of the measuring cycle (resilient distension) (Kapoor & Saraf, 2010:298).

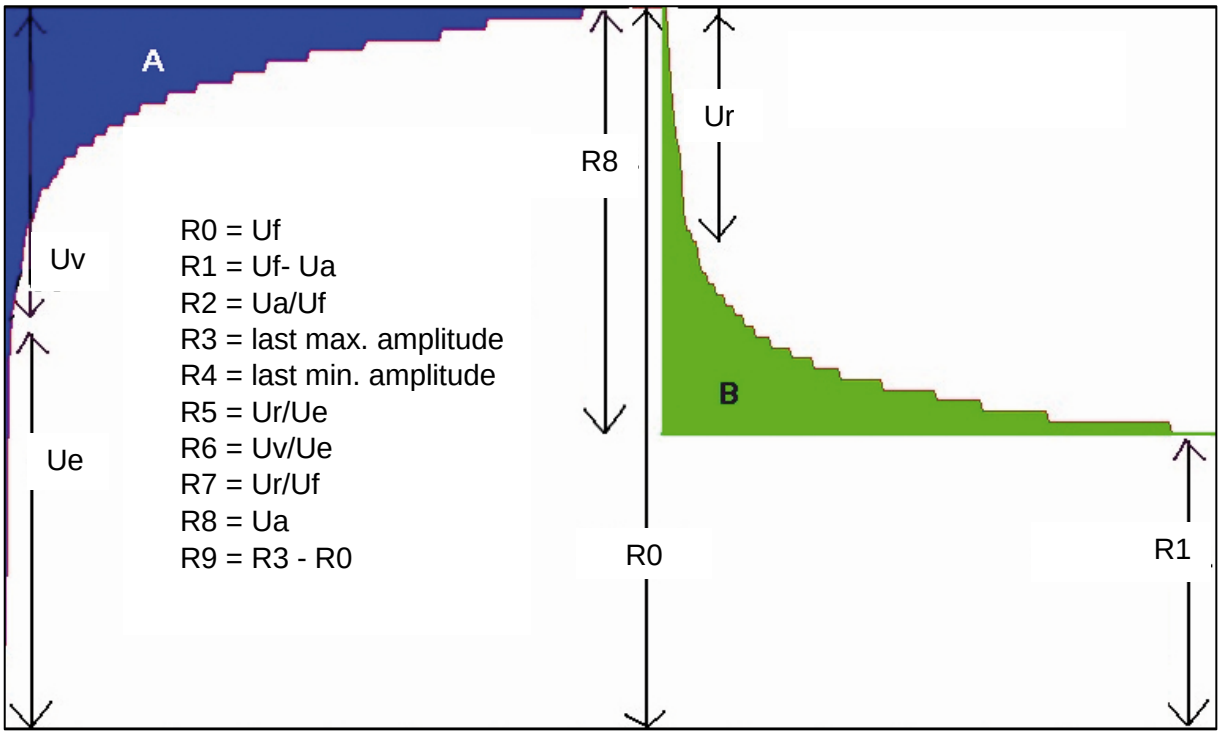

Figure C.3: Typical skin deformation curve generated from measurements with a Cutometer® MPA 580 (Adapted from Courage & Khazaka electronic GmbH, 2015b).

The mechanical R parameters are categorised into elastic and visco-elastic groups and further sub-divided into absolute and relative parameters (Akhtar *et al*., 2011:699). R parameters, as described in the following Table C.1, were utilised and evaluated during this study to assess the skin's visco-elastic properties.

Table C.1: Summary and clinical application of R parameters calculated from the Cutometer® MPA 580 deformation curves

R	Calculation	Description	Clinical application
R0	R0 = Uf	R0 represents the skin's distensibility [c] i.e. skin firmness, passive behaviour and resistance to the negative force applied [a, b]. R0 is illustrated as the first maximum (max.) amplitude and highest point of the first curve.	R0 is dependent upon skin thickness. ↑ R0 = ↑ skin firming = ↑ stratum corneum thickness.
R1	R1 = Uf-Ua	R1 represents the skin's ability to return to its original state and is determined by the first minimum (min.) amplitude and lowest point on the first curve [b].	↓ R1 = ↑ elasticity, or skin's capability to return to its original state [a].
R2	R2 = Ua/Uf	R2 is a relative elastic parameter representing the gross elasticity of the skin (including viscous deformation) [b, c].	The closer that R2 is to 1 (100%) = ↑ elasticity [b].
R3	R3 = last maximum amplitude	R3 is the last max. amplitude (highest point of the last curve), compared to the max. amplitude of the first curve [b], determined after repetition of the cycles.	R3 = ↑ amplitude after each suction indicate ↑ "tiring" effect of skin [b].
R4	R4 = last minimum amplitude	R4 is calculated using the last min. amplitude (last measuring point of the last curve), compared to the min. amplitude of the first curve [b]. R4 is determined after repetition of the cycles.	R4 = ↓ deformation ability indicate ↑ "tiring" effect of skin [b].
R5	R5 = Ur/Ue	R5 represents the net elasticity of the skin (without viscous deformation) [b, c] and reflects the skin's ability to recover to its original position after mechanical deformation. R5 values are independent of the skin's thickness.	The closer that R5 is to 1 (100%), the more elastic the curve where ↓ R5 = ↓ elasticity [b].
R6	R6 = Uv/Ue	R6 is a relative parameter, representing the ratio of visco-elasticity to elastic distension [c] of the skin, which is reflected on the elastic segment of the deformation curve [a, b].	↓ R6 = ↑ elasticity [b].
R7	R7 = Ur/Uf	R7 demonstrates biological elasticity [c], or "immediate retraction": "final" distension ratio where a portion of elasticity is compared to the complete curve [b].	The closer the value is to 1 (100%) the higher the skin's elasticity and firmness.
R8	R8 = Ua	Ua is the area under the suction segment [c] of the first curve, representing skin's total recovery [b].	The closer that R8 is to 0, the higher the skin's ability to return to its original condition [b, c]. ↓ R8 = ↑ elasticity [b].
R9	R9 = R3-R0	R9 represents the tiring effect of the skin (calculated after repeated suction of the skin) [b].	↓R9 = ↓ skin "tiring" effect [b].

a Akhtar *et al*., 2011:699; b Courage and Khazaka electronic GmbH, 2015b; c Kapoor and Saraf, 2010:298

C.2.2.3 Skin surface pH

The Skin-pH-Meter® PH 905 (Courage & Khazaka, Cologne, Germany) measures skin surface pH and consists of two glass electrodes combined into one probe, i.e. the hydrogen (H^+) ion sensitive electrode and an additional reference electrode. The probe, which is connected to a multi probe adapter (MPA), transmits measurement values directly to computer software (Courage & Khazaka electronic GmbH, 2015c).

Figure C.4: Skin-pH-Meter® PH 905 (Adapted from Courage & Khazaka electronic GmbH, 2015c).

C.2.2.4 Skin erythema assessments

The Skin Pigmentation Analyzer SPA99® (Courage & Khazaka, Cologne, Germany) measures the haemoglobin (erythema) and pigmentation (melanin) contents in the upper skin layer. The Skin Pigmentation Analyzer SPA99® measurements and the computerised software program are designed to make use of the reflection and absorption of light wave principles. A pre-determined, quantified wavelength light band is emitted, followed by a receiver that measures the light being reflected back into the probe by the skin. The quantity of light being absorbed by the skin is then subtracted from the quantity of light that is emitted by the probe, whereafter the result is calculated and quantified on a scale from 0 - 99. For the purpose of the anti-erythema study during this research, haemoglobin values and changes over time were measured.

Figure C.5: Skin Pigmentation Analyzer SPA99® (Adapted from Courage & Khazaka electronic GmbH, 2015d).

C.2.2.5 Skin surface morphology and digital imaging

The Visioscan® VC 98 (Courage & Khazaka, Cologne, Germany) was used to analyse skin topography changes and is designed with a high resolution camera and halogenide lightsource. Ultraviolet light illuminate the skin surface uniformly, preventing reflections from deeper layers in the skin. Images of the skin area (6 x 8 mm), taken with the built-in camera, are displayed in high resolution black and white images and can the wrinkles and skin surface properties be clearly distinguished. Computer software analyses the data and calculates various parameters, which are utilised to evaluate skin surface topography (Courage & Khazaka electronic GmbH, 2015e). The surface evaluations of the living skin (SELS) parameters, i.e. scaliness (SEsc), roughness (SEr), wrinkles (SEw) and smoothness (SEsm), as described in Table C.2, were evaluated during this study.

Table C.2: Summary of parameters calculated with Visioscan® VC 98 skin surface images

Parameter	Known as	Visioscan® VC 98 computer software analysis	Clinical significance
Surface evaluations of the living skin (SELS)			
SEsc	Scaliness	Image analysis that quantifies stratum corneum desquamation [b,c].	↓ SEsc = ↓ stratum corneum flaky skin cells = ↑ skin moisture [b,c].
SEr	Roughness	Is calculated from the grey colour levels above threshold, in comparison with the whole image [b, c].	↓ SEr = ↓ skin roughness [b,c].
SEw	Wrinkles	Wrinkle formation is determined by the average number/ average width of the wrinkles [b].	↑ SEw = ↑ number of wrinkles [a, c].
SEsm	Smoothness	Is computed, using the average width of the wrinkles [b].	↑ SEsm = ↑ moisturising or anti-ageing effect of formulation [b].
Rku	Curtosis	Is calculated from the histogram generated after image analysis [b].	The closer the value is to 3, the more ideal the curve of histogram = smoother skin [b].
Skin surface image analysis			
Surface	--	A measure of the size of the wavy skin surface: stretched skin surface [b].	(x:1) where similar or close values = ↑ smoother skin area [b].
Volume	--	The virtual amount of liquid needed to cover the average height of all mountains on the image is calculated [b].	↓ volume = ↑ smoother skin area [b].
Texture			
NRJ	Energy	Calculates the homogeneity of colour in the skin area image [b].	↑ NRJ = ↑ moisture, ↑ elasticity [b].
VAR	Variance	Calculates the variation in pixel values [b].	↑ VAR = ↑ roughness of skin [b].
CONT	Contrast	Calculates the difference between pixel grey values on the image [b].	↓ CONT = ↓ contrasts = ↑ skin condition [b]
ENT	Entropy	Determined by the frequency of a grey level combination occurring in a skin image [b].	↑ ENT = ↑ hydration level of skin [b].

a Choi *et al*., 2013:351; b Courage and Khazaka electronic GmbH, 2015e; c Ferreira *et al*., 2010:446

Figure C.6: Visioscan® VC 98 (Adapted from Courage & Khazaka electronic GmbH, 2015e).

C.2.2.6 Trans-epidermal water loss determinations

The VapoMeter® (Delfin, Finland) is a non-invasive, closed chamber instrument, which measures skin surface TEWL (Delfin Technologies Ltd, 2014). TEWL parameters indicate the degree of efficiency by intact skin to prevent uncontrolled water loss through evaporation from the stratum corneum. TEWL reflects permeability and efficient skin barrier function (Darlenski *et al.*, 2011:147; Darlenski & Fluhr, 2011:128).

