In vitro release and stability of rooibos tea extract in topical formulations

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"And whatever you do, whether in word or deed, do it all in the name of the Lord Jesus, giving thanks to God the Father through him."

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ABSTRACT

Aspalathus linearis (rooibos tea) is an indigenous plant that is commonly used to prepare a tea-based health beverage and aspalathin is the major flavonoid uniquely found in rooibos extract (RE). The use of RE in aqueous solutions is challenging, since aspalathin is chemically unstable at increasing temperatures and pH. There is limited research data available regarding the chemical stability improvement of aspalathin in RE topical formulations. Furthermore, the release of aspalathin from topical formulations has not yet been investigated. Aspalathin has previously been shown to exhibit poor absorption across biological membranes.

The purpose of this study was to develop different topical gel formulations containing aspalathin-enriched RE with the addition of selected anti-oxidants in order to improve the chemical stability of aspalathin over time. Eight experimental gel formulations containing aspalathin-enriched RE were manufactured, each containing selected anti-oxidants and a control gel without anti-oxidants. Stability studies were performed where gel formulations were subjected to long-term and accelerated storage conditions for three months. Chemical and physical properties were regularly evaluated and included aspalathin content assays, pH, viscosity (rheology) and visual inspection. Furthermore, *in vitro* diffusion studies were performed where the rate and extent of aspalathin release from all RE gel formulations were evaluated using synthetic polyvinylidene fluoride (PVDF) membranes mounted in a Sweetana-Grass diffusion chamber apparatus.

The stability studies demonstrated chemical degradation of aspalathin in all RE gel formulations at the specific storage conditions. Since no aspalathin could be quantified in the control gel formulation (containing no anti-oxidants) at the end of stability testing, while aspalathin was present in the experimental gel formulations, it was evident that the addition of anti-oxidants have improved the chemical stability of aspalathin in RE gel formulations. The gel formulation, which contained all three anti-oxidants (ascorbic acid, citric acid and sodium metabisulfite) exhibited the best chemical stability of aspalathin. With regards to physical stability evaluation, the pH remained stable within the pre-determined pH range (4.7 ± 0.3) in all gel formulations over time and was considered safe for topical application. No pronounced changes were observed in the viscosity of most of the gel formulations, except for the appearance of syneresis in gels, which contained ascorbic acid. The rheograms showed a pseudoplastic flow behaviour in all gel formulations that remained unchanged throughout the study. Pronounced colour changes were observed in most gel formulations at the end of stability studies. Gel formulations containing sodium metabisulfite exhibited the least colour change and it was suggested that the darker colour that occurred in the control and other gel formulations, could be attributed to flavonoid oxidation in the RE. *In vitro* studies revealed that aspalathin release could be

achieved in all gel formulations with a maximum cumulative amount of aspalathin release of 11.4%. The results showed that the extent of aspalathin release was inversely related to the viscosity of the formulations.

Different aspalathin-enriched RE topical gel formulations were prepared and evaluated with regards to chemical and physical stability, and *in vitro* release. Based on the literature review it is likely that these experimental results are the first to be reported regarding the chemical stability enhancement of aspalathin in RE topical gel formulations. Information regarding *in vitro* release studies of aspalathin from RE topical formulations also provides novel research reporting that can contribute to RE topical product development.

Key words: Rooibos tea extract, *Aspalathus linearis*, aspalathin, topical gel formulation, chemical stability, *in vitro* release, PVDF synthetic membrane

UITTREKSEL

Aspalathus linearis (rooibostee) is 'n inheemse plant wat algemeen gebruik word vir die bereiding van gesonde tee-gebaseerde drankies en aspalatien is die belangrikste flavonoïed wat uniek voorkom in rooibos-ekstrak (RE). Die gebruik van RE in waterige oplossings is uitdagend, aangesien aspalatien chemiese onstabieliteit toon met ’n toename in temperatuur en pH. Daar is beperkte navorsingsdata beskikbaar aangaande die bevordering van chemiese stabiliteit van aspalatien in RE topikale formulerings. Verder is die vrystelling van aspalatien vanuit topikale formulerings nog nie ondersoek nie. Daar is egter wel voorheen bewys dat aspalatien swak geabsorbeer word deur biologiese membrane.

Die doel van hierdie studie was om verskillende topikale jelformulerings te ontwikkel wat aspalatienverrykte RE bevat tesame met geselekteerde anti-oksidante om die chemiese stabiliteit van aspalatien te verbeter. Agt eksperimentele jelformules wat aspalatienverrykte RE bevat, is vervaardig, waarvan elk geselekteerde anti-oksidante bevat het en 'n kontrolejel, sonder anti-oksidante. Stabiliteitstudies is uitgevoer waar jelformulerings vir drie maande lank aan langtermyn- en versnelde bergingstoestande onderwerp was. Die chemiese en fisiese eienskappe was op ‘n gereelde basis geëvalueer tesame met aspalatieninhoudstoetsing, pH, viskositeit (reologie) en visuele inspeksie. Verder is *in vitro* diffusiestudies uitgevoer waar die tempo en omvang van die vrystelling van aspalatien vanuit al die RE jelformulerings ook geëvalueer was met behulp van sintetiese polivinilideenfluoried (PVDF) membrane wat gemonteer was in 'n Sweetana-Grass-diffusiekamerapparaat.

Die stabiliteitstudies het chemiese afbraak van aspalatien in al die RE jelformulerings getoon by die spesifieke stabiliteitsbergingstoestande. Aangesien geen aspalatien in die kontrolejel (wat geen anti-oksidante bevat het nie) aan die einde van die stabiliteitstoetse gekwantifiseer kon word nie, terwyl aspalatien wel teenwoordig was in die eksperimentele jelformulerings, was dit duidelik dat die toevoeging van anti-oksidante wel ‘n verbetering in die chemiese stabiliteit van aspalatien in RE-jelle verseker het. Die jelformule wat al die anti-oksidante (askorbiensuur, sitroensuur en natriummetabisulfiet) in kombinasie bevat het, het die hoogste chemiese stabiliteit van aspalatien getoon. Wat die fisiese stabiliteitsevaluering betref, het die pH stabiel gebly binne die voorafbepaalde pH-grense (4,7 ± 0,3) in al die jelformulerings met verloop van tyd en is dit veilig geag vir topikale toediening. Geen duidelike veranderinge was waargeneem in die viskositeit van die meeste jelformulerings nie, behalwe in jelle wat askorbiensuur bevat het waar sinerese plaasgevind het. Die reogramme het pseudoplastiese vloeigedrag getoon in al die jelformulerings wat gedurende die studie dieselfde gebly het. Duidelike kleurveranderinge was aan die einde van die stabiliteitstudies in meeste van die jelformulerings waargeneem. Jelformulerings wat natriummetabisulfiet bevat, het die minste kleurverandering getoon en

waarskynlik kon die donkerder kleur wat in die kontrole en ander jelformulerings voorgekom het, toegeskryf word aan die oksidasie van flavonoïde in die RE. *In vitro* vrystellingsstudies het bewys dat die vrystelling van aspalatien behaal was vanuit al die jelformules met 'n maksimum kumulatiewe hoeveelheid aspalatienvrystelling van 11.4%. Die omvang van die aspaltienvrystelling was omgekeerd verwant aan die viskositeit van die formulerings.

Verskillende soorte aspalatienverrykte RE topikale jelformulerings was suksesvol voorberei en jelformulerings was geëvalueer met betrekking tot chemiese en fisiese stabiliteit en *in vitro* vrystellingstoetsing. Op grond van die literatuuroorsig is dit waarskynlik dat hierdie eksperimentele resultate die eerste is wat gerapporteer word rakende die bewese verbetering van chemiese stabiliteit van aspalatien in RE topikale jelformulerings. Resultate rakende die *in vitro* vrystellingsstudies van aspalatien vanuit RE-bevattende topikale formulerings bied nuwe inligting wat van waarde kan wees vir die ontwikkeling van RE topikale produkte.

Sleutelwoorde: Rooibostee ekstrak, *Aspalathus linearis*, aspalatien, topikale jelformulering, chemiese stabiliteit, *in vitro* vrystelling, PVDF sintetiese membraan

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LIST OF ABBREVIATIONS

A	Long-term
ACE	Associated Chemical Enterprises
ANOVA	An analysis of variance
ANVISA	The National Health Surveillance Agency
ARC	Agricultural Research Council
Avg	Average
B	Intermediate
BP	British Pharmacopoeia
C	Accelerated
CO_2	Carbon dioxide
Da	Dalton
e.g.	*Exempli gratia* (for example)
G	Gel
g	Gram
g/mole	Molar mass (gram per mole)
GI	Geographical indication
GRE	Green rooibos extract
h	Hours
HMF	Hydroxymethylfurfural
HPLC	High performance liquid chromatography
HSD	Honest Significant Difference
i.e.	*Id est* (in other words)

ICH	International Conference on Harmonisation
LOD	Limit of detection
Log D	Partition coefficient
Log P	Lipid/water partition coefficient
LOQ	Limit of quantification
MCC	Medicine Control Council of South Africa
mg	Milligram
min	Minutes
ml	Millilitre
mm	Millimetre
MSDS	Material Safety Data Sheet
N/A	Not applicable
O_2	Oxygen
OECD	Organisation for Economic Co-operation and Development
OH	Hydroxide
p	Probability value
Pa	Pascal
Pa.s	Pascal seconds
P_{app}	Apparent permeability coefficient
pH	Potential hydrogen
pKa	Negative log of the acid dissociation constant
PVDF	Polyvinylidene fluoride
q.s.	*Quantum satis* (as much as needed)

R^2	Coefficient of determination
RE	Rooibos extract
RH	Relative humidity
ROS	Reactive oxygen species
rpm	Revolutions per minute
RSD	Relative standard deviation
s^{-1}	Shear rate (1/second)
SARC	South African Rooibos Council
SB	Solvent based
SC	Stratum corenum
SCCS	Scientific Committee on Consumer Safety
SD	Standard deviation
SOP	Standard operating procedure
T0	Initial value immediately after preparation
T1	After 1 month
T2	After 2 months
T3	After 3 months
T4	After 4 months
T5	After 5 months
T6	After 6 months/final times
TEWL	Transepidermal water loss
Tx	Specific time interval
USA	United States of America

UV	Ultraviolet
v/v	Volume per volume (ml/100ml)
Visc.	Viscosities
w/v	Weight per volume (g/100ml)
w/w	Weight per weight (g/100g)
WHO	World Health Organization
^{o}C	Degree Celsius
$[H^{+}]$	Hydrogen ion
cm^{2}	Square centimetre
m^{2}	Square metre
µg	Microgram
µm	Micrometre
µl	Microlitre
%	Percentage
% Asp	Percentage aspalathin

CHAPTER 1: INTRODUCTION

1.1 Background and justification

1.1.1 Topical formulations

The skin is the largest organ in the human body with an average total surface area of approximately 1.8 to 2.0 m^2 in adults. The major function of the skin is to protect the body and underlying tissues from the surrounding external environment (Godin & Touitou, 2007; World Health Organization (WHO), 2006). The skin is not uniform tissue, but it is composed of several different main layers of cellular strata, namely the epidermis, dermis and subcutaneous tissue (Gerber, 2012). The stratum corneum (SC) is the outside epidermis layer forming part of the skin barrier that is primarily responsible for protection against harmful exogenous substances (Roberts *et al.*, 2017). Most cosmetic skin formulations come into contact with the SC first before reaching the deeper skin layers. There are different pathways and mechanisms that facilitate the transport of active ingredients through the upper layers of the skin such as intercellular, transcellular and shunt routes (Williams, 2018).

A wide variety of formulation options are available for topical delivery applications such as simple solutions, lotions, ointments, common creams, patches and gels. Semisolid formulations consist of single and multiphase systems. Ointments and gels form part of single-phase semisolids, whereas emulsions (creams) are two-phase systems. A cream is an emulsion consisting of two immiscible phases dispersed together to produce a semisolid product. Ointments are oily preparations usually used on dry lesions, whether for treatment or as an emollient due to its occlusive properties (Williams, 2018). Creams contain more water than ointments and are thus cosmetically better tolerated. Lotions are watery suspensions with a drying, cooling effect and are usually composed as a water-in-oil emulsion. Gels are usually suspensions of soluble or insoluble active ingredients in water thickened with a gelling agent (Costa & Santos, 2017; Li & Chowdhury, 2017).

Gels are transparent semisolid preparations and are relatively easy to prepare by thickening a liquid phase (continuous phase) with other components that form a dispersed phase (Chang *et al*., 2013; Williams, 2018). Carbomers and xanthan gum are examples of thickening agents used to provide stiffness to gel preparations. The gelling agent is dispersed in a hydro-alcoholic medium or purified water together with the phase containing active ingredients and preservatives until a uniform dispersion is formed that is suitable for external application (Chang *et al.*, 2013; Mayba & Gooderham, 2017). Advantages of gels as dosage forms for application to patients are as follow: they are cosmetically elegant and non-greasy, are quick to dry when applied and easy to wash off (Mayba & Gooderham, 2017).

1.1.2 *Aspalathus linearis* (Rooibos) herbal tea

Aspalathus linearis (Rooibos) is a well-known herbal plant that is indigenous to South Africa that has a very successful history of commercialisation as a beverage, but also as an active compound in other products. According to the South African Rooibos Council (SARC), the annual global consumption of rooibos plant products is about 15 000 tons (SARC, 2017). The use of rooibos tea as beverage originated over 300 years ago in the Southern African Cederberg region (Beelders *et al.*, 2012; Huang *et al.*, 2008). Rooibos is claimed to exhibit profound health benefits that stimulated research and development of rooibos as a phytochemical. The use of rooibos has numerous advantages as it is considered to exhibit cardioprotective and hepatoprotective effects, immune system stimulation, as well as anti-inflammatory and anti-oxidant activities (Dludla *et al.*, 2014; Joubert *et al.*, 2008; Ku *et al.*, 2015).