Figure C.7: VapoMeter® (Adapted from Delfin Technologies Ltd, 2014).

C.2.3 General protocol design

The clinical studies and treatment protocols employed during this research were designed and aligned in accordance with the European Cosmetic, Toiletry and Perfumery Association (COLIPA) guidelines for the evaluation of the efficacy of cosmetic products, as well as with the European Group for Efficacy Measurements on Cosmetics and Other Topical Products (EEMCO), and with the International Conference on Harmonisation (ICH) Good Clinical Practice (GCP) guidelines. All participants signed an informed consent form and were permitted to discontinue their participation at any time during the study (Berardesca, 1997:129; Danby *et al.*, 2013:42; Dupont *et al.*, 2012:207; Katsarou *et al.*, 2000:85; Zhai *et al.*, 2000:77).

Since seasonal changes significantly affect skin conditions, it would be responsible for large variations in the skin parameter data being generated. Cold winter weather causes excessive dryness and scaliness of the skin, which may alter the skin's barrier function. Hot summer weather conditions, however, yield increased water content measurements, due to sweating and changes in cutaneous capillary blood flow. Seasonal variation should therefore be limited, or avoided if possible and is it therefore essential to plan clinical studies accordingly (Berardesca, 2011:92). To limit possible seasonal change influences during this research, all clinical efficacy studies were performed during the winter months from May to August. Clinical efficacy study measurements were performed in an environmentally controlled clinical laboratory, in which the temperature and humidity were maintained between 20 - 25°C and 40 - 60% relative humidity (RH), respectively. Participants were allowed to acclimatise for 30 min prior to the measurements (Berardesca, 1997:129; Wunderlich, 2011:70).

C.2.3.1 Participants

Due to the existence of many volunteer related variables and to minimise the effects thereof, clinical efficacy protocol design included detailed pre-determined inclusion and exclusion criteria related to gender, age, ethnicity or skin type, and health condition (Berardesca, 2011:93, 94). Gender differences correspond to certain structural skin differences and is dermal thickness generally higher for men than woman. To therefore limit possible variances due to gender differences, only female participants were included in this study (Berardesca, 2011:93, 94; Darlenski & Fluhr, 2011:135). To limit possible variation resulting from age differences, participants were divided into two age groups and specifically enrolled to participate in either the short-, or the long-term clinical study (Ryu *et al*., 2008:356). Because of inter-racial skin type differences, a group of Caucasian (Fitzpatrick skin type I-III) subjects were screened and enrolled in all of the clinical studies to be performed (Darlenski & Fluhr, 2012:270). To ensure the safety and well-being, all selected participants were to be in good physical health, not pregnant, nor breast feeding (Serup, 2001:143, 148).

Because of the nature of the clinical effects being investigated (anti-ageing and anti-inflammatory properties), no participants were allowed to topically apply, nor ingest either vitamin A (nor derivatives), or corticosteroid containing products within 3 months prior to commencement of the study, until after completion of the clinical study (Rawlings *et al*., 2012:26; Shlivko *et al.*, 2014:137; Zussman *et al*., 2010:511).

All participants had to refrain from unnecessary UV rays, or sun exposure for the duration of the study (Serup, 2001:148; Zussman *et al*., 2010:512). Participants were given clear instructions of all protocol procedures, which they were expected to strictly adhere to throughout the duration of the clinical study. A wash-out period started 7 days before commencement of each clinical study. Participants were supplied with pH neutral Dove® soap (Morelli & Weston, 1987:636). No other soap, lotion, nor perfume were to be used on either arm during the wash-out period and for the duration of the study.

Participants were to refrain from consuming any caffeine, or alcohol on days when measurements were taken. A list of prohibited medication, that could cause cutaneous microvascular vasodilatation, or -constriction and hence possible variations in measurements, were given to each subject (Henricson *et al*., 2009:204; Petersen *et al.,* 2010:501). Participants had to inform the investigator of any changes in their state of health, or about any pharmacological treatments taken during the study, as well as whether they experienced any adverse effects, related or non-related to treatment with the products being tested.

C.2.3.2 Protocols: Short- and long-term clinical studies

Open-label, non-randomised, comparative clinical studies were conducted. Short-term, single application (4 h) and a long-term, multiple application (84 days) studies were performed. The aim of these studies were to investigate the short-term moisturising effects and long-term anti-ageing effects of both the 100% rosehip seed oil (w/w) and an o/w emulsion, containing 20% of rosehip seed oil (w/w).

The participants were divided into two age groups and enrolled to participate in either of the short-term and long-term clinical studies (Ryu *et al*., 2008:356). Seventeen female, Caucasian participants, aged between 23 - 43 years, took part and completed the short-term study. Sixteen female Caucasian participants, aged between 35 - 53 years, were enrolled for the long-term study, of which two were unable to complete, due to personal reasons.

Baseline (T0) measurements were performed and the test formulations applied directly thereafter, while wearing a latex glove for the applications. The total duration of the short-term study was approximately 5 h (including acclimatisation of 30 min and the time needed to perform the baseline measurements). The short-term measurements were performed at

60 (T1), 120 (T2) and 240 min (T3) after application of the test product. The anti-ageing study was concluded over a period of 84 days and measurements were performed at days 28 (T1), 56 (T2) and 84 (T3).

Since skin water content measurements of contra-lateral anatomical skin sites exhibit indistinguishable differences, comparative left-right studies were performed, using the dominant and non-dominant volar forearms of each subject (Berardesca, 1997:129).

Different anatomical locations show variations in skin thickness, appendageal distribution, stratum corneum lipid composition, epidermal capillary network and skin structure, which would significantly influence measurement values (WHO, 2006:18, 23). To limit variation, therefore, a stencil and non-toxic skin marker were used to ensure that corresponding skin areas were measured for each subject, ensuring that subsequent measurements were performed at the correct areas. Two 8 cm^2 non-overlapping test locations were marked on each arm. The active ingredient's quantity was pre-calculated to range between 1 and 3 mg/cm^2 and was each applied to the marked skin areas.

Purposely for the long-term study, the test product multiple application process was demonstrated and the protocol procedures clearly explained to each subject. Rosehip seed oil was applied on the pre-marked area of the dominant volar forearm. The o/w emulsion was applied on the corresponding anatomical site, on the non-dominant arm. Test products were to be applied twice daily (between 06:00 and 08:00 and between 18:00 and 20:00) by each subject for the duration of the 84 days.

Three instruments, the Corneometer® CM 825, the Visioscan® VC 98 and the Cutometer® MPA 580 were used for measuring purposes. Each subject's own skin was used as control and each time interval value was evaluated relative to the initial baseline value. The short-term study comprised a skin hydration assessment, whereas skin hydration, topography and visco-elastic properties were assessed during the long-term study.

C.2.3.3 Protocol: Skin irritation patches (erythema assessments)

An open-label, non-randomised, comparative clinical study was performed. The aim of the erythema study was to assess the anti-inflammatory and skin-barrier repair properties of rosehip seed oil. 100% rosehip seed oil (w/w) and a cosmetic o/w emulsion formulation, containing 20% of rosehip seed oil (w/w), were evaluated.

The clinical effect following the application of the test products to pre-irritated skin areas, were compared to those of a 1% hydrocortisone cream (w/w) formulation (positive control), a 1% SLS (w/v) (negative control; induced skin irritation area) and untreated natural skin (control).

Fifteen female, Caucasian participants, aged between 20 - 30 years, took part in this study. One subject did not complete this study, due to personal reasons. The erythema study was performed over a period of 9 days. The first measurements (T0) were performed on specific untreated marked areas on the non-dominant inner arm of each subject. T0 data was used to determine the natural baseline redness (erythema) and TEWL values of each subject's skin, measured at five skin locations.

1% SLS (w/v) solutions were prepared by dissolving 0.5 g of SLS in 50 ml deionised water. Aluminium Finn Chamber® (8 mm inner diameter) on Scanpor® (SmartPractice®, Phoenix, USA) was used as the occlusive patch test device. Five Finn Chambers® were applied on the inner forearm. Four chambers, filled with 1% SLS (w/v) and one empty Finn Chamber® (control), were applied to the skin for a period of 24 h. Subjects were instructed to protect the skin test area from water contact and to refrain from excessive exercise for the next 24 h.

The Finn Chambers® were removed 24 h after application. The first 2 days of the clinical trial were used to induce skin irritation. T1 measurements were performed 48 h after the T0 measurements (thus 24 h following removal of the Finn Chamber® patch strip). T1 erythema measurements were reviewed and compared to the initial T0 outcomes to establish whether skin irritation had been induced. Following the T1 measurements, the test products were applied according to a pre-determined skin location diagram on the irritated skin areas:

- **Skin area 1** (pre-treated with 1% SLS (w/v)): treatment with 1% hydrocortisone cream (w/w) (positive control).
- **Skin area 2** (no pre-treatment with 1% SLS (w/v)): (empty Finn Chamber®) no test product treatment (untreated natural skin) (control).
- **Skin area 3** (pre-treated with 1% SLS (w/v)): treatment with o/w emulsion containing 20% rosehip seed oil (w/w).
- **Skin area 4** (pre-treated with 1% SLS (w/v)): no test product treatment (negative control).
- **Skin area 5** (pre-treated with 1% SLS (w/v)): treatment with 100% rosehip seed oil.

No test products were applied to skin areas 2 and 4, firstly to represent the natural skin condition (control = skin area 2) and secondly, to measure the natural healing process of irritated skin (negative control = skin area 4). T2 measurements were performed 48 h (2 days) after T1 and T3 measurements and 120 h (5 days) after T2.

The test products were applied directly following measurements. Subjects received an application diary and specially marked syringes filled with the test products. All subjects were instructed to apply each test product as demonstrated, twice daily. On measurement days,

subjects were instructed not to apply any test products before measurements were completed. Three instruments were used for data generation, i.e. the VapoMeter® (Delfin Technologies Ltd., Kuopio, Finland) for measuring TEWL, the Skin-pH-Meter® PH 905 (Courage & Khazaka, Cologne, Germany) and the Skin Pigmentation Analyzer SPA99® (Courage & Khazaka, Cologne, Germany) for measuring haemoglobin content (erythema).

C.2.4 Ethics

All clinical studies were conducted in accordance to the World Medical Association (WMA) Declaration of Helsinki (Ethical principles of medical research involving human subjects), consistent with the principles applied in Good Clinical Practise (GCP) (WMA, 2013). The clinical efficacy studies were approved under the umbrella application titled, "(*In vivo*) cosmetic efficacy studies" (NWU-00097-10-A5). Clinical efficacy studies were performed in conjunction with an independent clinical cosmetics testing laboratory, the Cosmetics Efficacy Laboratory (CEL) at the Centre for Pharmaceutical and Biomedical Service (North-West University, South Africa).

C.2.5 Statistical design

Interaction analyses were performed by employing a mixed analysis model, with fixed repeated measures concerning treatment and an analytical review (AR(1)) covariance structure. Statistical significance was determined at a 95% confidence interval and p-value of < 0.05. Restricted maximum likelihood value analyses (Type III tests of fixed effects) were used to determine the statistical significant differences among treatments, exposure times and interactions for time and treatment. It was assumed that the random sample subjects, used during this study, were equivalent and representative of a population who would normally apply these treatments.

Pairwise comparisons between treatments were performed, based upon an estimated marginal means (mean value for measurement values obtained from T0 - T3), using Sidak adjustments for multiple comparisons at a 95% confidence interval for differences ($p < 0.05$), to determine statistical significant differences.

Univariate F-tests, based upon the linearly independent pairwise comparisons between the estimated means of each treatment at each measurement time, were performed to determine the treatment effect size. In addition, Cohen's d-value was used to evaluate the practical significances of observed differences. An effect size (d) ≈ 0.20 is indicative of a small effect or practical non-significant difference, $d \approx 0.50$ points towards a medium effect, or practical visible difference, whereas $d \approx 0.80$ signifies a large effect, or practical significant difference (Ellis & Steyn, 2003:52).

Baseline values (T0) were measured for each treatment site prior to application of each test product. The percentage change (% Change) was calculated, relative to initial conditions (baseline value).

$$\% \text{ Change} = ((Tn-T0)/T0) \times 100 \qquad \textbf{Equation C.1}$$

Where:

Tn = Measurement value at given time with n = 1 (28 days), n = 2 (56 days) and n = 3 (84 days) for the long-term study and n = 1 (60 min), n = 2 (120 min) and n = 3 (240 min) for the short-term, single application study.

T0 = baseline value.

C.3 Results and discussion

C.3.1 Short-term (4 h) single treatment application studies

C.3.1.1 Skin hydration effects

Arbitrary Corneometer® values were obtained, indicative of the skin hydration levels. The results obtained after a single application of each test product, i.e. the 20% rosehip seed oil o/w emulsion (hereafter referred to as **ND2**) and the 100% rosehip seed oil (hereafter referred to as **D2**), are summarised in Table C.3.

Table C.3: Short-term skin hydration Corneometer® measurements (mean ± SD)

Time	ND2	D2	Untreated skin
T0	23.90 ± 4.92	23.60 ± 5.64	27.73 ± 5.75
T1	28.58 ± 3.89	31.48 ± 4.41	28.70 ± 4.54
T2	26.59 ± 5.92	28.19 ± 4.73	29.46 ± 5.32
T3	26.44 ± 4.66	28.14 ± 5.48	30.17 ± 5.14

ND2: 20% rosehip seed oil o/w emulsion; D2: 100% rosehip seed oil

C.3.1.1.1 Hierarchical linear models to test the effects of treatment, time and treatment-time interactions

Short-term Corneometer® analyses, performing restricted maximum likelihood (REML) data analysis (Type III test of fixed effects) at a 95% confidence interval, revealed statistical significant differences between time-treatment interactions ($p < 0.0001$), treatment ($p < 0.0001$) and time ($p < 0.0001$).

C.3.1.1.2 Pairwise treatment comparisons

Pairwise comparisons, based upon estimated marginal means, using Sidak adjustments for multiple comparisons, were performed to determine whether statistical significant differences ($p < 0.05$) were revealed when comparing (1) interactions among treatments and (2) interactions among treatments compared to untreated skin areas. Statistical significant differences were revealed among ND2 and D2 ($p = 0.003$), for interactions between ND2 and untreated skin ($p < 0.0001$) and with regards to interactions between D2 and untreated skin ($p = 0.007$). Statistical significant differences were therefore determined among the applied test products (between ND2 and D2), but also among the applied test products, ND2 and D2 each, compared to the untreated skin areas. These statistical significant differences being revealed were supported by the results, as illustrated in Figure C.8.

C.3.1.1.3 Effect sizes: treatments over time

Univariate F-tests, based upon linearly independent, pairwise comparisons of the estimated means of the effect of each treatment over time were performed and the magnitude of the effect (Cohen's d-value) determined. Noteworthy larger effects were obtained with D2, compared to ND2, indicating that D2 had increased skin hydration to a higher extent than ND2 at T1, T2 and T3 and did these results again concur with the results, as illustrated in Figure C.8 and summarised in Table C.4.

Table C.4: Effect sizes of each treatment relative to initial skin hydration level (T0)

Time	Effect size (Cohen's d-value) relative to T0		
	ND2	D2	Untreated skin
T1	1.33*	2.25*	0.28
T2	0.77*	1.31*	0.49
T3	0.72	1.29*	0.70

*d ≈ 0.8 signifies large effect, or practical significant difference

C.3.1.1.4 Treatment effects: mean percentage changes over time relative to baseline measurements

Both D2 and ND2 had increased skin hydration, relative to T0. Similarly, maximum mean percentage hydration increases were demonstrated for both ND2 and D2 after T1 (60 min). D2 had improved skin hydration (mean ± SD relative to T0) with 34.56 ± 15.82% after 60 min, followed by 20.46 ± 11.04% and 20.26 ± 17.14% at 120 min and 240 min, respectively (determined using Equation C.1). ND2 showed a similar trend as D2, although a smaller clinical effect and hydration increase were observed, i.e. ND2 had improved skin hydration by 20.29 ± 10.86% at T1 (60 min), 11.26 ± 8.27% at T2 (120 min) and 10.96 ± 9.41% at T3 (240 min), relative to T0 (Figure C.8).

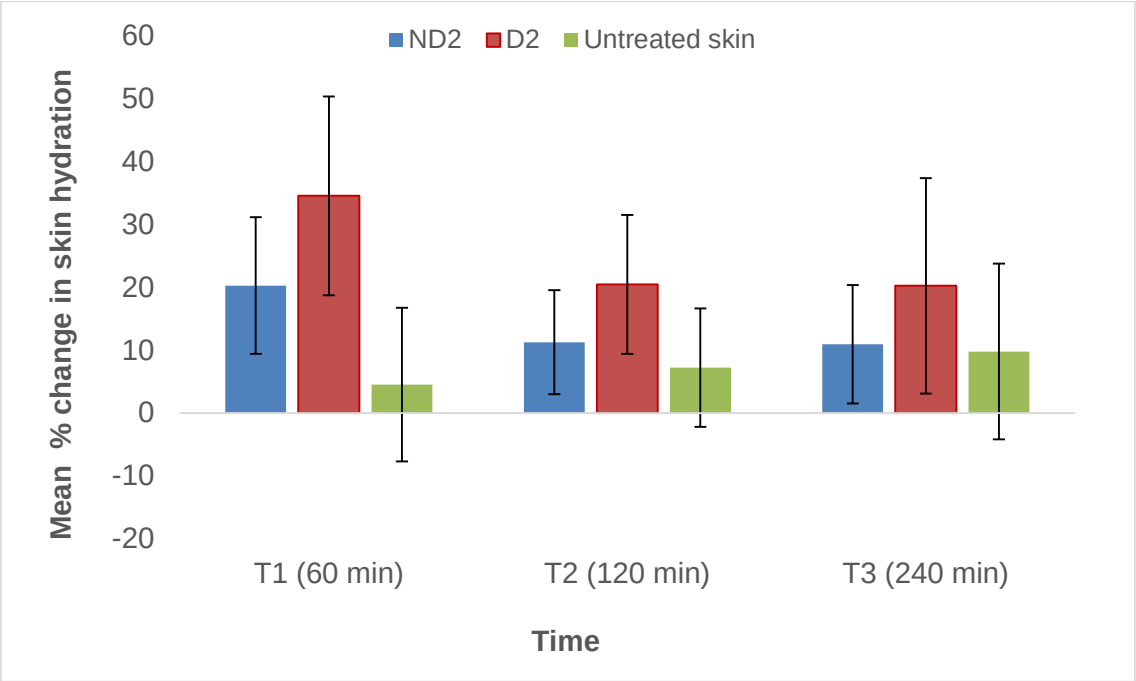

Figure C.8: Mean percentage changes in skin hydration relative to T0 for the short-term study.

Topically applied products increase skin hydration levels, either by supplying water directly to the skin surface, or indirectly through occlusion. Occlusion prevents TEWL from the stratum corneum into the atmosphere, by trapping water in the stratum corneum to subsequently increase skin hydration levels. It is therefore possible that the higher short-term skin hydration increase with regards to D2 could have been attributed to the occlusion properties of rosehip oil (Evangelista *et al*., 2014:101).

The initial skin hydration improvement that had been attained with ND2, may have been due to the o/w emulsion water component having been applied directly to the skin surface. The relatively smaller oil component of ND2, compared to D2, may have contributed to the fact that a similar occlusive effect had not been achieved with ND2. Subsequent evaporation of the water being applied to the skin was hence not hindered, due to the insufficient occlusive properties having been revealed by ND2. TEWL was consequently not reduced to a similar extend than by D2 (Riviere, 1993:117,118; WHO, 2006:20; Williams, 2013:687, 694; Zatz, 1993:128).

C.3.2 Long-term (84 days) multiple treatment application studies

Non-invasive bio-engineering methods were applied to measure skin water content levels by utilising a Corneometer®, skin topography assessments were done by evaluating the SELS parameters acquired from Visioscan® measurements, whilst R parameters were employed to assess skin visco-elastic properties with data obtained from the Cutometer® deformation curves. Measurements were performed at T0 (baseline) and again after 28 (T1), 56 (T2) and after 84 days (T3).

C.3.2.1 Skin hydration

Arbitrary Corneometer® values, indicative of skin hydration levels following long-term multiple applications of ND2 and D2, are reported in Table C.5.

Table C.5: Long-term Corneometer® measurements with regards to skin hydration (mean ± SD)

Time	ND2	D2	Untreated skin
T0	24.27 ± 3.56	23.72 ± 4.03	26.25 ± 5.15
T1	28.57 ± 6.63	27.08 ± 4.19	27.98 ± 4.93
T2	27.02 ± 6.07	25.48 ± 6.30	28.16 ± 5.51
T3	26.02 ± 4.10	28.37 ± 3.88	28.57 ± 3.75

ND2: 20% rosehip seed oil o/w emulsion; D2: 100% rosehip seed oil

C.3.2.1.1 Hierarchical linear models to test the effects of treatment, time and treatment-time interactions

Long-term Corneometer® analyses, applying REML data analysis (Type III test of fixed effects) at a 95% confidence interval, revealed statistical significant differences for interactions with regards to time and treatment ($p = 0.026$), treatment ($p \leq 0.0001$) and time ($p = 0.032$).

C.3.2.1.2 Pairwise treatment comparisons

Pairwise comparisons, based upon estimated marginal means, using Sidak adjustments for multiple comparisons, were performed and statistical significant differences ($p < 0.05$) were revealed for interactions between ND2 and untreated skin ($p = 0.001$), between D2 and untreated skin ($p = 0.012$), but not between ND2 and D2 ($p = 0.902$). Interactions between ND2 and D2 hence revealed similar effects with regards to interactions over time, which significantly differed from the untreated skin area.