Rooibos extract (RE) is incorporated into topical formulations to improve the skin, typically for its anti-oxidant properties and to reduce skin ageing (Huang *et al*., 2008). There are many cosmetic products containing RE claiming to alleviate skin eczema, have an anti-wrinkling effect and even inhibit skin tumour growth (Chuarienthong *et al.*, 2010; Joubert *et al.*, 2008; Marnewick *et al.*, 2005). Cosmetic industries use RE in their products for skin application, but scientific studies dealing with different aspects of RE in topical products such as content assay, stability, bioactivity and the release of phytochemicals are limited (Joubert & De Beer, 2011). The stability of certain components of RE in topical formulations raised concerns due to the inherent chemical instability of phytochemicals that are easily oxidised (De Beer *et al.*, 2012).

1.1.3 Product formulation and stability considerations

A topical product should be formulated in such a way that optimal stability and release of the active ingredient from the formulation is ensured. For most natural substances, there is a lack of information regarding the quality, standardisation and stability of the active phytoconstituents in topical formulations. Stability is important for cosmetic or pharmaceutical products, and stability testing has to be done to determine the influence of environmental factors (temperature and humidity) on the chemical stability of an active ingredient over time (ICH, 2003). In order to ensure chemical stability of the phytochemicals of RE in a topical formulation, various formulation additives may be considered to be included in the formulation.

In cosmetic products, anti-oxidants are mainly included to prevent oxidative deterioration of the active ingredients during storage (Eccleston, 2013). Vitamin E is an anti-oxidant, which is commonly used in topical formulations and also known to occur naturally as a lipophilic anti-oxidant in the dermis, epidermis and SC of the skin (Weber *et al*., 2009). Vitamin C (ascorbic

acid) is also a very important anti-oxidant of natural origin (Weber *et al*., 2009). Ascorbic acid acts as a reducing agent by reacting with free radicals (Loden, 2003).

This study is aimed at exploring the effects of the addition of selected anti-oxidants on the chemical and physical stability of topical gel formulations containing RE. There are several factors that can affect the stability of a topical gel formulation, including the ingredients used and the processing steps during formulation (Piriyaprasarth & Sriamornsak, 2011). Furthermore, the release of rooibos phytochemicals from topical formulations to become available for interaction with the skin, has not yet been investigated. A topical product must be formulated in such a way as to ensure release of active ingredients (e.g. aspalathin) to interact with the skin.

1.2 Research problem

The trend to include herbal extracts such as *Aspalathus linearis* (rooibos) extract in cosmetic products is increasing. Many cosmetic product owners and manufacturers claim that their products contain certain concentrations of RE with characteristic phytochemicals (e.g. aspalathin), without necessarily having the scientific data to proof the validity thereof. There are many publications describing the formulation of topical dosage forms, but little is known about the stability of RE, and more specifically, the aspalathin content of these formulations as a function of time (Joubert & De Beer, 2011).

There is also insufficient evidence regarding methods to improve the release of RE phytochemicals from the delivery vehicle to the skin during topical application. There is a general lack of information about the effects that excipients, such as gelling agents, may have on the release of aspalathin from topical gel formulations. Suitable ingredients that are compatible with RE (specifically the phytochemical aspalathin) that can be used to produce stable topical gel formulations need to be identified.

The research problem to be addressed in this study was therefore the formulation of topical gel formulations containing RE with improved stability and acceptable *in vitro* release of selected phytochemicals.

1.3 Aim and objectives

1.3.1 General aim

The main aim of this study was to develop different topical gel formulations containing aspalathin-enriched RE as active ingredient in order to improve the chemical stability of the

active ingredient over time (as measured by aspalathin concentration) and to ensure release of aspalathin from the topical RE formulations.

1.3.2 Objectives

The specific objectives of the study were to:

- conduct a literature review concerning the topical use of herbal teas, especially rooibos, a unique product of South Africa;
- incorporate three different anti-oxidants, either alone and in combinations, in topical gel formulations in order to stabilise the active ingredient (i.e. aspalathin);
- formulate seven different types of topical gel formulations with anti-oxidant properties, each containing aspalathin-enriched RE and the same gelling agent;
- formulate a control gel formulation with aspalathin-enriched RE that doesn't contain any anti-oxidants, in order to make accurate comparisons;
- chemically characterise an enriched RE by means of high performance liquid chromatography (HPLC) in order to determine the concentration of aspalathin in the extract;
- validate an existing HPLC analysis method for detecting aspalathin in an enriched RE against an aspalathin reference standard;
- perform stability tests at specific storage conditions on all the gel formulations to evaluate the content of aspalathin (assay) as a function of time, as well as the physical properties including visual appearance, viscosity (rheology) and pH; and
- conduct *in vitro* release studies with vertical customised Sweetana-Grass diffusion cells in order to determine the release (i.e. rate and extent) of the active ingredient marker (aspalathin) from each formulation across a synthetic membrane.

1.4 Ethics

The research proposal and project underwent ethical clearance procedures and were classified under 'no ethics'. No humans or animals were used in this study and therefore no ethical approval was necessary (Addendum A). All the materials for waste were disposed of according to the following pre-approved standard operating procedure (SOP): Pharmacen_SOP002_v02 Pharmaceutical waste handling.

1.5 Layout of dissertation

In this dissertation, Chapter 1 describes the rationale, motivation, as well as the aim and objectives of the study. Chapter 2 follows with an in depth look at the background and literature applicable to this study. The materials used and the experimental methods that were followed

are described in Chapter 3. Chapter 4 contains the results, discussions thereof and statistical analyses, presented as tables and figures. Final conclusions are made based on the results and recommendations for future studies are encompassed in Chapter 5.

CHAPTER 2: LITERATURE STUDY

2.1 Introduction

Treatment of various illnesses have been made possible through the successful delivery of active ingredients to the human body via several routes of administration such as oral, parenteral, rectal, sublingual, inhalation, topical, buccal and sub-lingual. Topical delivery is the application of an active ingredient formulated in a dosage form to the skin with the intention of achieving a pharmacological effect on the skin surface (Sahu *et al.*, 2016).

2.2 Skin

The human skin has a surface area of approximately 1.8 m^2 in a typical adult and is known as the largest organ in the human body constituting 10% body mass. It contains a number of layers and appendages that will be described below (WHO, 2006; Williams, 2018). The human skin forms a barrier and plays an important role in protecting the body and tissue from exogenous substances or injuries (Fox *et al.*, 2011; Godin & Touitou, 2007). This organ is also crucial in maintaining body temperature, and regulating electrolyte and fluid balances (Chuong *et al.*, 2002). Apart from its major functions, the skin also assists in metabolism, immunology and the endocrine and nervous system (Fox *et al.*, 2011; Ghafourian *et al.*, 2010).

2.2.1 Anatomy and function

Although the skin functions as a protective barrier, moderate permeation of chemicals can occur to allow for a transdermal entry route (WHO, 2006). Apart from protection, the skin also prevents the loss of water and nutrients, regulate body temperature and assist in the defence and repair mechanisms of wounds (WHO, 2006; Williams, 2018). The skin functions as the body's first line of defence against environmental exposure and it has many roles, ranging from barrier function to complex biochemical processes (McDaniel *et al*., 2010).

2.2.1.1 Skin layers

The skin consists of different layers, which include the epidermis, dermis and underlying subcutaneous tissue or hypodermis as schematically illustrated in Figure 2-1 (Ng & Lau, 2015).

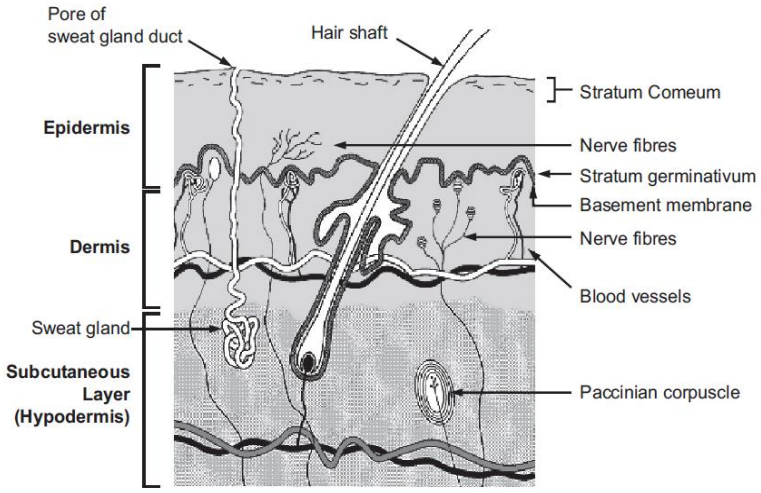

Figure 2-1: Structure and layers of the skin (WHO, 2006)

2.2.1.1.1 Epidermis

The epidermis itself consists of five or six layers, with the SC being the outermost layer of the skin. The majority of cells found in the epidermis are keratinocytes, but it also contains melanocytes and Langerhans cells (WHO, 2006; Williams, 2018). The SC is less than 20 µm thick (approximately 10 µm thick when dry) and consist of flat cells with corneocytes that are arranged parallel to the skin surface. These cells have no metabolic processes or nuclei and have a low moisture content (Hadgraft & Lane, 2016; Jellinek, 1971; Williams, 2018). The keratin in the corneal cells is responsible for chemical resistance and contributes to the SC's function to protect the body from external surroundings. The skin fat and sebaceous glands produce fatty substances with 'wax' components on the surface of the SC that helps regulate the skin's moisture content. In between the smooth skin surface, there are hair follicles covering most parts of the body (Hadgraft & Lane, 2016).

The SC functions as an impermeable membrane, except for a small amount of water loss that is important for the normal function of the SC. The lipids present in the SC are important for the barrier protection of the skin: it is not only the structures of the lipids that form the barrier, but also the 'brick-and-mortar' packing state in the flat corneocytes that are responsible for the skin's barrier function (Roberts *et al.*, 2017). The SC provides an 'acid mantle' that covers the skin surface which is vital for optimal functioning of skin tissue and inhibiting the growth or penetration of microorganisms into the skin (Matss & Rawlings, 2010).

Skin cleansing and moisturisation are processes that maintain skin health and barrier repair. The SC also functions to limit the amount of transepidermal water loss (TEWL), and dryness or

scaling take place when increased TEWL causes the water content in the SC to be less than 10%. The epidermal barrier consists of keratinised cells in the lipid bilayer that controls and maintains intercellular water movement and limit TEWL (Del Rosso, 2010).

2.2.1.1.2 Dermis

The dermis contains blood vessels, sensory nerves and a lymphatic system. The thickness of the dermis is about 3 – 5 mm, but it differs between race, the site on each individual's body and climatic conditions. This layer of the skin contains blood vessels and the blood flow in these vessels is essential in the regulation of body temperature. The dermis provides flexibility, stores water, provides nutrition to the vascular epidermis, and protects against infection (WHO, 2006; Williams, 2018).

2.2.1.1.3 Hypodermis

This small subcutaneous layer consists of adipose tissue and blood vessels that protects the skin against mechanical shock, which also contains high-energy molecules to store energy and insulate the body (Jellinek, 1971; Williams, 2018). The deeper layers of the skin (the hypodermis) contain appendages, such as sweat glands (eccrine and apocrine), sebaceous glands and hair follicles with their muscles. There is a variable amount of hair follicles on the skin, depending on the anatomical area, but there are typically 50 – 100 follicles per cm^2 (Williams, 2018; WHO, 2006). The sebaceous glands secrete sebum which lubricates the skin and maintains an acidic skin surface (pH of 5) (WHO, 2006). Healthy human skin has an acid mantle covering the surface with a pH generally between 4 and 6, and this acidic surface inhibits the growth of undesirable bacteria and fungi (Jellinek, 1971). The eccrine glands produce sweat when high temperatures are encountered and this secreted salt solution has a pH of about 5 (WHO, 2006).

2.2.1.2 Skin ageing

The appearance of healthy and younger looking skin is important for most individuals, but environmental factors exacerbate skin ageing. Skin ageing is a natural unavoidable process in all human beings, but a change in hormones can also influence the skin (Garg *et al.*, 2017). Environmental exposure to the sun and ultraviolet (UV) rays have been recognised for many years as a skin ageing precursor since sunburn causes photoageing, dryness, dark pigmentation and wrinkle formation with a leathery texture, whereas UV-A ray exposure can lead to skin cancer. Compounds from the ozone interact with lipids on the skin surface to form reactive oxygen species (ROS), which causes cellular stress cascades in the skin. Extrinsic skin ageing caused by the environment can be prevented by the use of sunscreens, and in fact can even be reversed through the application of topical vitamins C and E (Burke, 2018). The

general skin condition can be improved by the application of anti-ageing cosmetics, while benign wrinkles can be treated by applying hydrating and moisturising cosmetics to the skin. Severe skin ageing can also be prevented with the aid of collagen treatment, which increases the skin's thickness and improves elasticity (Chuarienthong *et al.*, 2010).

2.2.2 Topical delivery

Topical products are semisolid or liquid preparations intended to deliver an active ingredient to the skin to treat diseases or produce a desired effect (Shah *et al.*, 2015). To produce an effective topical or cosmetic formulation, it is important to deliver the active ingredient into the upper epidermal layers of the skin and keep the molecule at the target site for a desired local effect (Patravale & Mandawgade, 2008). The topical application of active ingredient-containing formulations is not always only intended for the local treatment of skin diseases. A systemic effect can also be attained with transdermal formulations (Nair *et al.*, 2013). Transdermal delivery is, however, not the purpose of this study, as systemic absorption was not desired. Transdermal delivery refers to the application of a skin formulation (i.e. a patch) to deliver an active ingredient through the skin into the systemic circulation, whereas the intention of topical delivery is to retain the active ingredient on the surface or within the skin to treat local disorders or to moisturise (as with cosmetics) (Williams, 2018).

The European Union defined the term cosmetics as "any substance or mixture intended to be placed in contact with the external parts of the human body" (Nohynek *et al.*, 2010). Dr. Albert Kligman defined the term cosmeceuticals as cosmetic products that contain a therapeutic agent with a pharmaceutical effect (Choi & Berson, 2006). Many cosmeceutical products are sold worldwide, claiming to have active ingredient-like effects without having the required scientific or clinical data to prove the claims (McDaniel *et al*., 2010).