C.3.2.1.3 Effect sizes: treatments over time

Univariate F-tests, based upon linearly independent, pairwise comparisons of the estimated means of the effect of each treatment over time were performed and the magnitude of the effect (Cohen's d-value) determined. Practical significant differences, indicative of hydration changes from T0 to T1 were revealed for both D2 and ND2 (Table C.6). The largest hydration effect (increase), relative to the baseline value for ND2 was obtained after 28 days, whereas the largest hydration increase for D2 was measured after 84 days.

Table C.6: Effect sizes of each treatment relative to initial skin condition (T0)

Time	Effect size (Cohen's d-value) relative to T0		
	ND2	**D2**	**Untreated skin**
T1	1.10*	0.86*	0.44
T2	0.70*	0.45	0.49
T3	0.41	1.15*	0.56

*d ≈ 0.8 signifies large effect, or practical significant difference

C.3.2.1.4 Treatment effects: mean percentage changes in skin hydration relative to baseline measurements

Practical significant differences were revealed for D2 with regards to improvement of skin hydration between T0 to T1 and T0 to T3 (Table C.6). Skin hydration levels (mean ± SD) relative to T0 were increased by 15.78 ± 16.90% after 28 days and 21.36 ± 26.33% after 84 days (determined according to Equation C.1).

ND2 achieved its maximum hydration effect after 28 days of twice daily applications and was baseline skin hydration increased by 18.52 ± 24.78%. After 56 days, analysis of ND2 data revealed a 12.31 ± 25.48% improvement of skin hydration, relative to T0. This hydration effect seemed to have decreased over time with no practical significant differences in skin hydration changes observed from baseline up to day 84 (Figure C.9 and Table C.6).

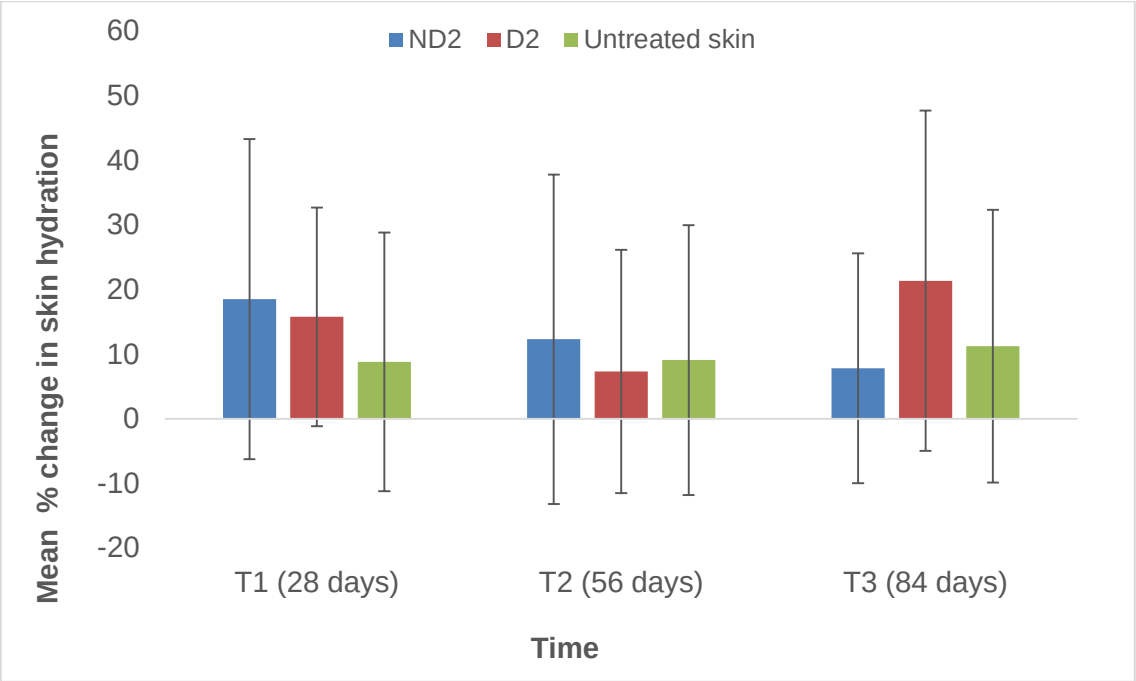

Figure C.9: Mean percentage changes in skin hydration relative to T0 for the long-term study.

C.3.2.2 Skin topography analyses

Visioscan® measurements were performed to investigate any possible skin topography changes with the application of the two formulations. The following SELS parameters were investigated: scaliness (SEsc), smoothness (SEsm), roughness (SEr) and wrinkles (SEw).

Table C.7: Long-term Visioscan® measurements of SELS parameters (mean ± SD)

Treatment	Time	SEsc	SEsm	SEw	SEr
ND2	T0	1.05 ± 0.41	63.46 ± 4.87	40.43 ± 5.08	2.61 ± 0.584
	T1	0.72 ± 0.23	57.73 ± 6.17	40.59 ± 5.21	2.99 ± 0.612
	T2	0.86 ± 0.41	52.31 ± 5.55	36.66 ± 4.09	2.70 ± 0.662
	T3	0.75 ± 0.18	54.28 ± 5.10	40.07 ± 3.92	2.93 ± 0.584
D2	T0	1.04 ± 0.46	67.92 ± 7.94	39.84 ± 4.00	2.98 ± 0.743
	T1	0.76 ± 0.19	63.04 ± 6.51	40.03 ± 4.09	3.01 ± 0.756
	T2	0.90 ± 0.24	60.16 ± 6.08	37.12 ± 4.38	2.60 ± 0.476
	T3	0.83 ± 0.22	63.22 ± 6.33	38.93 ± 6.06	2.98 ± 0.596
Untreated skin	T0	1.13 ± 0.51	53.44 ± 7.17	37.33 ± 3.95	3.16 ± 1.011
	T1	0.80 ± 0.38	51.24 ± 5.77	37.93 ± 4.37	2.73 ± 0.702
	T2	0.79 ± 0.36	49.88 ± 7.91	35.67 ± 3.29	2.58 ± 0.692
	T3	0.84 ± 0.37	51.13 ± 7.67	37.42 ± 3.96	2.98 ± 0.778

ND2: 20% rosehip seed oil o/w emulsion; D2: 100% rosehip seed oil

C.3.2.2.1 Hierarchical linear models to test the effects of treatment, time and treatment-time interactions

REML data analysis (Type III test of fixed effects) at a 95% confidence interval was performed and statistical significant differences established among treatments with regards to SEsm ($p < 0.0001$), SEw ($p < 0.0001$), time SEsm ($p < 0.0001$) and SEw ($p = 0.019$). No statistical significant differences were determined among SEsc and SEr treatments and therefore, together with the statistical significant differences ($p < 0.05$) being revealed over time, a possible time dependent effect for both SEsc and SEr (Table C.8) was suggested.

Table C.8: Type III Test of fixed effects for long-term measurements, evaluating SELS parameters

	Statistical significant difference (p-value)			
	SEsm	**SEsc**	**SEw**	**SEr**
Treatment	< 0.0001*	0.612	< 0.0001*	0.666
Time	< 0.0001*	0.001*	0.0190*	0.015*
Time-treatment interaction	0.0830	0.714	0.7950	0.027*

* Statistical significant difference $p < 0.05$

C.3.2.2.2 Pairwise treatment comparisons

SEsm: Pairwise comparisons, based upon estimated marginal means over time, using Sidak adjustments for multiple comparisons, revealed statistical significant differences ($p < 0.05$) between ND2 and D2 ($p < 0.0001$), as well as between each ND2 and D2 ($p < 0.0001$), compared to the control areas. This comparison indicated that statistical significant differences existed between the measured clinical effects of ND2 and D2 over time, as well as among the applied treatments, between ND2 and D2, compared to untreated skin respectively.

SEw: Similar statistical analyses being performed with regards SEw revealed statistical significant differences ($p < 0.05$) for each ND2 and D2, when compared to untreated control skin areas, whilst no statistical significant differences existed between ND2 and D2. These results implicated that similar clinical effects were obtained over time by both applied treatments, with statistical significant differences regarding their clinical effects, compared to those of untreated skin areas (Table C.9).

SEsc and SEr: Results obtained after pairwise comparisons (Table C.9) between treatments with SEsc and SEr concurred with the results obtained in Table C.8. No statistical significant differences were established between untreated skin and each ND2 or D2, nor between ND2 and D2 with regards to SEsc and SEr. Indicating that SEsc and SEr results, may have been indicative of a time dependent effect.

Table C.9: Pairwise comparisons between treatments and between each treatment and the control

Pairwise comparisons between treatments and the control		Statistical significant difference (p-value)			
		SEsm	SEsc	SEw	SEr
ND2	D2	< 0.0001*	0.877	0.813	0.769
Untreated skin	ND2	< 0.0001*	0.695	< 0.0001*	0.861
	D2	< 0.0001*	0.996	0.001*	0.987

* Statistical significant difference $p < 0.05$

C.3.2.2.3 Effect sizes: treatments over time

Table C.10: Effect sizes of treatments over time relative to initial skin condition (T0)

Treatment	Time	Effect size (Cohen's d-value) relative to T0		
		ND2	D2	Untreated skin
SEsm	T1	0.92*	0.79*	0.35
	T2	1.80*	1.25*	0.57
	T3	1.45*	0.73*	0.35
SEsc	T1	0.54	0.47	0.55
	T2	0.31	0.24	0.56
	T3	0.48	0.33	0.47
SEw	T1	0.04	0.05	0.16
	T2	1.00*	0.72*	0.43
	T3	0.12	0.26	0.00
SEr	T1	0.36	0.02	0.40
	T2	0.09	0.36	0.55
	T3	0.31	0.00	0.17

*d ≈ 0.8 signifies large effect, or practical significant difference

Practical significant differences were revealed, using univariate F-tests, based upon linearly independent, pairwise comparisons among the estimated means of the effects of each treatment over time. Effect sizes (Cohen's d-value) were calculated (Table C.10). The largest effect size (practical significant difference) and maximum clinical effect changes, relevant to baseline values were established with regards to SEsm for ND2 (d-value = 1.80) and D2 (d-value = 1.25), both starting from T0 to T2 (day 56). As per Table C.10, the established d-values for ND2 exceeded those of D2. Practical significant differences for SEw were observed from between T0 to T2 (56 days) for both ND2 and D2. Clinically significant effects with regards to decreasing wrinkle appearances with ND2 and D2 were revealed. For both the SEsm and SEw parameters, the largest effect sizes were achieved for measurements performed between T0 to T2 (56 days).

C.3.2.2.4 Treatment effects: mean percentage changes over time relative to baseline measurements

Table C.11: Percentage Visioscan® SELS parameter changes relative to T0 for measurements performed after multiple product application (mean ± SD)

Treatment	Time	Percentage Visioscan® SELS parameter change [a]			
		SEsc	SEsm	SEw	SEr
ND2	T1	-24.80 ± 24.59	-8.73 ± 10.17	1.72 ± 17.73	17.51 ± 24.36
	T2	-10.92 ± 37.40	-17.31± 9.09	-7.65 ± 17.61	7.93 ± 37.43
	T3	-19.58 ± 26.59	-14.35 ± 7.92	0.09 ± 16.01	17.09 ± 36.29
D2	T1	-15.22 ± 34.83	-6.68 ± 8.64	1.03 ± 11.18	3.43 ± 22.01
	T2	-3.89 ± 32.77	-10.61 ± 11.74	-6.25 ± 12.04	-9.44 ± 20.14
	T3	-5.77 ± 32.59	-4.36 ± 14.86	-2.86 ± 13.49	4.78 ± 27.37
Untreated skin	T1	-20.80 ± 39.08	-3.43 ± 10.05	2.34 ± 13.75	-10.22 ± 23.54
	T2	-17.91 ± 45.89	-6.53 ± 9.71	-3.57 ± 12.32	-14.31 ± 24.41
	T3	-18.22 ± 31.27	-4.08 ± 9.79	0.70 ± 9.29	-0.18 ± 30.07

a using Equation F.1

Visioscan® SEsm parameter values were determined by the average width of the wrinkles, where positive changes and higher SEsm parameter values suggested increased moisturising effects (Courage & Khazaka electronic GmbH, 2015e). Negative changes were determined for SEsm, whereas smoothness decreased with both ND2 and D2. These unexpected results did not mirror the long-term skin hydration/moisturising results, obtained with the Corneometer®.

As seen in Table C.2, negative changes for the Visioscan® SEsc, SEr and SEw parameters were indicative of improvements in skin condition, through a reduction in skin scaliness, roughness and wrinkle appearances. Visioscan® SEw parameter values were determined, following computerised calculation of the average number and average width of the wrinkles, and were the decreasing SEw values indicative of reduced wrinkle appearances over time (Courage & Khazaka electronic GmbH, 2015e). Wrinkle appearances practical significantly decreased following multiple application for both ND2 and D2 until day 56. After day 56, only D2 continued to decrease wrinkle appearances (Table C.11).

C.3.2.3 Visco-elastic properties

Table C.12: Long-term Cutometer® measurements of R parameters (mean ± SD)

Treatment	Time	R0 ± SD	R2 ± SD	R6 ± SD	R7 ± SD	R8 ± SD
ND2	T0	0.263 ± 0.06	0.839 ± 0.07	0.685 ± 0.11	0.458 ± 0.08	0.221 ± 0.05
	T1	0.267 ± 0.06	0.863 ± 0.04	0.631 ± 0.15	0.476 ± 0.07	0.231 ± 0.06
	T2	0.251 ± 0.05	0.878 ± 0.04	0.670 ± 0.12	0.486 ± 0.06	0.220 ± 0.05
	T3	0.292 ± 0.05	0.797 ± 0.03	0.531 ± 0.08	0.412 ± 0.04	0.233 ± 0.04
D2	T0	0.223 ± 0.06	0.818 ± 0.08	0.776 ± 0.15	0.437 ± 0.09	0.182 ± 0.05
	T1	0.242 ± 0.03	0.840 ± 0.05	0.696 ± 0.08	0.451 ± 0.06	0.203 ± 0.03
	T2	0.227 ± 0.05	0.858 ± 0.06	0.760 ± 0.10	0.476 ± 0.08	0.194 ± 0.04
	T3	0.286 ± 0.04	0.781 ± 0.07	0.579 ± 0.08	0.405 ± 0.07	0.224 ± 0.04
Untreated skin	T0	0.245 ± 0.05	0.808 ± 0.08	0.646 ± 0.13	0.408 ± 0.08	0.200 ± 0.05
	T1	0.245 ± 0.05	0.840 ± 0.07	0.694 ± 0.12	0.429 ± 0.06	0.206 ± 0.04
	T2	0.227 ± 0.04	0.830 ± 0.05	0.718 ± 0.11	0.437 ± 0.06	0.188 ± 0.03
	T3	0.274 ± 0.03	0.777 ± 0.06	0.550 ± 0.08	0.389 ± 0.06	0.213 ± 0.03

ND2: 20% rosehip seed oil o/w emulsion; D2: 100% rosehip seed oil

Evaluation of Cutometer® R0 (distensibility), R2 (gross elasticity), R6 (stretch capacity), R7 (elastic recovery) and R8 (total recovery) parameters, as described in Section F.2.2.2, was performed to assess the skin's visco-elastic property changes, following long-term, multiple application treatments, using the ND2 and D2 test products. The Cutometer® results obtained are reported in Table C.12.

C.3.2.3.1 Hierarchical linear models to test the effects of treatment, time and treatment-time interactions

Table C.13: Type III test of fixed effects for long-term measurements evaluating Cutometer® R parameters

	Statistical significant difference (p-value)				
	R0	**R2**	**R6**	**R7**	**R8**
Treatment	< 0.0001*	0.0030*	< 0.0001*	< 0.0001*	< 0.0001*
Time	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	0.0140*
Time-treatment interaction	0.4030	0.9000	0.0380*	0.8730	0.5290

* Statistical significant difference $p < 0.05$

Cutometer® analyses by means of REML data analysis (Type III test of fixed effects) at a 95% confidence interval were performed. All R-parameters being investigated revealed statistical significant differences among treatments, as well as for fixed time intervals, whereas statistical significant differences for time-treatment interactions were only revealed with regards to R6 ($p = 0.038$) (Table C.13).

C.3.2.3.2 Treatment effects: mean percentage changes over time relative to baseline measurements

R0 (distensibility) represents skin firmness and the skin's passive resistance to negative pressure applied. R0 is dependent upon skin thickness, where positive change indicates improvement of skin firming and increased stratum corneum thickness (Courage & Khazaka electronic GmbH, 2015b). During this study, D2 improved skin firmness by 12.12 ± 18.77% (after 28 days), 5.24 ± 21.83% (after 56 days) and 32.81 ± 28.54% (after 84 days), relative to T0. Treatment with ND2 did not improve the skin's condition and clinical effectivity though. As illustrated in Figure C.10, ND2 and the untreated skin areas followed a similar clinical effect trend, which may have been indicative of the possibility that the ND2 application had not influenced skin distensibility.

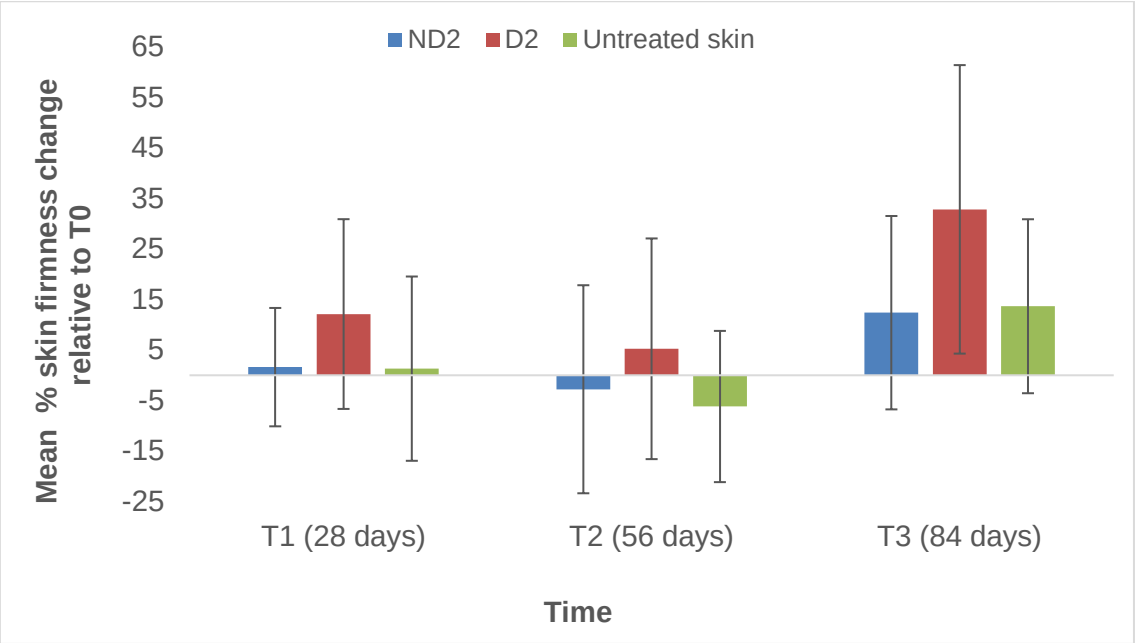

Figure C.10: Percentage skin firmness changes (R0 parameter) relative to T0.

The Cutometer® R2 parameter is a relative elastic parameter (R2 = Ua/Uf), representing gross, or overall elasticity of skin with R2 closer to 1 (100%) reflecting higher skin elasticity properties (Courage & Khazaka electronic GmbH, 2015b). Positive change is indicative of an improvement in skin elasticity. ND2 and D2 exhibited similar clinical effect trends by increasing skin elasticity, according to measurements on days 28 and 56. This trend was also revealed by untreated skin areas, indicating the possibility that the R2 parameter changes were neither due to the application of ND2, nor D2 (Figure C.11).

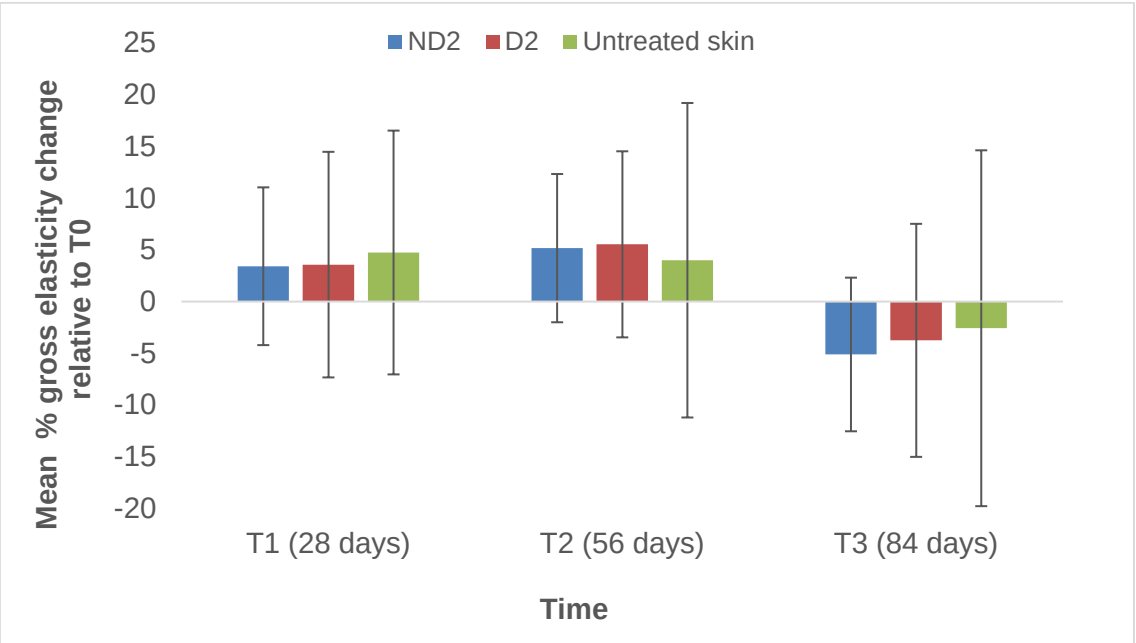

Figure C.11: Percentage gross elasticity changes (R2 parameter) relative to T0.