2.2.2.1 Rationale and advantages of topical delivery

Advantages of the topical route of administration include convenience and improved patient compliance, controlled release, reduced risk of side effects and bypassing of first pass metabolism and gastrointestinal tract (Li & Chowdhury, 2017; Pastore *et al.*, 2015; Sivaraman *et al.*, 2017). Topical delivery has a low risk, less fluctuations of active ingredient concentrations in patients and less absorption complications (pH and gastric enzymes) than oral administration. It is also easier to achieve efficacy with a lower dosage through continuous active ingredient input (Sahu *et al.*, 2016).

2.2.2.2 Considerations of topical delivery

The ability of an active ingredient to penetrate into the skin determines the product's effectiveness. The age and hydration of the skin can influence topical active ingredient delivery, for example, substances may enter aged skin more easily due to changes in the dermal matrix. When emollients are applied for skin hydration purposes, they may increase active ingredient penetration into the skin after topical administration (Li & Chowdhury, 2017). When developing a topical formulation, it is important to take into account the properties of the active ingredient, as well as the vehicle type, the stability of the formulation, and the compatibility of the ingredients with human skin (Williams, 2018).

For an active ingredient to enter the skin, it needs to be in the molecular state and this penetration is driven by a concentration gradient between the topical formulation and the site of action on the skin (Chang *et al*., 2013). Thermodynamic activity is essential for an active ingredient to be successfully delivered to the skin and it can be described as the tendency or drive for an active ingredient to be released from the cosmetic vehicle formulation to the skin (Williams, 2018). The thermodynamic activity is directly proportional to the flux of active ingredient to the skin, and if the cosmetic vehicle is compatible with the skin, a saturated formulation will also promote active ingredient release from the vehicle (Costa & Santos, 2017; Williams, 2018).

Experimental tests can be performed to assess the safety of ingredients, spreadability, colour changes, homogeneity, pH, phase separation, consistency or rheological properties and effect of storage temperatures on the product (Costa & Santos, 2017; Mohamed, 2004). Many cosmetic preparations can influence the moisture content of the corneal layer of the SC, which could lead to disruption in its structure, elasticity or chemical resistance functions (Jellinek, 1971). Almost every cosmetic ingredient can cause a physiological change in human skin, for example, certain natural products may produce skin irritation, sensitisation or phototoxicity. The use of natural or organic products are regarded as healthy in the consumer market, but it is important to note that the term 'natural' does not necessary assure safety (Nohynek *et al.*, 2010).

2.2.2.3 Factors influencing topical delivery

For a molecule to be released and transported into and across the skin, there are several factors to consider. For instance, the molecule has to overcome the protection properties of the keratinised hydrophobic SC (Godin & Touitou, 2007). Hence, the SC is considered to govern the rate of dermal permeation of most substances (Gee *et al.*, 2014; Hadgraft & Lane, 2016). Another factor that can influence absorption into the skin is molecular size, where molecules

larger than 500 Daltons (Da) exhibit a lower degree of penetration across the SC (Bos & Meinradi, 2000). Lipophilicity is a key factor in the permeation and flux of compounds across human skin and the lipophilic value of a compound can assist in the prediction of skin permeability (Levin & Maibach, 2009; Schröder, 2009). The lipid/water partition coefficient (Log P) is a physicochemical property that is used to describe the hydrophilicity or lipophilicity of a substance by comparing the partitioning of a substance between a polar and non-polar solvent (Huang *et al.*, 2008). A lipid/water partition coefficient equal to one or higher (Log P ≥ 1) is required for optimal active ingredient permeability across the skin (Nair *et al.*, 2013). The active ingredient should have a log P value of around 2 or range between 1 and 4, in order to be considered as reasonably soluble in both water and oil (Oliveira *et al.*, 2012).

The pH of the formulation can also affect active ingredient penetration into the skin. Natural skin has an acidic surface with a pH of mostly 5. Cosmeceutical formulations must be prepared in such a way that the skin's pH remains unchanged, in order to maintain the normal physiology of the skin (Ansari, 2009). The pH of the vehicle can also influence release of the active ingredient from the formulation to the skin. At a particular pH (depending on the pKa of the active ingredient), the degree of ionisation can be altered in such a way that the unionised species will be most prevalent and permeation through the lipophilic SC is promoted (Nair *et al*., 2013). A pH that is close to human skin pH (near 5) is deemed appropriate for topical formulations to prevent harm to the skin (Ansari; 2009).

Other factors that can influence active ingredient absorption are the composition of the formulation, temperature, skin enzymes and hydration (Nair *et al*., 2013). The extent of active ingredient absorption across the skin barrier is in general dependent on the physicochemical properties of the active ingredient, the formulation's stability, the compatibility between the active ingredient and excipients, and the rate and extent of active ingredient release from the dosage form (Williams, 2018). When developing a topical formulation, it is important to take into account the properties and concentration of the active ingredient, as well as the vehicle type, the stability of the formulation, and compatibility of the ingredients with human skin (Li & Chowdhury, 2017; Williams, 2018). To conclude, for optimal diffusion through the SC barrier, it is beneficial to use active ingredients with molecules smaller than 500 Da that possess both lipophilic and hydrophilic character traits (Roberts *et al.*, 2017).

2.2.3 Formulations for topical delivery

Pharmaceutical and cosmetic formulations for topical application can range from solid systems, e.g. transdermal patches and powders; to semisolids, e.g. creams (emulsions), ointments, lotions and gels; or simple liquid systems, e.g. aqueous solutions (Otto *et al.*, 2009). Several semisolid topical formulations are currently on the market due to more selective application,

self-administration, reduced systemic side effects and fewer fluctuations in active ingredient levels (Sivaraman *et al.*, 2017). The different types of topical formulations are briefly discussed below.

2.2.3.1 Creams

A cream is a semisolid formulation intended for application on the skin or mucous membranes and is usually composed of an emulsion, either water-in-oil or oil-in-water (Garg *et al.*, 2017; Williams, 2018). An emulsion can be defined as a dispersion of two immiscible liquids or phases that are mixed together and formulated in such a way to produce a semisolid product. One phase is distributed uniformly as fine droplets (disperse phase) in the other liquid (continuous phase) (Eccleston, 2013). Basic emulsion formulations include oily and aqueous ingredients, humectants, surfactants, thickeners, stabilisers, chelating agents, preservatives, perfumes, neutralisers and pharmaceutical agents (Mitsui, 1997; Nohynek *et al.*, 2010). An important function of creams is to hydrate and moisturise the skin. When a cream is rubbed onto the skin, the different vehicle constituents in the formulation can either evaporate or be absorbed into the skin (Mayba & Gooderham, 2017).

2.2.3.2 Ointments

An ointment is a semisolid dosage form intended for external application (Mayba & Gooderham, 2017). Ointments contain more oil than creams and are fatty preparations generally used for dry skin lesions. The active ingredient can be dissolved or dispersed in the greasy base and used for superficial treatment. Otherwise, ointments can also be used as an emollient to smooth, soothe and hydrate the skin. Due to their oiliness, ointments are very occlusive, which increases hydration of the SC and provides prolonged active ingredient delivery through longer residence time of the formulation on the skin. Ointment formulations usually consist of liquid paraffin to generate hydrocarbon bases, however, the thick greasy texture of ointments is less ideal and may deter patients from using the product (Garg *et al.*, 2017; Li & Chowdhury, 2017; Williams, 2018).

2.2.3.3 Lotions and liniments

Lotions are watery liquid preparations with low to medium viscosity, and more convenient to use on unbroken skin or hairy and large body surfaces (Li & Chowdhury, 2017). Lotions are liquid suspensions or emulsions with an aqueous vehicle consisting of more than 50% water and volatiles (Mayba & Gooderham, 2017). Solvent evaporation provides a cooling effect when applied and is beneficial for exudative dermatoses (Garg *et al.*, 2017). Lotions are thin and opaque with a non-greasy texture and thus cosmetically elegant. Creams or lotions are usually more spreadable and less viscous than ointments or pastes (Mayba & Gooderham, 2017).

According to the British Pharmacopoeia (BP) (2019), liniments are liquids intended for cutaneous application that should be applied to unbroken skin with friction. Liniments are semiliquid or liquid preparations of alcohol or oil that is usually rubbed on the skin. The vehicle can be soap, oil or alcohol based, and these topical solutions can be used for soothing, as a stimulant, or for dilation of capillaries (Garg *et al.*, 2017; Williams, 2018).

2.2.3.4 Gels

The term gel originated in the late 1800's to classify semisolid formulations in which polymer molecules in the liquid phase is crosslinked with a polymeric matrix (Dragicevic-Curic & Maibach, 2015; Rathod & Mehta, 2015). The dispersion phase, which can contain organic or inorganic substances, is dispersed in an aqueous or hydro-alcoholic liquid phase to form a three-dimensional structural matrix (Asija *et al.*, 2013; Sivaraman *et al.*, 2017). The cross-linked network of colloidal particles in the surplus liquid provides the rigidity in a gel structure (Ajazuddin *et al.*, 2013; Asija *et al.*, 2013). There is an interlinking of particles and this physical or chemical force provides the structure and properties of a gel, which make it rigid (Rathod & Mehta, 2015; Sivaraman *et al.*, 2017). A gel is colloidal as the large amount of liquid (> 90%) is immobilised with the high surface tension that forces a macromolecular network between the liquid and the gelling substance (Panwar *et al.*, 2011). The attraction forces can range from strong covalent bonds to weaker hydrogen bonds or Van der Waal forces (Rathod & Mehta, 2015). The high water content makes gels very similar to natural tissue and provides a degree of flexibility (Tavano, 2015).

2.2.3.4.1 Advantages of gels

Gels can be applied locally to produce a desired effect on the skin, eyes or mucous membranes. Gels can also be used in intramuscular products, tablet binding, suppositories and cosmetics like hair care, dental products or skin preparations. The application of gels are non-invasive, cosmetically elegant, promote good patient compliance, require less dosing than oral dosage forms, are more economic, and have a stable active ingredient delivery profile (Babar *et al.*, 1991; Velissaratou & Papaioannou, 1989). The less greasy and non-occlusive texture of gels makes removal of formulations from the skin easier than ointments or creams (Rathod & Mehta, 2015; Saroha *et al.*, 2013; Tas *et al.*, 2003). Gel formulations are also easily spreadable, emollient, thixotropic, water-soluble and compatible with several excipients (Ajazuddin *et al.*, 2013; Panwar *et al.*, 2011). These formulations provide a cooling effect when applied to the skin, has a good safety profile with a quick onset of action and a long-lasting effect (Kurian & Barankin, 2011). When a gelling agent is incorporated, the system can become thixotropic. The latter is a reversible structural transition and refers to a viscous gel becoming more liquid when shearing is applied (Mewis & Wagner, 2009; Singla *et al.*, 2012).

2.2.3.4.2 Classification of gels

Gels may be divided according to the nature of the colloid phase (organic or inorganic) or the solvent (aqueous or non-aqueous) (Saroha *et al.*, 2013). In pharmaceutical applications, aqueous or hydro-alcoholic gels are most commonly used (Rathod & Mehta, 2015). Pharmaceutical gels are classified according to the microstructural network as (i) covalently bonded, (ii) physically bonded or (iii) well-ordered gels (Saroha *et al.*, 2013).

Hydrogels are gel systems where the polymer network chain is extensively swollen in water as the dispersion medium. The polymeric material doesn't dissolve in the water, but has the ability to absorb a fraction of water through hydrophilic functional groups that are attached to the backbone of the polymer (Ahmed, 2015). Hydrogels exhibit bio-adhesive properties, good viscosity, without irritating actions on the skin, and can therefore be used in many cosmetic and pharmaceutical applications (Rathod & Mehta, 2015; Realdon *et al.*, 1998; Tas *et al.*, 2003).

Gels can be classified as natural or synthetic based on the origin of the polymer used as gelling agent. Natural hydrogels were replaced by synthetic hydrogels due to their long service life, high gel strength and capacity to absorb more water. Natural gelling agents have a bigger risk for microbial contamination and degradation, while synthetic hydrogels have the advantage of being able to remain stable during excessive temperature fluctuations (Ahmed, 2015; Ajazuddin *et al.*, 2013).

2.2.4 Formulation components of gel preparations

2.2.4.1 Active ingredient

The onset, duration and extent of the therapeutic response of an active ingredient after topical administration depend on a series of processes. These processes include the release of the active ingredient from the dosage form and the diffusion of the active ingredient to the site of action through the SC (Shah *et al.*, 2015). Certain excipients in cosmetic and pharmaceutical products (e.g. penetration enhancers, propylene glycol, ethanol and water) can have an impact on the absorption of the active ingredient into and across the skin (Hougeir & Kircik, 2012).

2.2.4.2 Appropriate vehicle or solvent

A vehicle in the topical delivery system is included to carry the active ingredient to the target area. In a cosmeceutical product, the delivery system should be able to retain the active ingredient in the superficial skin layers (Costa & Santos, 2017). The delivery vehicle can also influence consumer acceptance of the product and subsequently compliance, therefore a formulation with a pleasant feel should be prepared (Hougeir & Kircik, 2012).

Distilled water is usually used as vehicle in gel preparations, but other co-solvents can be used to improve the solubility of ingredients or facilitate dispersion; e.g. alcohol, propylene glycol, glycerol or mineral oil (Chang *et al.*, 2013). Ethanol can be used as a co-solvent with water to solubilise active ingredients and increase skin penetration. Alcohols are popular solvents in topical formulations because it can partition into the skin and provide a reservoir for active ingredient absorption. Alcohol may also provide a better diffusion coefficient for the active ingredient in the SC. Another benefit of using alcohol as solvent is the effect that evaporation provides, namely it leaves the formulation saturated with the active ingredient and a finite-dose application is achieved (Williams, 2018).