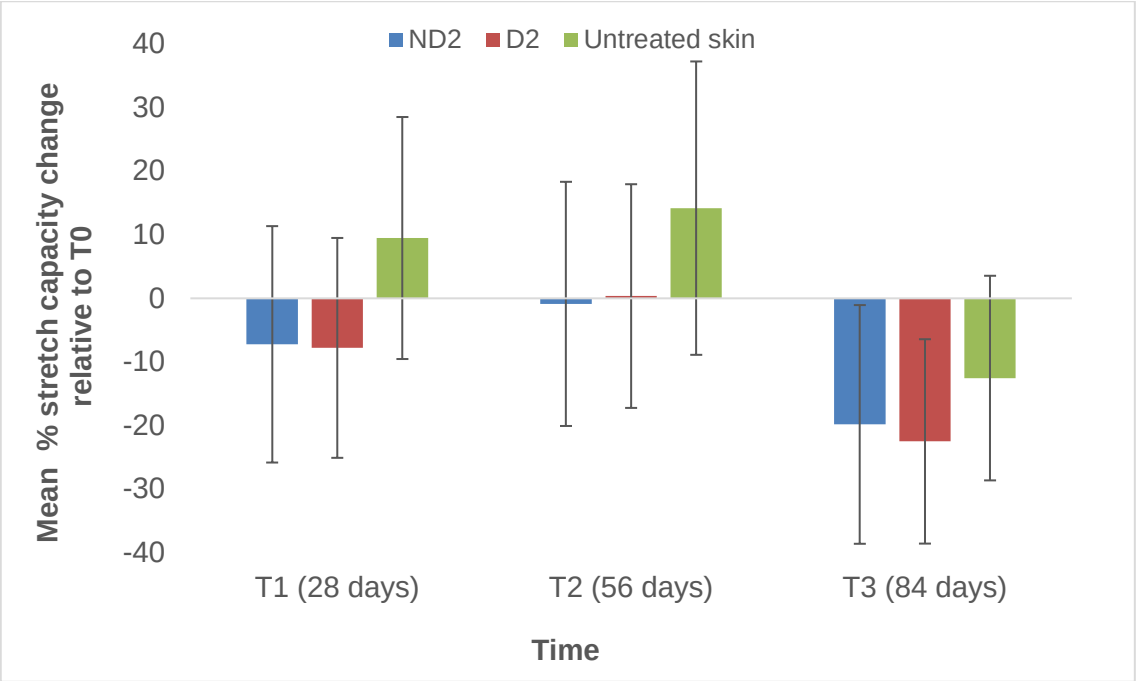

Figure C.12: Percentage stretch capacity changes (R6 parameter) relative to T0.

The Cutometer® R6 parameter reflects the stretch capacity of the skin and a negative change would be indicative of an improvement in skin condition (Courage & Khazaka electronic GmbH, 2015b). ND2 and D2 exhibited a similar trend in improving the skin's stretch capability, with maximum improvement relative to T0 achieved after 84 days (Figure C.12).

The Cutometer® R7 parameter represents the degree of elastic recovery after total deformation, where the "immediate retraction" equals the "final" distension ratio and is a portion of elasticity hence compared to the complete curve (R7 = Ur/Uf). Values closer to 1 (100%) reflect proportionally higher elasticity properties (Courage & Khazaka electronic GmbH, 2015b). Positive change demonstrates improvement in the skin's elasticity properties. Positive changes were revealed for ND2 and D2 measurements, performed relative to T0 after 28 and 56 days, although a similar clinical trend was also measured for untreated skin areas (Figure C.13).

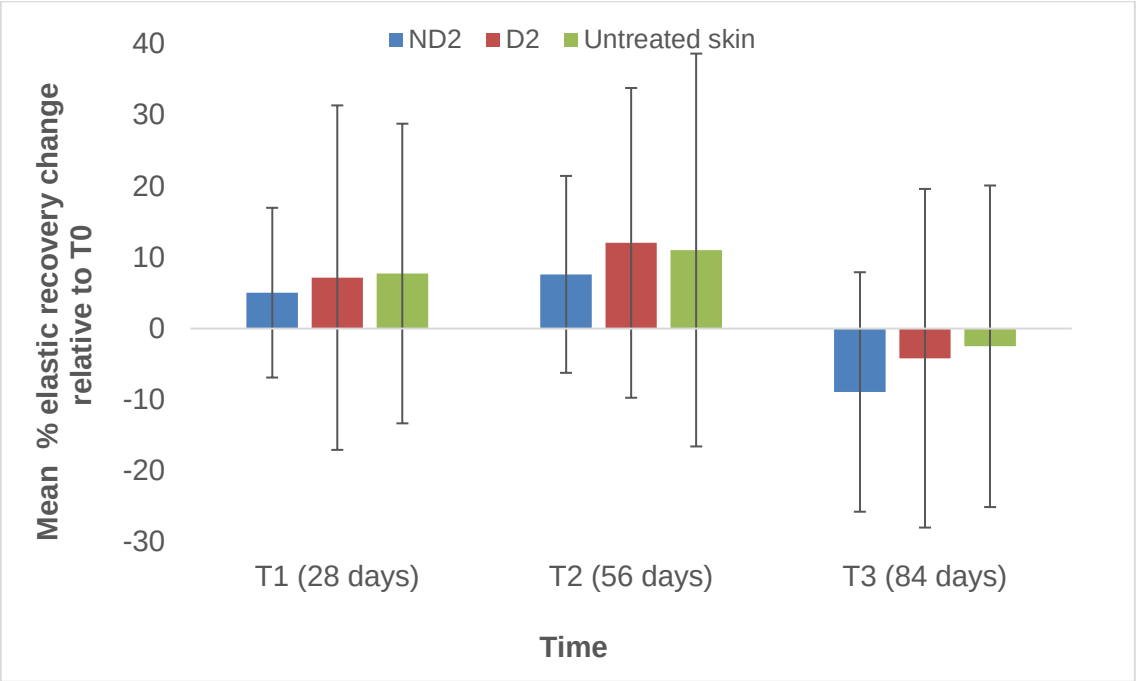

Figure C.13: Percentage elastic recovery changes (R7 parameter) relative to T0.

The Cutometer® R8 parameter represents the skin's total recovery. The closer R8 is to 0, depicted by a negative effect change, the better the skin's ability to return to its original condition (Courage & Khazaka electronic GmbH, 2015b). A similar clinical effect trend was followed by both ND2 and D2, but neither of the applied treatments had demonstrated improvement in skin elasticity (Figure C.14).

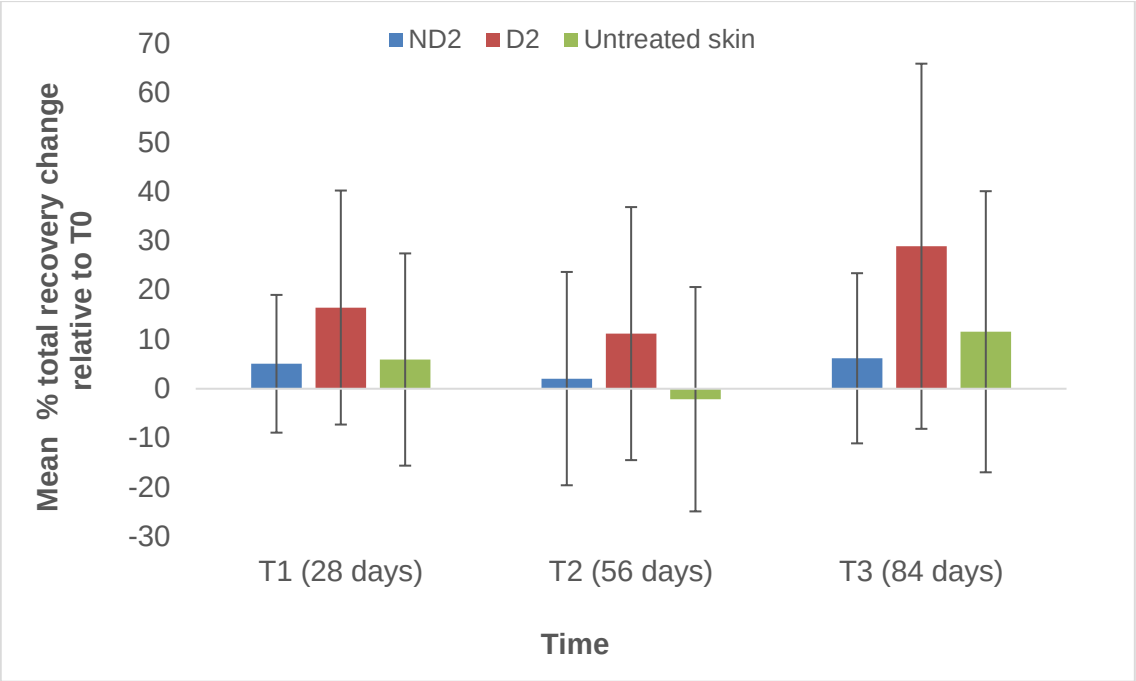

Figure C.14: Percentage total recovery changes (R8 parameter) relative to T0.

C.3.3 Skin irritation patch tests (9 days)

Table C.14: SPA99® and VapoMeter® measurements (mean ± SD)

Treatment	Time	SPA99® (haemoglobin)	VapoMeter® (TEWL)
ND2	T1	41.71 ± 4.55	33.08 ± 8.81
	T2	41.57 ± 5.27	31.46 ± 8.92
	T3	37.79 ± 5.04	25.46 ± 10.86
D2	T1	42.71 ± 4.97	39.09 ± 17.00
	T2	43.21 ± 5.07	35.86 ± 10.50
	T3	40.00 ± 5.97	27.10 ± 11.05
Positive control, 1% hydrocortisone cream (w/w)	T1	46.43 ± 4.96	44.18 ± 14.93
	T2	44.93 ± 6.60	37.86 ± 17.45
	T3	39.64 ± 4.85	25.86 ± 8.93
Negative control	T1	42.29 ± 5.92	38.22 ± 18.42
	T2	41.71 ± 7.35	34.95 ± 15.26
	T3	38.36 ± 5.71	25.99 ± 10.75
Untreated skin	T1	40.93 ± 5.12	22.99 ± 5.58
	T2	40.93 ± 5.78	24.16 ± 5.12
	T3	37.50 ± 4.60	22.56 ± 10.69

ND2: 20% rosehip seed oil o/w emulsion; D2: 100% rosehip seed oil

The skin irritation patch test, as described in Section C.2.3.3, was performed to assess the anti-erythema and skin barrier repair properties of an o/w emulsion, containing 20% (w/w) of rosehip seed oil, 100% rosehip seed oil and 1% hydrocortisone cream (w/w).

C.3.3.1 Hierarchical linear models to test the effects of treatment, time and treatment-time interactions

SPA99® and VapoMeter® analyses, performing REML data analysis (Type III test of fixed effects) at a 95% confidence interval, revealed statistical significant differences. SPA99® haemoglobin measurements revealed statistical significant differences between interactions for both treatment ($p < 0.0001$) and time ($p < 0.0001$), possibly indicative thereof that skin erythema changes may have been time dependent. TEWL data analysis revealed statistical significant differences for interactions over time and treatment ($p = 0.005$), treatment ($p < 0.0001$) and time ($p = 0.006$).

C.3.3.2 Pairwise treatment comparisons

Table C.15: Pairwise comparisons between treatments and between each treatment and the control with regards to measured erythema changes

Treatment / Control		SPA99® (haemoglobin)
ND2	D2	0.0340*
	Positive control	< 0.0001*
	Negative control	0.9970
	Untreated skin	0.9700
D2	ND2	0.0340*
	Positive control	0.0220*
	Negative control	0.2640
	Untreated skin	0.0010*
Positive control, 1% hydrocortisone cream (w/w)	ND2	< 0.0001*
	D2	0.0220*
	Negative control	< 0.0001*
	Untreated skin	< 0.0001*
Negative control	D2	0.2640
	ND2	0.9970
	Positive control	< 0.0001*
	Untreated skin	0.5060
Untreated skin	ND2	0.9700
	D2	0.0010*
	Positive control	< 0.0001*
	Negative control	0.5060

* Statistical significant difference $p < 0.05$; ND2: 20% rosehip seed oil o/w emulsion; D2: 100% rosehip seed oil

C.3.3.2.1 Anti-inflammatory responses

As expected, pairwise comparisons, based upon estimated marginal means, using Sidak adjustments for multiple comparisons, revealed statistical significant differences ($p < 0.05$) among the positive and negative controls' erythema (haemoglobin) values, which demonstrated that the positive control had been effectively reducing erythema, compared to SLS irritated skin's natural anti-inflammatory response.

Unfortunately, statistical significant differences were revealed between the positive control and ND2 ($p < 0.0001$) and again between the positive control and D2 ($p = 0.022$), which were indicative thereof that erythema had not reduced with a similar anti-inflammatory effect, as had been demonstrated by the positive control (Table C.15). It was therefore concluded that neither test products ND2 and D2 had exhibited anti-inflammatory effects compared to the 1% hydrocortisone cream (w/w).

C.3.3.2.2 Skin barrier repair

Table C.16: Pairwise measurement comparisons among TEWL treatments

Treatment		VapoMeter® (TEWL)
ND2	D2	0.1260
	Positive control	0.0030*
	Negative control	0.4540
	Untreated skin	< 0.0001*
D2	ND2	0.1260
	Positive control	0.9220
	Negative control	1.0000
	Untreated skin	< 0.0001*
Positive control, 1% hydrocortisone cream (w/w)	ND2	0.0030*
	D2	0.9220
	Negative control	0.5240
	Untreated skin	< 0.0001*
Negative control	D2	1.0000
	ND2	0.4540
	Positive control	0.5240
	Untreated skin	< 0.0001*
Untreated skin	ND2	< 0.0001*
	D2	< 0.0001*
	Positive control	< 0.0001*
	Negative control	< 0.0001*

* Statistical significant difference $p < 0.05$; ND2: 20% rosehip seed oil o/w emulsion; D2: 100% rosehip seed oil

Pairwise comparisons, based upon estimated marginal means, using Sidak adjustments for multiple comparisons, revealed no statistical significant differences ($p < 0.05$) between the positive and negative controls, showing that the positive control had not been more effective in reducing TEWL, compared to SLS irritated skin's natural skin barrier repair response.

As expected, statistical significant differences ($p < 0.05$) were revealed between untreated skin and all SLS irritated areas, indicating that the SLS induced skin barrier damage and the exhibited changes in TEWL measurements over time, did indeed occur.

No statistical significant differences ($p < 0.05$) were revealed among the negative control and ND2, between the negative control and D2, nor between the negative and positive controls, which may have suggested that the skin barrier repair exhibited over time, had followed a similar effect than that of the natural healing process occurring in skin. No applied test product had therefore altered the TEWL, nor promoted skin barrier repair (Table C.16).

C.3.3.3 Effect sizes: treatments over time

Practical significant differences were revealed using univariate F-tests, based upon linearly independent, pairwise comparisons among the estimated means of the effect of each treatment over time. Effect sizes (Cohen's d-value) were calculated.

C.3.3.3.1 Anti-inflammatory responses

Practical significant differences were revealed for each treatment from between T1 to T3. As per Table C.17, the positive control area depicted the largest effect size. Both ND2 and D2 revealed a smaller effect size, compared to the positive control, therefore no anti-inflammatory effect, larger than the positive control, was revealed for either test product.

C.3.3.3.2 Skin barrier repair

Once again, practical significant differences were revealed and did the positive control exhibit the largest effect size. Both ND2 and D2 revealed smaller effect sizes, compared to the positive control and therefore yet again, no larger clinical effect with regards to reducing TEWL, compared to the positive control, was revealed for either test product.

Since the largest effects, or practical significant differences (d-values) were revealed for the positive control, for both erythema and TEWL measurements from T1 to T3, it was concluded that neither test product had revealed superior clinical effects to the positive control being utilised during this study (Table C.17).

Table C.17: Effect sizes of treatments over time relative to T1

Treatment	Time	Effect size (Cohen's d-value) relative to T1				
		ND2	D2	Positive control	Negative control	Untreated skin
SPA99® (haemoglobin)	T2	0.04	0.13	0.39	0.15	0.00
	T3	1.02*	0.71*	1.77*	1.02*	0.89*
VapoMeter® (TEWL)	T2	0.16	0.31	0.61	0.32	0.11
	T3	0.74*	1.16*	1.77*	1.18*	0.04

***d ≈ 0.8 signifies large effect, or practical significant difference

C.3.3.4 Treatment effects: mean percentage changes over time

Table C.18: Percentage changes relative to T1

Treatment	Time	Percentage change relative to T1				
		ND2	D2	Positive control	Negative control	Untreated skin
SPA99® (haemoglobin)	T2	-0.24	1.46	-3.22	-1.56	0.05
	T3	-9.47	-6.20	-14.30	-9.03	-8.15
VapoMeter® (TEWL)	T2	-1.08	-1.17	- 9.48	-2.29	14.48
	T3	-17.93	-24.57	-35.93	-23.89	9.48

As discussed in the above sections, even though rosehip products are believed to possess anti-inflammatory action, no such effect, superior or equivalent to the positive control, had been revealed during this study (Cohen, 2012:496).

TEWL is not an obvious indicator of skin damage, like the level of skin redness, or if present, distinct skin blistering. TEWL changes are only established once TEWL measurements are performed. TEWL values are highly sensitive and directly influenced by the degree of skin barrier damage. Following SLS irritant application, therefore, markedly higher TEWL values should be measured. For this reason, the degree of TEWL changes is indicative of changes in the skin barrier condition, whether its deterioration, or healing (Evangelista *et al*., 2014:100; Tupker *et al*., 1997:57).

Free fatty acids, such as oleic and linoleic acid that are located in the skin's lipid matrix, are present in rosehip seed oil. The expected clinical benefit and skin barrier repair effect had not been achieved following topical application during the TEWL investigation (Adamczak *et al.,* 2011:57; Barros *et al.,* 2011:2235; Concha *et al.,* 2006:773; Nowak, 2005:232; Menon *et al*., 2012:6).

Various sizes of Finn Chambers® are available to perform skin patch testing. The small Finn Chamber® (diameter 8 mm) was used for the skin irritation patch tests during this study. The small chamber with maximum fill capacity of 20 µl may have contributed to the inconclusive results having been obtained during this study. The irritant skin reaction may have been inadequate, due to the small skin application area and small quantity, or dose of SLS that had been applied to the skin. According to guidelines drafted by Tupker *et al*. (1997:59), larger chamber sizes (diameter 12 or 18 mm) are recommended for performing SLS irritant test models, whilst the use of small chambers should be reserved for allergy testing procedures.

Complete occlusion skin patch systems are well-known and widely used methods for increasing exposure to applied test products to amplify the treatment effect. Complete occlusion had been

attempted when performing the skin patch studies. As stated above, the participants were clearly instructed to act in accordance with the well explained protocol, to prevent contact with moisture, or sweat on the test areas. SLS containing Finn Chambers® were removed by the participants at home, without supervision by the investigator. No inspection was hence performed to confirm whether the Finn Chambers® were fully occluded and held intact for the required 24 h, and may non-adherence have contributed towards the inconclusive results (McNamee *et al*., 2008:27).

The irritation patch test results may therefore be inconsistent with existing literature and results from previous studies, resulting from protocol violation and could a repeat of these tests under better supervised conditions achieve different outcomes.

C.4 Conclusion

The results obtained from the clinical studies demonstrated that both test products, i.e. the 20% rosehip seed oil o/w emulsion (w/w) (rosehip emulsion) and the 100% rosehip seed oil (rosehip oil), practical significantly improved skin moisture levels and furthermore demonstrated beneficial anti-wrinkle and skin visco-elastic properties. Unfortunately, results with regards to an improvement of the skin barrier repair properties and anti-inflammatory effects remained inconclusive.

C.4.1 Skin hydration effects

Long-term multiple application and short-term single application illustrated practical significant improvements in skin hydration levels by both the rosehip emulsion and rosehip oil.

- Results obtained from the short-term study indicated that statistical significant increases in skin hydration had been achieved by both the rosehip emulsion and rosehip oil, after single application, compared to untreated skin areas. Maximum hydration increase effects were achieved by both test products, 60 min after single application. Results further revealed that better hydration had been achieved by rosehip oil at 60, 120 and 240 min after single application, than the rosehip emulsion.

- The long-term study results indicated that although both test products showed beneficial skin hydration effects, the rosehip emulsion and rosehip oil did not follow similar clinical effect trends. The rosehip emulsion had achieved maximum hydration after 28 days, after which all subsequent measurements constantly decreased, whereas the rosehip oil demonstrated an opposite clinical trend, with the increase in skin hydration having reached a maximum after 84 days of treatment. Statistical significant skin hydration

increases were revealed for both the rosehip emulsion and rosehip oil, when compared to the untreated skin areas.

C.4.2 Skin topography changes

Visioscan® clinical SELS parameters were evaluated to assess skin surface changes and the clinical efficacy of the two test products. For an improvement in the skin's condition, negative changes in SEsc, SEr and SEw parameters, as well as a positive change with regards to SEsm should have been achieved.

- Both rosehip oil and rosehip emulsion had decreased skin wrinkle formation and had the skin's condition therefore been improved, following multiple application over long-term use. Maximum wrinkle improvement effects revealed practical significant differences relative to T0 after 56 days (- 6.25%) for rosehip oil and rosehip emulsion (- 7.65%).

- Contrary to the beneficial results obtained regarding skin wrinkle formation and skin hydration, the evaluation of skin smoothness yielded an unfavourable clinical effect. Results showed that both the rosehip emulsion and rosehip oil had decreased skin smoothness at all measurement intervals, relative to T0. The true mechanism of each effect had not been studied prior to performing the experiments and should they be investigated beforehand as part of future studies.