2.2.4.3 Preservatives

Anti-microbial preservatives such as parabens, phenoxyethanol or phenolics are added to semisolid preparations to prevent microbial growth, protect consumers against contamination and improve the shelf life of a product (Epstein, 2009; Stahl, 2015). Parabens, especially methyl- and propylparaben, are widely used in topical as well as oral pharmaceutical formulations, food products and cosmetics. The addition of propylene glycol not only assists in the solubility of parabens, but also improves preservative efficacy. Parabens function over a wide pH range and are most stable in aqueous solutions with a pH of 3 – 6. In the European Union, a cosmetic product is generally regarded as safe if the total amount of parabens in the product does not exceed 0.8% (Rowe *et al.*, 2009).

2.2.4.4 Anti-oxidants

Anti-oxidants are commonly included in topical products. Certain ingredients are prone to oxidative degradation and anti-oxidants can be added to improve the chemical stability of these substances by minimising oxidative deterioration (Saib, 2010). A study demonstrated that the application of a topical formulation containing a mixture of various anti-oxidants can improve the anti-oxidative action in the skin. The results indicated that topical products containing anti-oxidants remained in the SC for a relatively short duration due to desquamation, textile contact, washing and environmental exposure that cause depletion of the anti-oxidant substances to negligibly low quantities (Darvin *et al.*, 2011). Anti-oxidants are selected for inclusion in topical products based on their stability, solubility and compatibility with other ingredients in the formulation (Saib, 2010). Examples of anti-oxidants commonly used in topical preparations are butylated hydroxytoluene, sodium metabisulfite, citric acid and vitamin E or C (Rowe *et al.*, 2009). Gels are mostly aqueous and thus water-soluble anti-oxidants are regularly used in these topical preparations (Chang *et al.*, 2013).

Vitamin C is a vital anti-oxidant required in the human body and is also known as ascorbic acid. Topical vitamin C protects the skin against solar damage by primarily acting as an anti-oxidant, which reacts against free radicals from UV damage. Studies have also proved that vitamin C has anti-inflammatory and anti-ageing effects (Sheraz *et al.*, 2011). Furthermore, vitamin C not only moisturises the skin, but it also improves the skin's protective barrier function (Burke, 2015). L-ascorbic acid is regularly used in aqueous pharmaceutical formulations with maximum stability at a pH of about 5.4. The oxidation of l-ascorbic acid can be accelerated by light and heat (Rowe *et al.*, 2009).

Citric acid is widely used in food, cosmetic and pharmaceutical products due to its safety and solubility in water. It can function as an anti-oxidant, buffer, chelating, sequestering, and acidifying agent (Rowe *et al.*, 2009). Citric acid acts as an intermediate in chemical synthesis and permits the formation of reactive products and several complex molecules. The pH adjusting and chelating properties of citric acid improves the stability of food products by enhancing the anti-oxidant action (Soccol *et al.*, 2006).

Sodium metabisulfite is used in food products and also in oral, parenteral and topical formulations in the pharmaceutical industry at concentrations of 0.01 – 1.0% (w/v). It is water soluble and used as an anti-oxidant and preservative at a more acidic pH. When sodium metabisulfite is exposed to moisture and air, the crystal structure disintegrates and it is slowly oxidised to sodium sulphite. In water, sodium metabisulfite is quickly converted to sodium and bisulfite ions (Rowe *et al.*, 2009).

2.2.4.5 Buffers

As mentioned earlier, the skin has a natural pH of about 5, and to avoid the risk of dermal irritation, topical preparations should be formulated close to the pH of human skin in a range of 4.5 to 6 (Ansari; 2009; Lucero *et al*.,1994; Nair *et al*., 2013). To maintain a proper pH, acidifying or alkalising agents can be incorporated, e.g. citric acid, hydrochloric acid, triethanolamine or sodium hydroxide (Chang *et al.*, 2013; Rowe *et al.*, 2009). Buffer solutions consist of a weak acid with its conjugate base and are included in gel formulations to ensure that the pH of the formulation remains relatively constant within a specified range. Examples include phosphate, citrate or acetate buffers (Calbiochem®, 2006; Draganoiu *et al.*, 2009; Rathod & Mehta, 2015; Rowe *et al.*, 2009).

2.2.4.6 Gelling agents

Gelling agents are also known as thickeners, and are used to produce a gel structure or can be used as viscosity modifiers in other formulations (Stahl, 2015). Gel forming substances are usually polymeric macromolecules used to increase the viscosity of the system, and are

essential in forming the main structure of a gel and can also be used to control the rheology or texture of the formulation (Chang *et al.*, 2013; Saroha *et al.*, 2013).

Gels are relatively easy to prepare and the formulation of gels require the use of polymers, which can be classified as natural, synthetic, or semi-synthetic polymers (cellulose derivatives), surfactants and inorganic substances as listed in Table 2-1 (Rathod & Mehta, 2015; Saroha *et al.*, 2013).

Table 2-1: Classification of different types of gelling agents with examples (Lubrizol, 2011; Rathod & Mehta, 2015; Saroha *et al.*, 2013)

Classification	Gel forming substance	Examples
Natural polymers	Proteins	Collagen Gelatin
	Polysaccharides	Agar Alginate acid Cassia tora Gellum Gum Guar Gum Pectin Sodium or Potassium carageenan Tragacanth Xanthan Gum
Synthetic polymers	Carbomer	Carbopol® - 940, 934P, 940, 974PNF, 971P NF & 71G NF
	Poloxamer	
	Polyacrylamide	
	Polyethylene and its co-polymers	
	Polyvinyl alcohol	
Semi-synthetic polymers	Cellulose derivatives	Carboxymethyl cellulose Hydroxypropyl cellulose Hydroxypropyl (methyl cellulose) Hydroxyethyl cellulose Methylcellulose
Surfactants	Cetostearyl alcohol	
	Brij® – 96	
	Dodecyl pyridinium iodide	
	Lecithin	
	Sodium lauryl sulphate	
	Sorbitan mono-oleate	
Inorganic substances	Aluminium hydroxide	
	Bentonite	

Synthetic Carbopol® polymers have been incorporated into topical pharmaceutical products for more than 50 years and are widely used to thicken formulations such as creams, lotions or gels and to adjust the rheology of water-based systems. Carbopol® are polymers of acrylic acid that are cross-linked with polyalkenyl ethers or divinyl glycol (Barreiro-Iglesias *et al.*, 2003; Lubrizol, 2011). Each polymer molecule forms a network structure where polymer chains are interconnected by crosslinks (Lubrizol, 2011). The formulation becomes more stable when polymers are completely swollen and swelling can be achieved through neutralisation or hydrogen bonding. Carbopol® polymers have the ability to swell in water almost 1 000 times their original volume when the formulation is neutralised. The addition of a base in the acidic colloidal dispersion produces a highly viscous gel (Draganoiu *et al.*, 2009). When it is challenging to increase the pH of the final formulation, thickening of the formulation may be achieved through hydrogen bonding (Lubrizol, 2011).

Advantages associated with the use of aqueous carbomer gelling agents include: (i) high viscosity can be achieved at a low concentration, (ii) gels have a broad viscosity interval with characteristic flow behaviour, (iii) good bioadhesive properties, (iv) compatible with numerous active ingredients, (v) thermal stability, and (vi) favourable organoleptic characteristics (Islam *et al.*, 2004).

2.2.5 Manufacturing methods of gels

Gels are manufactured by thickening a liquid phase with other components in the dispersed phase (Williams, 2018). Carbomer powders should be dispersed in water while stirring (mechanical stirring) and care should be taken to avoid the formation of indispersible agglomerates. When a gel is prepared, the solution should be agitated gently by stirring at low revolutions to allow swelling and to avoid the formation of air bubbles or aggregation. Other excipients can be added if necessary, and after dissolution of the polymer is achieved, stirring can be stopped. The pH of the final formulation should be adjusted to the desired value and a buffer added (Draganoiu *et al.*, 2009; Rathod & Mehta, 2015). According to literature, complete swelling is achieved when the carbomer water dispersion is left to stand and rest for some time (Al-Suwayeh *et al.*, 2014; Dantas *et al.*, 2016; Ghorpade *et al.*, 2012; Shukr & Metwally, 2013).

Hydro-alcoholic gels can be prepared with ethanol if it is necessary to improve the solubility of certain active ingredients. These gels promote a more aesthetic clear product, whereas if a solubiliser is used instead of ethanol, the product feels sticky or rubbery. The formulation of hydro-alcoholic gels entails the same methodology as for the manufacturing of a hydrogel, where the carbomer is slowly dispersed in water followed by the addition of ethanol to the solution while continuously stirring. Neutralisation of the hydro-alcoholic gel can be accomplished with the addition of a base to the formulation: when the product has a high

ethanol content, triethanolamine is usually recommended to increase the gel's pH (Lubrizol, 2011).

2.2.5.1 Factors influencing gel formation

During formulation of a gel, it is important to avoid high shear mixing or the use of a homogeniser, as high shear can break down the polymer structure and result in a reduction of gel viscosity. Aqueous gels generally have a higher viscosity than hydro-alcoholic gels, because more hydrogen bonds are present. If higher viscosity of a hydro-alcoholic gel is required, the amount of carbomer polymer should be increased (Lubrizol, 2011).

Anti-oxidants and other ingredients may also influence the structure of the gel. It is well known that maximum gel strength is achieved near a neutral pH, and anti-oxidants have been shown to severely affect the functionality of gelling agents in an aqueous system (ANVISA, 2004; Deuschle *et al.*, 2015; Mitchell *et al.*, 1992). The gel strength or apparent viscosity is directly proportional to the effective crosslink density of a gel, but increasing temperature may change the viscosity, and this occurrence is dependent on the molecular interactions between the solvent and polymers (Rathod & Mehta, 2015). Researchers studied the effect of additives on gelation temperature and gel strength and they concluded that ethanol, hydrochloric acid and propylene glycol decrease gel strength and bioadhesive force (Choi *et al.*, 1999). Carbomer polymers are sensitive to the addition of certain electrolytes (especially ionised ingredients), which can decrease gel viscosity. However, the viscosity reducing effects of electrolytes can be minimised or prevented by increasing the carbomer content in the gel formulation (Lubrizol, 2011).

2.2.6 Evaluation of gel formulations

According to the Scientific Committee on Consumer Safety (SCCS) (2016), when developing a cosmeceutical formulation, it is important to conduct a stability study of the test substance under storage and experimental conditions. The stability of a gel system can be affected by several factors, such as the pH of the dispersion, type of polymer used and its concentration, processing temperature, duration of swelling, type of vehicle, and the addition of cations or anions (Piriyaprasarth & Sriamornsak, 2011). Relevant physical and chemical tests should be performed and gel formulations should be evaluated based on the general parameters, which are discussed below.

2.2.6.1 Stability assessment

According to the International Conference on Harmonisation (ICH) Guidelines Q1A (R2) (ICH, 2003), the purpose of stability testing is to investigate how environmental factors, such as

temperature, light and humidity, affect the quality and content of the active ingredient in the formulated product. The guidelines suggest that testing should be done every three months over the first year, every six months in the second year and then annually for longer studies (ICH, 2003; Medicines Control Council (MCC), 2012). Long-term testing for a time period of twelve months requires the following storage conditions: 25 ± 2 °C/60 ± 5 % relative humidity (RH) (MCC, 2012). When intermediate storage testing conditions are required for a twelve-month study, testing should be done at a minimum of four time intervals, including the initial and final times. The required temperature and humidity conditions for intermediate testing are 30°C ± 2°C and 65 ± 5 % RH, respectively. For accelerated studies evaluations are required at month zero, three and six. Products for accelerated testing are subjected to 40°C ± 2°C and 75 ± 5 % RH for a minimum time period of six months (MCC, 2012).

According to the MCC (2012) and ICH (2003), a significant change in an active ingredient product's properties during stability testing can be described as:

- A 5% loss or change in the active ingredient from the initial value;
- Any degradation products exceeding the specified acceptance limit;
- Failure of product to remain within the specified pH range; and
- Failure to meet the acceptance criteria in terms of appearance and physical characteristics, e.g., colour changes, phase separation and hardness.

2.2.6.1.1 Chemical quantification

A chemical compound can be identified with an analytical technique such as UV/Vis spectroscopy or HPLC with UV diode array detection (Chang *et al.*, 2013). Briefly, HPLC is used to analyse and separate chemical compounds through a mass-transfer of analytes located between stationary and mobile phases. In order to accurately determine the active ingredient content in the prepared gel formulations, a validated HPLC analytical method is an acceptable analytical technique (Singh, 2013).

2.2.6.1.2 pH

The pH value describes the degree of basicity or acidity of an aqueous solution and a pH unit provides an indication of the hydrogen ion concentration, $[H^+]$. The pH of a solution can range between values of 0 and 14, where 7 is neutral, a value below 7 is acidic and above 7 is basic (Hach Company, 2018). In the pharmaceutical industry, pH is an important consideration in formulations since it can have numerous effects on the solubility of active ingredients, the degree of ionisation of active ingredients, and active ingredient absorption into blood and body tissues (Aulton, 2018). Many active ingredients are weak acids or bases that exist as ionised or unionised species in an equilibrated mixture (Calbiochem®, 2006). pK_a is usually used to

express the ionisation constants of acid and base active ingredients, and the degree of ionisation is controlled by the pH of the environment. There is a link between the pH and pK_a of an active ingredient; a weak acid, for example, is highly ionised at high pH values and highly unionised at low pH values. It is the unionised form that is easily absorbed across biological membranes (Aulton, 2018).

It is important to measure the pH of the formulation, as a change in pH can affect the properties of the semisolid product, as well as the stability of the active ingredient in the product. The pH of the product may also play a role in the viscosity and effectiveness of other ingredients in the formulation and pH measurements can be used to determine stability of the formulation over time (ANVISA, 2004; Chang *et al.*, 2013).

2.2.6.1.3 Viscosity (rheology)

Viscosity is a variable that is used to characterise a formulation in terms of its rheological properties. In the pharmaceutical industry, viscometers with various geometries are used to monitor product consistency. By measuring a gel formulation's viscosity over time, it allows the formulator to evaluate the behaviour and stability of the product (ANVISA, 2004). Apart from determining viscosity as a function of shear rate, a rheometer can also display the flow behaviour of a gel formulation (Chang *et al.*, 2013).

2.2.6.1.4 Visual inspection

After the gel is formulated, it can be observed macroscopically to evaluate product stability (ANVISA, 2004). Visual inspection enables the formulator to identify possible homogeneity issues, any foreign matter inclusion, colour changes, phase separation or syneresis (this occurs when water exudes from a gel). If the active ingredient disperses in the product, it is useful to inspect each batch visually (Chang *et al.*, 2013).

2.2.6.2 *In vitro* assessment for active ingredient release

Artificial membranes can be used to measure the release of an active ingredient from a topical formulation (Williams, 2018). Cellulose acetate, for example, is a synthetic membrane that has been used extensively over the past few years in diffusion studies (Haq *et al.*, 2018). The transport and *in vitro* release of an active ingredient can be assessed with the aid of vertical type diffusion cells to evaluate if the marker molecule has been released sufficiently from the gel formulation in order to reach the skin (Rathod & Mehta, 2015).

2.2.7 The use of herbs in topical cosmeceutical products

The incorporation of herbs and other natural compounds in topical cosmeceutical products is becoming more prevalent. For many years, the advantages of herbal skin product application have been acknowledged and clinical studies have demonstrated the possibility of treatment and prevention of atopic dermatitis with natural herbal topical medicines (Cho *et al.*, 2011; Hu *et al.*, 2015). Herbal medicines may be considered as an alternative in the treatment and management of dermatoses due to lower incidences of adverse events and sometimes lower cost (Man *et al.*, 2011). A topical herbal product containing *Wrightia tinctoria* oil extract has been used successfully in the treatment of specific skin conditions such as psoriasis, dermatitis, pigmentation, eczema and inflammation (Kanaujia *et al.*, 2010). Another study also reported that *Pinhão* extract incorporated in a topical emulgel formulation exhibited anti-oxidant potential due to certain phenolic compounds that are present in the extract (Daudt *et al.*, 2015).

Oils from the African oil palm, baobab tree, Cape mahogany and sesame seeds are often used in South Africa in products to moisturise the skin. Other popular plants are frequently used in skin creams such as Aloe, Cape chestnut, white milkwood, honeybush- and rooibos tea to improve skin appearance by alleviating the effects of ageing, wrinkling, acne and skin hyperpigmentation (Lall & Kishore, 2014).

2.2.7.1 Aspalathus linearis (Rooibos tea)

The leguminous shrub, *Aspalathus linearis* also known as rooibos, is an indigenous South African plant that is globally used to prepare a herbal tea known as rooibos tea (Joubert *et al*., 2008). Photographs of the *A. linearis* plant that are used to make rooibos tea are shown in Figure 2-2.

Figure 2-2: Rooibos tea plants (Joubert, 2019)

2.2.7.1.1 History

The history of the use of the rooibos plant dates back to 1772, when a botanist, Carl Thurnberg, discovered that the Khoi people crudely processed rooibos plants into a beverage. In 1904, Benjamin Ginsberg started marketing rooibos for commercial herbal tea use. After the Second World War, the demand for rooibos tea had increased even more and in 1948, the Clanwilliam Tea Co-operative Company set out to improve the marketing for rooibos tea (Joubert & De Beer, 2011; Snijman, 2007). Processed rooibos plant material was mostly exported for use as a tea, and since 1987, rooibos was sold in Japan for its 'anti-ageing' properties (Joubert & Schulz, 2006). The international market for rooibos grew steadily and the annual demand had increased from 750 tons in 1993 to 5 633 tons in 2010 (Beelders *et al.*, 2012). Rooibos tea accounts for 10% of the global herbal tea market, and in South Africa the rooibos production was 12 000 tons by 2014 (Santos *et al.*, 2016). Furthermore, the worldwide demand for rooibos export is increasing annually. According to the SARC (2017), rooibos tea is exported to more than 30 countries with a current global consumption of 15 000 tons per year. In the food and beverage industries, aqueous extracts and extract powders that are prepared from fermented rooibos tea are used, for example, in many ready-to-drink iced teas (Joubert & Schulz, 2006).

2.2.7.1.2 Botany and geographical distribution

Aspalathus linearis (Burm.f.) Dalgh (Genus *Aspalathus*; Family Fabaceae; Tribe Crotalarieae) (rooibos) is a shrub-like leguminous bush endemic to the Cederberg Mountains in the Western Cape Province, South Africa (Beelders *et al.*, 2012; Gouws *et al.*, 2014; Ku *et al.*, 2015). Rooibos forms a unique part of the fynbos biome, which can only be found in South Africa (Joubert *et al.*, 2008; Joubert & De Beer, 2011). The genus *Aspalathus* consists of more than 200 species, of which the *linearis* subspecies occurs commonly and the 'red type' is mostly used for tea production (Gouws *et al*., 2014; Joubert & Schulz, 2006). Rooibos has developed geographical indication (GI) to prevent any cultivation outside South Africa so that intellectual property rights can be protected. This label enables manufacturers to reserve the characteristic and defining qualities of rooibos within the products' geographical location and necessitate strict quality criteria to be applied (Joubert & De Beer, 2011).

2.2.7.1.3 Tea industry: harvest and fermentation

Rooibos herbal tea can be prepared from the unfermented (green) or fermented (oxidised) plant materials, although the latter product is traditionally more commonly used. Leaves and fine stems are processed to produce this herbal drink and the oxidation process forms the characteristic woody, floral and honey taste of rooibos (Koch *et al.*, 2013). The processing of

rooibos tea entails the planting of seedlings between June and August and the first harvest takes place 18 months later in summer (from December until early autumn).

After the branches are harvested, the tea shoots are shredded and bruised and then collected in heaps for further processing. Water is added to accelerate fermentation and to extract polyphenols, and the shredding initiates enzymatic oxidation which causes a change in colour. As soon as the right aroma develops, the plant material is spread open to dry until a moisture content of no more than 10% is attained. The plant material is then steam-pasteurised to eliminate microbial growth (Beelders, 2011; Joubert & Schulz, 2006; Santos *et al.*, 2016).

2.2.7.1.4 Characteristics of rooibos tea

Phenolic compounds exhibit anti-oxidant activity, which is achieved by reacting with ROS to ameliorate molecular damage. Rooibos tea contains phenols and the total polyphenol content determines the anti-oxidant activity of rooibos tea infusions and extracts (Di Mambro & Fonseca, 2005; Ku *et al.*, 2015). Flavonoids exhibit anti-oxidant activities through various mechanisms, which may involve metal chelation, free radical scavenging and/or oxygen removal through enzyme inhibition (Marnewick, 2009). Aspalathin, a C-C linked dihydrochalcone glucoside (Figure 2-3), is a unique flavonoid, which is only found in rooibos and can be used as a chemical marker for analytical purposes (Joubert & De Beer, 2011; Marnewick *et al.*, 2003). Aspalinin and nothofagin are also unique phenolic compounds, which are found in rooibos tea. Rooibos tea also contains flavones (orientin, isoorientin, luteolin, vitexin, chrysoeriol, isovitexin), flavonoles (quercetin, isoquercetin, hyperoside, rutin), and flavanones (dihydro-orientin, hemiphlorin, dihydro-isoorientin) (Beelders, 2011; Joubert & De Beer, 2011; Muller *et al.*, 2012; Santos *et al.*, 2016). Minerals, such as sodium, calcium, potassium, zinc and magnesium can also be found in rooibos tea (Beelders, 2011; Ku *et al*., 2015).

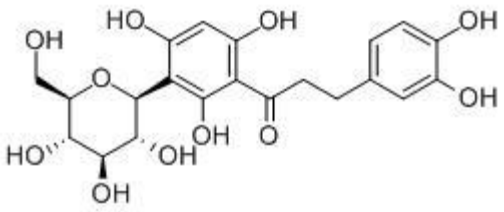

Figure 2-3: Chemical structure of aspalathin (adapted from HWI, 2018)

Rooibos tea is popular as a beverage due to the absence of caffeine and low tannin content (Santos *et al.*, 2016; Snijman, 2007). The fermentation process turns the rooibos leaves into the characteristic red-brown colour due to enzymatic oxidation of the polyphenols (Snijman,

2007). When rooibos plant material is prepared for fermentation, it is exposed to air and the fermentation process induces oxidation. This results in a loss of phenolic compounds and there is especially a rapid decrease in aspalathin after fermentation (Joubert, 1996; Walters *et al.*, 2017). Unfermented rooibos leaves and stems remain green in colour and have higher anti-oxidant capacity than fermented rooibos plant material (Snijman, 2007; Von Gadow *et al.*, 1997; Walters *et al.*, 2017).

According to the Material Safety Data Sheet (MSDS) (2017), rooibos tea exhibits the following characteristics: pH of 4.0 – 6.5, specific gravity of 1.26, and soluble in water. According to Huang *et al*. (2008), aspalathin is a hydrophilic molecule that is highly soluble in water. The physiochemical properties of aspalathin are as follow: it has a molecular weight of 452.1 g/mole; partition coefficient (log D) of 0.13 at pH 7.4, which indicates low lipophilicity and is highly soluble in hydrochloric acid and saline (Bowles *et al.*, 2017). Studies have shown that aspalathin is absorbed in the human body after rooibos tea ingestion and an intact methylated form of aspalathin can be found in human urine, but with very low traces in human plasma (Bowles *et al.*, 2017; Breiter *et al.*, 2011; Courts & Williamson, 2009). Recent studies reported that aspalathin is unstable in an aqueous solution and at a physiological pH (7.4), with a slightly higher stability in buffer at pH 6.0 (Bowles *et al.*, 2017; Monsees & Opuwari, 2017). Commercialised rooibos iced teas were investigated at different pH values and the results showed that a better aspalathin stability was achieved at lower pH values (De Beer *et al.*, 2012).

2.2.7.1.5 Health promoting effects and clinical uses

Traditionally, rooibos tea has been used to treat asthma, eczema, nausea, colic, headache and mild depression, promote smooth muscle relaxing effects, improve immune response and exhibit anti-microbial activity (Gouws *et al.*, 2014; Marnewick, 2009). RE is widely used in the food industry as an anti-oxidant since it reduces oxidative stress (Joubert & De Beer, 2011; Marnewick *et al.*, 2003). The use of rooibos beverage has numerous advantages as it is considered to exhibit cardioprotective and hepatoprotective effects, stimulates the immune system and displays anti-inflammatory activity (Dludla *et al.*, 2014; Joubert *et al.*, 2008; Ku *et al.*, 2015). Muller *et al.* (2012) demonstrated that RE with high levels of aspalathin had therapeutic anti-diabetic potential by decreasing blood glucose levels in diabetic rats. The study done by Ku *et al.* (2015) showed that aspalathin can be used to reduce vascular inflammation and atherosclerosis complications in diabetics. There are claims that the use of aspalathin, obtained from RE, may be beneficial for the treatment for certain central nervous system disorders, including dementia, depression and Parkinson's disease (Frank & Dimpfel, 2010). A recent study demonstrated the phytoestrogenic activity of rooibos tea, where it was discovered that 5% unfermented rooibos tea can increase the weight of the uterus significantly, which may be beneficial for female fertility (Monsees & Opuwari, 2017). Sanderson *et al.* (2014) also

established that regular intake of rooibos tea infusions promoted hypolipidemic effects through the inhibition of adipogenesis.

A study done in April 2019 evaluated the effect of green rooibos extract (GRE) against fermented RE on the ageing of fat cells (adipocytes). Cosmetic appearance can be preceded by adipocyte ageing, meaning that changes such as skin folds, sunken eyes and wrinkles can appear and are caused by a loss in adipose tissue (Hattingh *et al.*, 2019; Tchkonia *et al.*, 2010). GRE contains significantly higher total polyphenols and aspalathin than fermented RE and treatment with GRE showcased higher functional capacity of pre-adipocyte cells, resulting in less oxidative stress and prevention of cellular ageing. This research proves that GRE can counteract the ageing process in adipocytes, which can be beneficial in cosmetic appearance (Hattingh *et al.*, 2019).

Several laboratory studies showed promising health benefits of rooibos tea, but limited clinical studies are available. Black tea (*Camellia sinensis*), a popular beverage among consumers, was reported to cause a reduction in iron absorption (Joubert *et al*., 2008). In contrast, two studies in human subjects showed that the intake of traditional rooibos tea (200 ml) had no detrimental effect on the iron absorption status of the candidates (Breet *et al.*, 2005). The first clinical intervention study was done in South Africa on adults at risk for heart diseases to monitor the effects of traditional rooibos tea on oxidative stress, and it was concluded that rooibos protects the human body against oxidative damage (Marnewick, 2009). Another clinical study reported a significant improvement in the levels of several biomarkers and a reduction in oxidative stress related to cardiovascular disease (Marnewick *et al.*, 2011). An infusion of rooibos tea was also reported to have a positive effect on patients with atopic dermatitis, showing reduced itching and inflammation, as well as a decreased incidence in *Herpes simplex* infection (Joubert *et al.*, 2008; Shindo & Kato, 1991).

2.2.7.1.6 Topical formulations containing rooibos extract

The use of RE in cosmeceutical products increased globally since Dr. Annetjie Theron introduced an extensive range of RE containing products (e.g. Annique® range of products) to the cosmetics market (Joubert & Schulz, 2006). Topical RE applications are believed to reduce dermatological problems, e.g. acne, eczema and nappy rash (Joubert *et al.*, 2008; Joubert & Schulz, 2006). RE is used in cosmeceutical formulations typically for anti-oxidant properties and to reduce skin ageing (Chuarienthong *et al.*, 2010; Gouws *et al.*, 2014; Huang *et al.*, 2008). A formulation containing RE and tea (*C. sinensis*) tested topically on women's skin delivered the best efficacy in wrinkle reduction compared to other formulations and it exhibited a slight improvement in skin smoothness (Chuarienthong *et al*., 2010). However, it remains to be investigated whether or not rooibos was responsible for the anti-wrinkling effects that were

observed. Studies indicated that topically applied RE may inhibit tumour growth and might possibly be used together with other skin treatments to assist in the prevention of skin cancer (Joubert *et al.*, 2008; Marnewick *et al.*, 2005; Na *et al.*, 2004). Glynn (2009) discovered during clinical trials that topical application of combined botanical extracts containing rooibos helped to improve hair regrowth in male baldness. A patent exists which claims that a cosmetic preparation containing RE, in combination with protective agents, can be used to achieve exfoliation of the skin and to minimise adverse effects of estrogen deficiency on menopausal skin (Reinhart *et al*., 2006).