C.4.3 Visco-elastic effects

The assessment of the Cutometer® R0, R2, R6, R7 and R8 parameters suggested potential visco-elastic changes following long-term multiple applications.

- R0 assessment revealed improved skin firming and increased stratum corneum thickness by rosehip oil, but a similar clinical effect had not been achieved by the rosehip emulsion.

- Rosehip oil demonstrated a better improvement in skin stretch capacity (R6) than rosehip emulsion and a maximum clinical improvement effect for rosehip oil had been observed after 84 days.

- Neither rosehip oil, nor rosehip emulsion applications had improved the skin's total elastic recovery, as seen after evaluating the R8 parameter.

- Similar clinical effects were demonstrated by untreated skin areas after evaluating gross elasticity (R2) and elastic recovery (R7), and hence, as supported by the statistical

significant differences having been revealed with regards to the fixed effect of time R2 and R7 ($p < 0.0001$), none of the applied treatments altered R2, nor R7.

C.4.4 Skin barrier repair and anti-inflammatory effects

- Although skin erythema and TEWL values had been reduced by both the rosehip emulsion and rosehip oil, it had not improved the skin's condition significantly better than the negative control. The improvement by the two treatments had therefore not exceeded the clinical effects being exhibited by the skin itself.

- During this study, it was found that neither the rosehip emulsion, nor the rosehip oil had accomplished any beneficial clinical effect regarding reducing erythema or decreasing TEWL, superior to the positive control, i.e. the 1% hydrocortisone (w/w).

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Appendix E

Poster presentation

The following Appendix is a copy of the poster accepted and presented at the 2015 *Society of Cosmetic Chemists South Africa* (Coschem) annual scientific conference.

The Coschem conference took place from 2-3 September 2015, at the Bytes Conference Centre Bytes Business Park, Block C, Third Rd, Midrand, South Africa.

The formatting hence differs from the technical structure which was followed throughout the dissertation.

CONCENTRATION ASSAY OF ALL-*TRANS*-RETINOIC ACID IN COMMERCIALLY ACQUIRED *ROSA RUBIGINOSA* ROSEHIP SEED OIL

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PURPOSE

To investigate the all-*trans*-retinoic acid concentration present in commercially available *Rosa rubiginosa* rosehip seed oil. This study was conducted to extend cosmeceutical natural raw materials monographs.

BACKGROUND

Rosa rubiginosa is the only rose species documented to contain the vitamin A derivative all-*trans*-retinoic acid. Cosmeceuticals concentrating on the anti-ageing segment are presuming that *Rosa rubiginosa* rosehip seed oil contains the biologically-active all-*trans*-retinoic acid (or tretinoin) (Concha *et al*., 2006:771; Ioele *et al*., 2005:251). Analysis of seed oils extracted through different cold press methods revealed that different extraction methods consequently influence the concentration of the all-*trans*-retinoic acid present in the oil and range between 0.051 and 0.324 mg/L (Concha *et al*., 2006:772). Natural ingredients are not standardised, presenting difficulty when formulating clinically effective cosmeceuticals. Although widely used, only a small percentage of herbal or natural cosmetic raw materials are well documented (Kumar, 2005:1270; Nohynek *et al*., 2010:243).

METHODS

Three different batches of rosehip seed oil from the same rose species *Rosa rubiginosa* was commercially obtained from three different eco-regions namely Lesotho, Spain and Chile. High performance liquid chromatography (HPLC) methods were developed and validated to conduct the all-*trans*-retinoic acid assay. The instrument used was an Agilent 1200 series HPLC consisting of a quaternary gradient pump, autosampler and variable wavelength UV detector and Chemstation v10.02 control software (Agilent, Palo Alto, CA). HPLC wavelength was set at 349 nm; the mobile phase comprised of 100.0% HPLC grade acetonitrile with 0.5% acetic acid. The mobile phase was degassed before set up on the HPLC with the flow rate set at 1 ml/min and an injection volume of 10 µl. A Venusil XBP C18, 150 x 4.6 mm column was used (Agela Technologies, Newark, DE). Three samples of each oil batch and a standard solution were prepared according to current GLP (good laboratory practice) methods before the assays were performed. Sample solution (10 µl) was injected twice. The method was linear over the range of 0.04 – 97.70 mg/L with recovery of 98.94% and inter-day precision of 2.13%.

Tretinoin assay studies were performed on *Rosa rubiginosa* rosehip seed oil spiked with tretinoin. Tretinoin assays were conducted at T0 (day 1). Tretinoin / *Rosa rubiginosa* seed oil was stored and a tretinoin assay was conducted again at T1 (after 10 days).

RESULTS AND DISCUSSION

No tretinoin was found in any of the commercially available oils. The T1 tretinoin assay performed in the tretinoin / *Rosa rubiginosa* solution indicated a 45% tretinoin concentration decrease within 10 days after solution construction (T0).

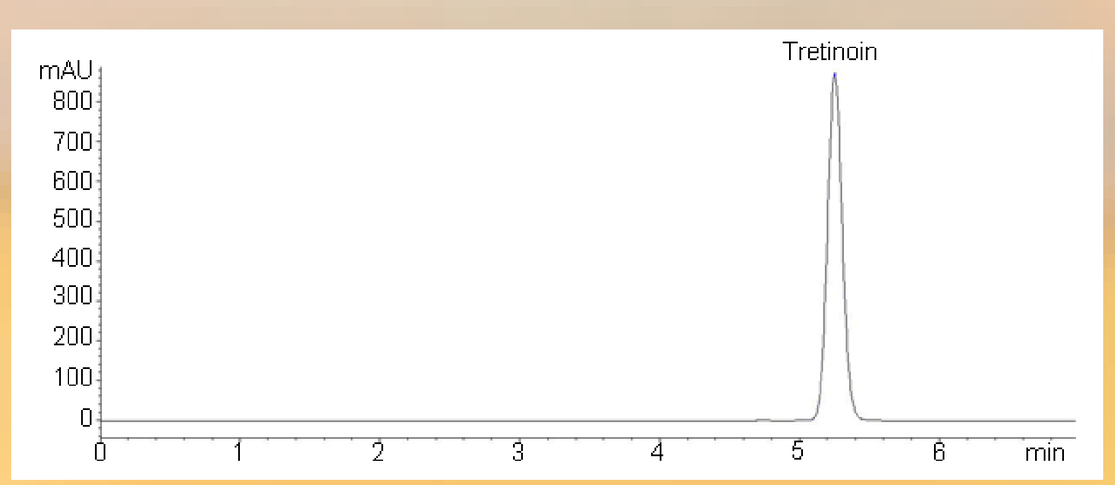

Fig 1: HPLC Chromatogram (349 nm) tretinoin standard solution (1:10) acetonitrile

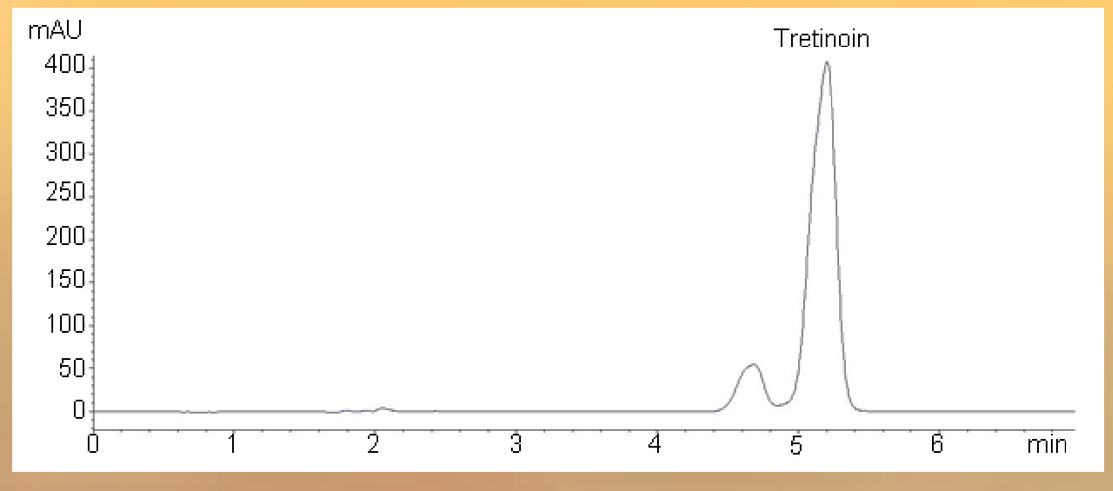

Fig 2: HPLC Chromatogram (349 nm) T1 *Rosa rubiginosa* rosehip seed oil spiked with tretinoin (1:25) tetrahydrofuran solution

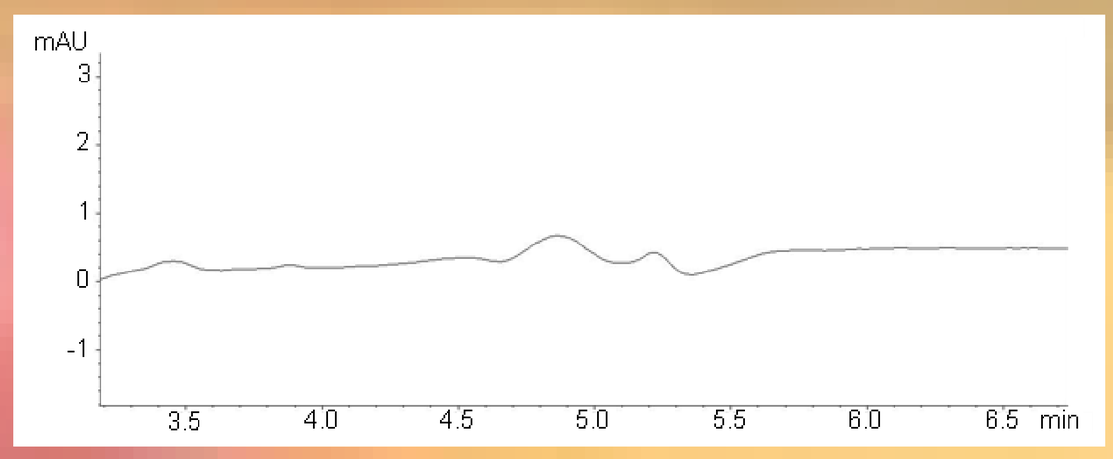

Fig 3: HPLC Chromatogram (349 nm) *Rosa rubiginosa* (1:25) tetrahydrofuran solution

The hypothesis is that commercially available *Rosa rubiginosa* rosehip seed oil may contain, if extracted according to optimised cold press conditions, all-*trans*-retinoic acid directly after extraction of the oil. Following extraction, rosehip oil is exposed to a chain of diverse handling and storage conditions. These conditions may vary in temperature and different light environments. Since all-*trans*-retinoic acid is heat labile and light labile, even if it was present directly after extraction, it is not present when reaching the manufacturer or with even a smaller probability, the consumer.

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