2.2.7.1.7 Stability considerations

A recent study proved that the duration of the extraction process and the extent of temperature variations during extraction of rooibos plant material had an effect on the quantity of the bioactive compounds in rooibos (Santos *et al.*, 2016). In another study, the effect of steam pasteurisation on rooibos caused a significant alteration in the soluble solids, total phenolic and aspalathin content. During the pasteurisation process, fermented rooibos leaves are steamed at 96 °C to reduce microbiological activity. It was suggested that chemical changes may be a result of aspalathin's instability at high temperatures (Koch *et al.*, 2013). A concern regarding the process of rooibos plant fermentation is the oxidation of aspalathin, which can in turn reduce the content of the phenolic composition in the final product (Joubert & De Beer, 2011; Snijman, 2007). In the food industry, De Beer *et al.* (2012) acknowledged that the amount of aspalathin reduced with increasing temperature and pH values during the production of iced teas infused with rooibos and that the addition of aspalathin-enriched RE may reduce this problem.

Research showed that unfermented rooibos tea or GRE exhibited more pronounced anti-oxidative activity than the traditional form. The development of 'green rooibos' increased in the international market and unfermented RE are typically included in nutraceutical and cosmeceutical products, as it contains higher concentrations of the marker molecule, aspalathin (Beelders, 2011; Joubert & Schulz, 2006; Snijman, 2007). During processing, the greatest challenge to prepare unfermented rooibos is to minimise the oxidative changes in order to contain the green leaf colour and aspalathin content (Beelders, 2011).

Like most plant products, rooibos plant material also contains high microbial load as the long fermentation times, open air exposure, high temperatures and moisture content during production provides ideal conditions for the growth of bacteria and moulds (Gouws *et al.*, 2014). Another concern involving product formulation and storage was raised in 1986, when Du Plessis and Roos (1986) found traces of *Escherichia coli* and *Salmonella enteritidis* during the fermentation process (Gouws *et al*., 2014). Improper storage conditions is another factor that can cause deterioration in the composition of rooibos containing products. For a product to

exhibit extended shelf-life, heat treatment is required, but heat together with extended storage time can cause a loss in the amount of phenolic compounds. To resolve this problem, ascorbic acid (anti-oxidant) and citric acid (acidifier) are added to rooibos iced teas to enhance the stability of aspalathin (De Beer *et al*., 2012).

A clinical study done by Chuarienthong *et al*. (2010) tested the anti-wrinkling effect of a topical formulation containing tea (*C. sinensis*) and RE, but the formula caused skin roughness and scaliness under participants. This may have been caused by the decomposition of flavonoids and a reduction of anti-oxidant efficacy despite the addition of an anti-oxidant to the product. Furthermore, it appeared as if the flavonoids had oxidised in the preparations as was evident in the change in colour of the product (Chuarienthong *et al.*, 2010).

2.3 Summary

Rooibos, a native plant product from South Africa, is commonly consumed in the form of a herbal tea, which has been reported in the literature to provide numerous health benefits. Many commercially available cosmetic products claim to contain RE with beneficial effects on the skin, without scientific results to prove what quantities of RE are contained in these products. Aspalathin is a flavonoid, which is only found in rooibos and has been proven to exhibit anti-oxidative effects. Aspalathin however, is not chemically stable and several factors (pH, temperature and exposure to light) may cause degradation of aspalathin and subsequently also a reduction in its anti-oxidative capacity. Researchers have recently developed unfermented GRE, which contains more aspalathin that may enhance the beneficial effects of rooibos.

Topical delivery may be achieved using a variety of different formulations to deliver active ingredients to the skin to achieve a desired effect. Gels are semisolid formulations that allow molecules to be dispersed in a hydro-alcoholic or aqueous liquid phase for topical application. The strong polymer network formed in gel formulations promotes a more stable product during excessive temperature fluctuations. Anti-oxidants are included in topical formulations to improve the chemical stability of substances in a product and anti-oxidants also improve skin function and appearance. Various formulations can be prepared with different additives, like anti-oxidants, to produce a stable topical formulation that retains the active ingredient content for an extended time period.

The cosmetics market is expanding and the development, evaluation and stability testing of cosmeceutical products are essential. Stability evaluation is important for cosmetic and pharmaceutical products containing active ingredients, especially with natural substances, as they are more prone to instability. Specific pharmaceutical aspects must be assessed to determine if a stable topical formulation containing RE has been formulated. Some of these

stability assessments, which may be performed on gel formulations include: content assay, pH, viscosity and visual appearance.

Apart from stability, release of the active ingredient from the vehicle is also an important consideration. Diffusion studies are performed to determine if an active ingredient is sufficiently released to the skin and it has been reported that aspalathin shows limited permeation across human abdominal skin. Research regarding the improvement in chemical stability of aspalathin in gel formulations and release of aspalathin from the gel vehicle is essential to help develop an ideal RE containing formulation, which may be applied to the skin.

CHAPTER 3: MATERIALS AND METHODS

3.1 Introduction

Rooibos tea is a popular beverage with potential health benefits, which led to research on rooibos in the nutraceutical industry. The inclusion of RE in cosmetic products is rising, but information in the literature regarding the stability of phytochemicals of RE (e.g. aspalathin) in these products is limited (Joubert & De Beer, 2011). Furthermore, very little information is available regarding suitable excipients, which may be included in gel formulations containing RE to improve the chemical and physical stability of the formulation. It was previously reported that aspalathin has a relatively low penetration into human skin and research into improving the release of aspalathin from topical RE gel formulations was therefore necessary (Huang *et al.,* 2008).

The research problem to be addressed in this study was to investigate the quality and stability of different extemporaneously prepared gel formulations containing RE and to evaluate the gel formulations with regards to specific pharmaceutical properties and also the rate and extent of aspalathin release from the gel formulations.

Preliminary studies were performed, which entailed the manufacturing and evaluation of RE gel formulations containing selected gelling agents in combination with various anti-oxidants. The preliminary studies showed that it was possible to manufacture RE containing gel formulations with favourable chemical and physical stability characteristics.

Based on the results of the preliminary studies, eight different RE gel formulations were prepared, each containing a different selected anti-oxidant system. The aim was to produce a topical formulation containing aspalathin-enriched RE that will remain stable for an extended period of time. To evaluate the stability, the gel formulations were subjected to specific stability storage conditions while their physical and chemical properties were regularly evaluated regarding aspalathin content, pH, viscosity (rheology) and visual appearance.

The *in vitro* aspalathin release potential (i.e. rate and extent) of the gel formulations were also evaluated using synthetic membranes mounted in a Sweetana-Grass diffusion chamber apparatus to determine the release of the active ingredient marker (aspalathin) from each gel formulation.

3.2 Materials

The materials used in this study for the formulation of gels, HPLC method of analysis and diffusion studies are listed in Table 3-1.

Table 3-1: Materials used in this study, supplier information and batch numbers

Ingredient	Supplier	Batch/LOT number
Materials used for formulation of gels:		
Deionised water	In house	No batch number
Asplathin-enriched RE	Agricultural Research Council (Stellenbosch, South Africa).	SB-1
Methylparaben	Clariant Produkte GmbH, Germany	BM1611711
Propylparaben	Clariant Produkte GmbH, Germany	BP15091411
Carbopol® 974	Noveon® (Cleveland, United States of America (USA))	CC77KAB716
Carbopol® 974P NF	Lubrizol (Kentucky, USA)	0102067942
Noveon®- AA 1	Noveon® (Cleveland, USA)	CC75LAW051
Avicel® RC/CL	FMC Corporation (Pennsylvania, USA)	ENO9820100
Propylene glycol	Associated Chemical Enterprises (ACE) (Johannesburg, South Africa)	35553
Triethanolamine	ACE Chemicals (Johannesburg, South Africa)	30066
Sodium acetate anhydrous	ACE Chemicals (Johannesburg, South Africa)	27652
Hydrochloric acid 32 % (v/v)	ACE Chemicals (Johannesburg, South Africa)	25204
Ethanol (industrial) 99.9 % (v/v)	ACE Chemicals (Johannesburg, South Africa)	35160
Sodium metabisulfite	UniLAB (SAARchem Pvt. Ltd.)	22881
Citric acid monohydrate	ACE Chemicals (Johannesburg, South Africa)	36114
Ascorbic acid	Rochelle Chemicals (Johannesburg, South Africa)	180416AC
L - ascorbic acid	ACE Chemicals (Johannesburg, South Africa)	33542

Table 3-1 (continued)

Ingredient	Supplier	Batch/LOT number
Sodium l-ascorbyl phosphate (vitamin C Stay-C 50®)	Chempure, DSM (Pretoria, South Africa)	0280593
Mettler Toledo pH calibration buffer solutions, InLab®	Microsep (Johannesburg, South Africa)	1D102B (pH 7.00) 1D162D (pH 4.01)
Materials used for HPLC:		
Aspalathin reference standard	HWI pharma services (Ruelzheim, Germany)	HWI00319
Acetic acid (glacial)	Sigma Aldrich, SAFC (Johannesburg, South Africa)	GLAA132-35
Acetonitrile (Chromatographic grade)	Merck (Johannesburg, South Africa)	I0910330735
HPLC grade water, MilliQ®	In house	No batch number
Materials used for diffusion studies:		
Dow Corning® high vacuum grease	Sigma Aldrich (Johannesburg, South Africa)	9121909
Sodium acetate anhydrous	ACE Chemicals (Johannesburg, South Africa)	27652
Acetic acid (glacial)	Sigma Aldrich, SAFC (Johannesburg, South Africa)	GLAA132-35
Ethanol (industrial) 99.9% (v/v)	ACE Chemicals (Johannesburg, South Africa)	35160
Synthetic membrane filters	Stargate scientific (Roodepoort, South Africa)	CD01U0612

3.3 High performance liquid chromatography validation

3.3.1 Introduction

In order to chemically characterise the aspalathin content in the aspalathin-enriched RE, an HPLC method was validated and employed. The method used was adapted from Beelders *et al*. (2012), Muller *et al.* (2012) and Walters *et al.* (2017), and is described below. Aspalathin reference standard was purchased and used to assist in the validation of the HPLC analytical method (Addendum B). Validation was done to ensure that the analytical method produced accurate and reliable results according to ICH validation requirements (ICH, 2005).

3.3.2 Chromatographic conditions

The chromatographic conditions of the validated analytical method for the analysis of aspalathin were as follow:

Table 3-2: Summary of the chromatographic conditions used to analyse the aspalathin content in experimental samples

Analytical instrument and specific parameters	Description
Analytical instrument	Hitachi® Chromaster (Figure 3-5) equipped with a 5410 UV detector, 5260 auto sampler and 5160 pump. Chromaster System Manager data acquisition and analysis software version 1.0
Column	Luna 5 µ (C18) 150 x 4.6 mm 5 micron (Phenomenex)
Mobile phase	Phase A: HPLC grade water and 2% (v/v) glacial acetic acid Phase B: Acetonitrile
Flow rate	1 ml/min
Injection volume	10 µl
Detection	UV absorbance at 288 nm
Retention stop time	45 min

The mobile phase consisted of two components and was applied by means of gradient elution (Table 3-3).

Table 3-3: Gradient conditions of the mobile phases used in the analytical method

Time (min)	% Mobile phase A (HPLC grade water and 2% (v/v) glacial acetic acid)	% Mobile phase B (Acetonitrile)
0	90	10
2	90	10
10	50	50
15	0	100
25	0	100
30	90	10
45	90	10

3.3.3 Validation parameters

3.3.3.1 Linearity

Linearity describes the ability of an analytical method to produce accurate response values from sample analysis, which are directly proportional to the magnitude of the analyte concentration in the samples within a specified concentration range. Calculation of the coefficient of determination (R^2) entails the measurement of a series of three to nine samples using a minimum of five concentrations. According to the literature, an R^2 value of more than 0.99 should be attained before an analytical method may be considered to be acceptable in terms of linearity (Shabir, 2003).

To determine the linearity of the HPLC method employed in this study, a concentration range of sample solutions were prepared by dissolving approximately 8 mg of aspalathin standard in 10 ml HPLC grade water. A volume of 1 ml of the standard solution was transferred to a 100 ml volumetric flask and made up to volume with HPLC grade water to prepare a stock solution of aspalathin. Volumes of 3, 4, 5, 8 and 9 ml were each transferred from the stock solution to 10 ml volumetric flasks and made up to volume with HPLC grade water in order to produce a series of aspalathin standard solutions. These dilutions yielded standard solutions with concentrations of 2.4, 3.2, 4.0, 6.4 and 7.2 µg/ml of aspalathin. The standard solutions were each transferred into amber HPLC auto-sampler vials and a volume of 10 µl of each dilution was injected into the HPLC in duplicate and the standard solution containing 6.4 µg/ml of aspalathin was injected six times, in order to provide more replicates (Shabir, 2005). The samples were analysed to obtain peak area values for each concentration in the specified range.

The measured peak area values of the standard solutions were then plotted as a function of aspalathin concentration, after which linear regression of the curve was performed using Microsoft Excel® software in order to determine the slope, *y*-intercept and R^2 value.

The slope and *y*-intercept values of the standard curve were used to calculate the magnitude of the aspalathin concentrations in the test samples from the chromatogram peak area values, which were obtained from the stability and diffusion studies (assays). The aspalathin concentration in the experimental samples was calculated by using Equation 3.1:

$$\text{Concentration in sample (µg/ml)} = \frac{\text{(peak area of sample - y-intercept)}}{\text{slope}} \qquad \textbf{Equation 3.1}$$

3.3.3.2 Accuracy and precision

Accuracy can be defined as the closeness of the results obtained from the analytical method to the true value. A minimum of nine determinations over three concentration levels are required to document accuracy. Accuracy is reported as a percentage recovery value based on the assay of a sample which contains a known amount of a specific analyte. A mean percentage recovery value between 90 to 110% of the theoretical value is considered to be adequate to prove that the analytical method is sufficiently accurate (Shabir, 2003).

Repeatability and precision of an analytical method is assessed over a specified range covering a minimum of nine determinations followed by the calculation of the mean percentage recovery, standard deviation (SD) and percentage relative standard deviation (RSD) values. During repeatability evaluations, the precision criteria should deliver an RSD value of ≤ 1% for instrument precision and ≤ 2% for intra-assay precision (Shabir, 2003).

Samples of aspalathin-enriched RE were prepared in concentrations according to ICH guidelines Q2 (R1) to determine accuracy and precision (ICH, 2005). Three solutions of RE were prepared by weighing 45.25, 45.22 and 45.26 mg, respectively, and dissolving it in three different 10 ml volumetric flasks with 50:50 ethanol and HPLC water as solvent. Each solution was filtered with a 0.45 µm nylon syringe filter (Anatech (Pty) Ltd) and transferred to HPLC vials. The solvent based (SB)-1 RE contained 18.44% (w/w) aspalathin (Muller *et al*., 2012). Thus, the three RE solutions contained 834.41, 833.86 and 834.59 µg/ml aspalathin, respectively. An aspalathin reference standard solution of 835 µg/ml, representative of the aspalathin concentration in the RE solutions, was also prepared. A volume of 10 µl of each of the three RE samples and the aspalathin reference standard solution were each injected five times and analysed with the HPLC method. The set of RE concentrations were analysed against the standard aspalathin regression curve.

3.3.3.3 Limit of quantification and limit of detection

The limit of quantification (LOQ) is defined as the minimum concentration of an analyte that can be determined and quantitated in a sample, whereas limit of detection (LOD) is the lowest concentration of an analyte which can be detected in the sample. The LOD can be determined by preparing samples via serial dilution followed by analysis of the samples until a point is reached where the analytical method can no longer detect evidence of the specific analyte in the diluted samples (Eurachem, 2014). The standard solutions that were used during the linearity evaluation, as described in section 3.3.3.1, were used. Further dilutions were made by taking 1 and 5 ml of the lowest concentration (2.4 µg/ml) used for linearity evaluation and separately diluting each solution to 10 ml with HPLC grade water. A volume of 10 µl of each

dilution (1.25 and 0.25 µg/ml) was injected in duplicate and analysed by means of the HPLC method described above. At each concentration, the SD of the resultant peak areas was calculated. The percentage (%) RSD was calculated in the concentration range from 0.25 to 8.35 µg/ml with Equation 3.2 and used to determine the LOD and LOQ values analytically (Singh, 2013).

$$\% \text{ RSD} = \frac{\text{standard deviation}}{\text{average of peak areas}} \qquad \textbf{Equation 3.2}$$

3.3.3.4 Specificity

To determine the specificity of an HPLC method, the instrument response should be evaluated in the presence of the pure analyte (aspalathin), compared to a mixture of other components in the experimental samples, which may potentially interfere with the analytical results (Singh, 2013). The specificity of the HPLC method was evaluated by using sample solutions of each ingredient used to prepare the gel formulations. The solutions included: propylene glycol, methyl- and propylparaben, citric acid, sodium metabisulfite, ascorbic acid, solvent used for RE (ascorbic acid and water), triethanolamine and an acetate buffer solution. The sample solutions were prepared, filtered and then analysed with the specific HPLC method. Another sample containing a placebo mixture (i.e. no RE) of the gel ingredients were also prepared and analysed. To determine the retention time of the aspalathin peak in the RE, a solution was prepared where RE was spiked with aspalathin standard. The chromatogram was inspected to check if the aspalathin peak retention time was the same in RE compared to the peak, which was obtained from the pure aspalathin standard solutions.

The acceptance criteria for specificity are that the excipients or any other components in the sample must not generate disturbances (peaks) or interfere with the analysis of the target analyte (Shabir, 2003). The chromatograms of the individual substances and placebo mixture were compared with chromatograms of aspalathin reference standard and RE solution and inspected to ensure that no peaks interferences were present near the retention time of the aspalathin peak. If an interfering peak was found at the same retention time, the delivered peak intensity of the substance should be small enough to not have an effect on the intensity of the aspalathin peak.

3.4 Formulation development process of topical gels containing aspalathin-enriched rooibos extract

3.4.1 Using aspalathin-enriched rooibos extract as an active ingredient

RE enriched with aspalathin (SB-1) from green plant material was obtained from Dr. C.J. Malherbe at the Agricultural Research Council (ARC) Post-Harvest and Agro-processing Technologies Division, Infruitec-Nietvoorbij (Stellenbosch, South Africa). The GRE was prepared according to a patented process (Gruner-Richter *et al.*, 2008). The process entailed the extraction of green rooibos plant material at room temperature with 80:20 ethanol and water mixture, filtration and vacuum drying. To reduce the chlorophyll content, the RE powder was extracted with ethyl acetate, filtered and vacuum-dried to render the SB-1 extract. The phenolic composition of SB-1 was determined and found to contain 18.44% (w/w) of aspalathin in the RE (Muller *et al*., 2012).

Aspalathin is a unique flavonoid found in RE and is regarded as water-soluble. However, the extraction process of SB-1 entailed solvent extraction and this made the extract poorly soluble in water (Gruner-Richter *et al.*, 2008; Muller *et al.*, 2012). Prior to formulation, a solubility study was performed to determine the best solvent for RE. The RE dissolved poorly in deionised water, even after ultrasonication (Figure 3-1 A). Usually heat treatment allows an active ingredient to dissolve more easily (Aulton, 2018), but as established from the literature study, aspalathin is unstable at high temperatures. Based on preliminary solubility studies (data not shown), ethanol was selected as co-solvent. The most suitable solvent for RE, that is still safe for topical application, was determined to be a 50:50 ethanol and deionised water mixture (Figure 3-1 B).

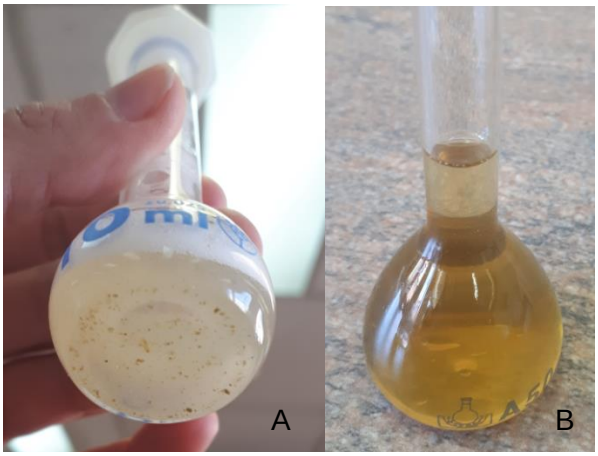

Figure 3-1: Photographs illustrating rooibos extract with (A) poor solubility in deionised water and (B) good solubility in a 50:50 water/ethanol mixture

3.4.2 Pre-formulation studies

After the most applicable solvent for RE was established, the topical formulation type had to be chosen. RE is sensitive to light and heat exposure and aspalathin degradation is initiated at temperatures ranging from 30 to 37 °C (Joubert *et al.*, 2008). Most topical semisolid dosage forms such as cream formulation methods require the use of high temperatures, and therefore it was decided to use a cold process system where no ingredients were subjected to any heat source during the formulation process in order to retain the most bioactivity of the active ingredient (Koch *et al.*, 2013). Furthermore, the formulation of creams requires the use of relatively large amounts of ethanol to help dissolve RE. When alcohol was incorporated into preliminary RE cream formulations, the emulsion underwent phase separation (Figure 3-2 A) and the RE migrated to the surface of the cream (Figure 3-2 B). Another type of semisolid dosage form had to be considered to formulate a stable topical product containing RE.

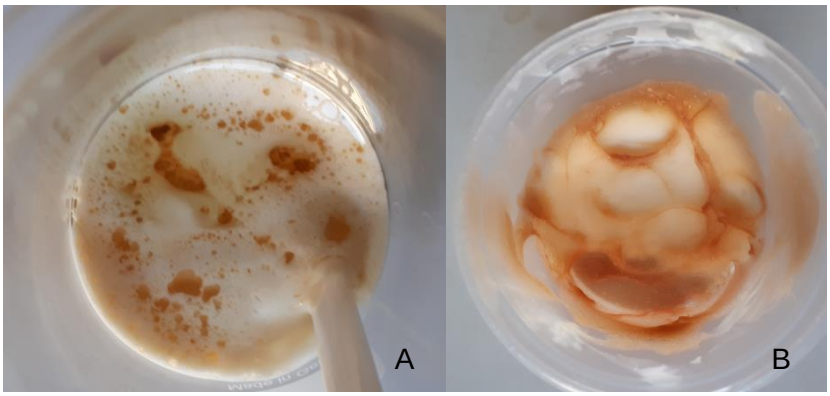

Figure 3-2: Photographs illustrating rooibos extract in creams indicating (A) phase separation when rooibos extract and ethanol was added and (B) migration of rooibos extract to the top of the cream

As described in chapter 2, section 2.2.3.4, topical gels can accommodate a large amount of water/ethanol in the water phase of the formulation and are compatible with several water-soluble excipients. Due to the large volume of solvent required to dissolve RE, it was more practical to formulate a gel preparation. Gels are able to retain their polymeric structure in the presence of ethanol and this allowed the formulation of a hydro-alcoholic gel. There is a wide variety of polymers available that can be used in gel formulations (Table 2-1) and a variety of these gelling agents were investigated in the pre-formulation study (data not shown) in order to select the best candidate for use in the RE gel formulations. Carbopol® 974P NF was chosen as gelling agent for the RE gel formulations as it delivered gels with a stable polymeric structure and favourable viscosity (or texture for easy application to the skin).

3.4.2.1 Pre-formulation considerations

For an anti-oxidant to be effective in a hydrogel formulation, it is a pre-requisite that the anti-oxidant must be sufficiently water soluble. A literature study was conducted and anti-oxidants were selected based on their anti-oxidative activity, aqueous solubility, compatibility with other ingredients and stability in a specific pH range (Rowe *et al.*, 2009). The following anti-oxidants were selected to be incorporated into the RE gel formulations in this study: ascorbic acid, citric acid and sodium metabisulfite.

Based on the information available in the literature, a topical formulation should be formulated and buffered to maintain a pH value between 4.5 and 6 to be considered safe to use on the skin (Lucero *et al.*, 1994). It is also known that aspalathin is chemically unstable and is prone to chemical degradation at pH values of 5 and higher (De Beer *et al.*, 2012; Human, 2019). Therefore, it was decided to formulate gels at a pH of 4.7 to ensure better chemical stability of aspalathin, while still remaining within the recommended pH safety limits for skin products (Lucero *et al.*, 1994). A combination of acetic acid and sodium acetate in water (Calbiochem®, 2006) was also incorporated in the gel formulation to act as a buffer to maintain the formulation's pH at 4.7.

The inclusion of certain excipients such as anti-oxidants, ethanol and propylene glycol may decrease the viscosity of a gel formulation. Apart from increasing the amount of Carbopol® 974P NF polymer in the RE gel formulations, it was also observed that the viscosity of the gels had in most cases increased to some extent when left to hydrate for one week. This occurrence may be attributed to the complete swelling of the polymer structure in the presence of water after a prolonged contact period (Neves *et al.*, 2009).

3.4.3 Preparation of preliminary gel formulations

To formulate a gel with sufficient RE content that is completely in solution, it was decided to formulate gels containing 0.3% (w/v) aspalathin-enriched RE. This concentration resulted in good RE solubility and it was possible to obtain a firm gel regardless of the high water/ethanol solvent phase.

Preliminary pilot gel formulations were prepared and selected based on their viscosity, texture, excipient solubility and visual appearance. For the pilot gel formulations, four different formulations (2000 ml) were prepared, each containing 0.3% (w/v) RE. The four preliminary gel formulations were subjected to specific storage conditions and chemical stability tests (assays) were performed for a period of six months. Pilot gels were formulated according to the ingredients and concentrations shown in Table 3-4.

Table 3-4: Composition of the four preliminary pilot gel formulations (% w/v)

	Gel 1	Gel 2	Gel 3	Gel 4
Gelling agent/viscosity enhancer:				
Carbopol® 974P NF	3		3	
Noveon® AA-1		1.5		1.5
Avicel® RC/CL		4.5		4.5
Anti-oxidants:				
Sodium metabisulfite	1	1		
Citric acid monohydrate			0.5	0.5
Ascorbic acid				0.5
Sodium l-ascorbyl phosphate		0.5	0.5	
Preservatives:				
Methylparaben	0.02	0.02	0.02	0.02
Propylparaben	0.02	0.02	0.02	0.02
Buffer and pH adjustment:				
Sodium acetate (anhydrous)	0.159	0.159	0.159	0.159
Acetic acid	0.175	0.175	0.175	0.175
Triethanolamine	0.8	0.95	0.8	1.5
Hydrochloric acid	0.075	0.55	0.00	0.35
Others:				
Rooibos extract (SB- 1 RE)	0.3	0.3	0.3	0.3
Solvent (50% water:ethanol)	50	50	50	50
Propylene glycol (solvent and humectant)	1.5	1.5	1.5	1.5
Deionised water (q.s.) to total:	100	100	100	100

3.4.3.1 Gel preparation

The preservatives were dissolved in propylene glycol and added to deionised water in a clean glass bowl. The respective anti-oxidants were weighed and mixed into the preservative solution with a Heidolph® overhead mechanical mixer RZR-2000 (Germany) stirrer at maximum speed (±570 rpm) until all components were dissolved. The gelling agent was then gradually added

and slowly mixed into the water phase until a gel was formed which contained no undissolved components when visually inspected. The gel was mixed on moderate speed (300 – 500 rpm) for approximately two hours to avoid the formation of lumps or air bubbles. To increase the pH of the gel to 4.7, triethanolamine was added and mixed slowly into the formulation, which produced a relatively clear and viscous gel (Saija *et al.*, 1998). A buffer solution was then prepared by mixing the buffer salt with water and acetic acid and subsequently incorporated into the gel while continuously stirring. The base gels were then covered with parafilm and one week was allowed for the gels to swell and hydrate completely (Figure 3-3) (Neves *et al.*, 2009).

After complete hydration of the gels, the RE was added to the formulations (Figure 3-4). RE was accurately weighed and added to the specified volume of a 50:50 mixture of deionised water and ethanol as specified in Table 3-4 while stirring until a solution was obtained that contained no undissolved RE upon visual inspection. The RE mixture was slowly added to the base gel formulations under moderate stirring. The gel was mixed for an hour and a half to ensure that the RE solution was distributed homogeneously throughout the gel formulation. Deionised water was used to rinse the remaining RE in the volumetric flask, which was also stirred into the gel. After mixing, each gel was left to stand for one hour to allow the ingredients to diffuse evenly in the gel structure, and for the pH to reach equilibrium. Thereafter, the pH of the gel formulation was measured with a Mettler Toledo SevenMulti™ pH/conductivity meter (Schwerzenbach, Switzerland). Minor pH adjustments were made where necessary by adding triethanolamine drop wise into the gel formulations until a pH of 4.7 was obtained. The correct amount of deionised water was added and mixed into the gel formulations to make each gel formulation up to a total volume of 2000 ml.

Figure 3-3: Photograph of the base gel before rooibos extract was added

Figure 3-4: Photographs of (A) rooibos extract in solution and (B) gel formulation after rooibos extract solution was added and mixed into the gel

3.4.4 Formulation of final topical gel formulations containing aspalathin-enriched rooibos extract for experimental purposes

For experimental purposes, seven different final gel formulations were prepared, all containing the same gelling agent (Carbopol® 974P NF), but in combination with different selected anti-oxidants at pre-determined concentrations. Three gel formulations contained each of the selected anti-oxidants alone and the other four gel formulations contained different combinations of the selected anti-oxidants (Table 3-6). A control gel formulation, which only contained RE (no anti-oxidant) was also prepared for reference purposes.

3.4.4.1 Composition of the experimental gel formulations

The ingredients that were used to prepare the experimental gel formulations (1000 ml of each) and the respective concentrations (% w/v) are provided in Table 3-5. The RE concentration was kept constant in all of the experimental gel formulations. The anti-oxidant concentration of each gel formulation is provided in Table 3-6.

Table 3-5: Composition of the experimental gel formulations

Ingredient	Function	% (w/v)
Carbopol® 974P NF	Polymer (gelling agent)	2.5
Methylparaben	Antimicrobial preservative	0.02
Propylparaben	Antimicrobial preservative	0.02
Propylene glycol	Solvent and humectant	1.5
Sodium acetate	Buffer salt	0.159
Acetic acid	Acid for buffer	0.175
Water (buffer)	Solvent	3.0
Triethanolamine	Alkali for pH adjustment / neutraliser	0.8
50% deionised water/ethanol	Solvent for active ingredient	50
Rooibos extract (SB-1)	Active ingredient	0.3
Deionised water	Solvent (vehicle)	q.s.

Table 3-6: Concentrations (% w/v) of the anti-oxidants in the experimental gel formulation

Anti-oxidants	Gel 1	Gel 2	Gel 3	Gel 4	Gel 5	Gel 6	Gel 7	Control gel
Sodium metabisulfite			1	1	1		1	0
Citric acid monohydrate		1			1	1	1	0
Ascorbic acid	1			1		1	1	0

3.4.4.2 Preparation of the experimental gel formulations

The same method as described in 3.4.3.1 regarding preparation of the preliminary pilot gel formulations, was employed to formulate the final experimental gel formulations. Additional triethanolamine was added, where required, to adjust the pH of the gel formulations to a value of 4.7.

3.5 Evaluation of experimental gel formulations

Immediately after each gel was prepared, the stability study commenced and the different parameters related to the aspalathin content, viscosity, pH and visual appearance of each gel formulation was measured every 30 days. For the stability studies, 2 000 ml of the initial four

pilot gel formulations were prepared according to the method described in section 3.4 and for the final experimental gel formulations, 1 000 ml of each of the seven different gel formulations as well as the control gel formulation were prepared.

3.5.1 Stability studies

For the purpose of stability testing, all the gel formulations were transferred to 125 ml glass containers. Each gel was divided into four equal parts and transferred to labelled containers for the respective assessments: assay, pH, viscosity and visual appearance. Each glass container was tightly closed with screw caps containing liners that are impermeable to water. The experimental gel formulations were subjected to stability storage conditions as described in section 2.2.4.3.1 and the temperature and humidity were maintained according to ICH Q1A guidelines (ICH, 2003). The specific storage stability conditions were as follow: A, long-term (25°C ± 2 °C/60 ± 5 % RH); B, intermediate (30°C ± 2°C/65 ± 5 % RH); and C accelerated (40°C ± 2°C/75 ± 5 % RH).

The experimental gel formulations were evaluated over a period of three months and were subjected to long-term (A) and accelerated storage conditions (C) (ICH, 2003). Stability assessments were performed once every month, and are described as T0 (initial value immediately after preparation), T1 (after 1 month), T2 (after 2 months) and T3 (after 3 months). The pilot gel formulations were subjected to long-term (A), intermediate (B) and accelerated (C) storage conditions. Chemical stability testing (assays) of the pilot gel formulations were performed monthly over a period of six months, including the initial (T0), T1, T2, T3, T4 (after 4 months), T5 (after 5 months) and month 6 (T6). The following assessments, as discussed below, were performed on the final experimental gel formulations every 30 days: chemical quantification (assay), pH, viscosity (rheology) and visual inspection.

3.5.1.1 Assay (quantification of aspalathin content)

As RE exhibited a tendency to migrate to the surface of the gel formulations, causing the upper layer in the container to be darker in colour than the rest of the gel, each gel was stirred thoroughly before samples were collected. A volume of 1 ml of each gel formulation, stored at each of the previously mentioned storage conditions, was withdrawn with a syringe and transferred into a 25 ml volumetric flask. Care was taken to avoid entrapment of air bubbles in the gel sample. Each volumetric flask was made up to volume with a 50:50 mixture of HPLC grade water and ethanol. Ascorbic acid (10% w/v) was added to the contents of the volumetric flask according to a previously published method (Muller *et al.*, 2012) to maintain stability of aspalathin during HPLC analysis. After the gel samples had completely dissolved in the

solvent, the sample solutions were filtered (0.45 µm syringe filter) and transferred into amber HPLC vials and analysed by means of the validated HPLC method described above.

Before the gel samples were analysed, a standard solution of RE was injected at 3 mg/ml (concentration of RE in the gel) and 0.12 mg/ml (concentration of RE in the diluted gel sample), which constituted of 553 and 22 µg/ml aspalathin, respectively. Chromatograms of the standard RE solutions were used to verify the retention time of the aspalathin peak in each gel assay during stability studies.

The aspalathin concentration in the gel formulations was calculated from the measured peak areas and resulting aspalathin standard regression curve, obtained from the HPLC method validation (Equation 3.1). The percentage aspalathin content in the samples that were obtained from the stability and diffusion studies was calculated as indicated below:

$$\% \text{ Content} = \frac{(\text{experimental value of aspalathin content})}{(\text{theoretical value of aspalathin content})} \times 100 \qquad \textbf{Equation 3.3}$$

Figure 3-5: Hitachi® Chromaster HPLC instrument

The percentage aspalathin content was plotted as a function of time for each gel formulation at the different storage conditions. Equation 3.4 was used to calculate the % reduction in aspalathin content in each gel for the respective time intervals, relative to the initial content (T0):

$$\% \text{ change} = \frac{(Tx-T0)}{T0} \times 100 \qquad \textbf{Equation 3.4}$$

Where T0 is the baseline measurement and Tx the measured aspalathin content at respective time intervals during the stability studies.

3.5.1.2 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 gel sample (Figure 3-6). The instrument was calibrated in all instances prior to pH measurement using Mettler Toledo pH buffer solutions to calibrate at pH 4.01 and 7.00. Starting point (T0) pH values were determined after preparation of the gel formulations. All pH measurements for each test sample at all test intervals were performed with four readings, and the average of the last triplicate measurements were taken. Between each pH measurement the previous gel sample was rinsed from the electrode with HPLC grade water and ethanol and dabbed dry using an absorbent paper towel.

To evaluate possible pH fluctuations in the experimental gels over time during the stability assessment, pH limits was set at 4.7 ± 0.3 (during stability studies). The SD and %RSD was also calculated with Equation 3.2 for each experimental gel at the respective months of stability assessment and specific storage conditions.

Figure 3-6: Photograph showing the Mettler Toledo SevenMulti™ pH meter used in this study

3.5.1.3 Viscosity and rheology

The viscosity and rheology of the experimental gel formulations were determined with a rheometer (ARES-G2; TA Instruments, New Castle, USA. Serial no: 4010-0677). A flow sweep test was performed in duplicate at 25 °C with stainless steel cone-and-plate geometry, with a diameter of 40 mm, a cone angle of 0.03521 rad and a trim gap offset of 0.05 mm (Terescenco *et al.*, 2018). Before sample load, the geometry was calibrated by allowing the cone-and-plate to reach a zero fixture. The gel sample was carefully loaded with a spatula at a loading gap of 25 mm and the truncation gap was set at 0.045 mm (Figure 3-7). After the geometry gap was reached, each sample was left to equilibrate for two minutes (min) prior to any measurements to allow the gel to recover to its original structure (Ozkan *et al.*, 2012). During flow sweep testing, the flow properties were obtained by recording shear stress (Pa) and viscosity values (Pa.s) at shear rates increasing from 0.01 to 100 s^{-1} (Smith, 2018). In between each measurement, the fixture and cone-and-plate geometry were cleaned thoroughly with deionised water and 70% (v/v) ethanol. Logarithmic sweep modes were run and ten points per decade were measured and displayed in Trios software (TA instruments, version 4.3.1).

Each set of data was exported to Microsoft Excel® software and the average of duplicate readings was used. Graphs were plotted for each gel of the viscosities as a function of shear rate over the stability test time intervals (T0 – T3) to determine if there was a change in the gels' viscosity. To compare the viscosities of the different gel formulations, their viscosities at minimum shear rate were tabulated for each month and the percentage change between T3 and T0 was calculated with Equation 3.4. Rheograms were also constructed where shear rate was plotted as a function of shear stress and the graphs were used to determine the flow behaviour of each gel formulation over time (T0 – T3).

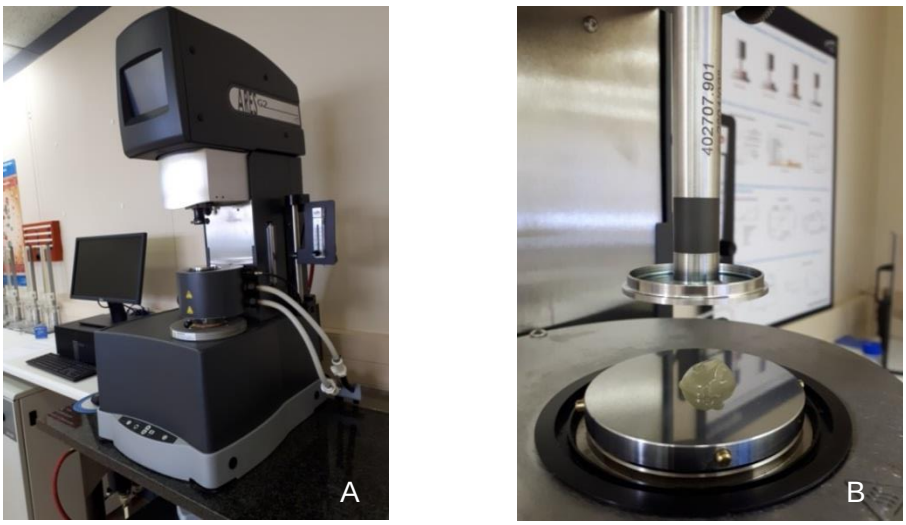

Figure 3-7: Photographs depicting (A) ARES-G2 rheometer and (B) a gel sample on the sample plate

3.5.1.4 Visual inspection

Macroscopic analyses were performed on gel samples in a controlled room with no natural light present. The gel containers were visually inspected and photographic images were taken of the front, back and top views of the gels. A Canon EOS 650 D (Canon Inc, Tokyo, Japan) digital camera was used. Each photo was taken with flashlight in order to keep the lighting constant. Paint colour charts were used to compare the colour change in the gels more objectively. Images following immediate formulation (T0) were compared to formulations after being subjected to the specified storage conditions and stability assessment time points.

3.6 Diffusion studies

3.6.1 *In vitro* assessment for aspalathin release across membranes using Sweetana Grass diffusion apparatus

For the purpose of measuring the rate and extent of aspalathin release from the RE gel formulations, synthetic polyvinylidene fluoride (PVDF) hydrophilic membrane filters (47 mm, 0.45 µm) were mounted between the half-cells of a Sweetana-Grass diffusion chamber apparatus (Grass & Sweetana, 1988) and all aspalathin release studies were performed in six-fold.

The diffusion chamber apparatus consisted of six chambers or cell blocks in between heating blocks in contact with the front and back surfaces of the chambers. The heating blocks are required to maintain the desired temperature with the aid of a heated water bath which circulates pre-heated water through the heating blocks. Each chamber consists of two half-cell

blocks containing metal pins in between where the membrane can be clamped into position (Figure 3-8). To allow maximum exposure of the test formulation (gel) to the membrane, gas flowed from a circular pathway into each half-cell which circulated the buffer through the chambers (Joubert, 2014).

Acetate buffer was used as receptor fluid for all diffusion studies. The pH was set to 4.7 and 25% (v/v) ethanol was added to allow the RE to dissolve in the fluid. Prior to the diffusion studies, the buffer was pre-heated in the water bath to 32 °C (temperature for skin) (SCCS, 2016). Each gel was also allowed to equilibrate to 32 °C before commencing with the release studies. The synthetic membrane filters were cut to fit on the 1.78 cm^2 surface area of the half-cell and mounted on the metal pins. To prevent any leakage, the circumference of each membrane was sealed with Dow Corning® high vacuum grease and the matching half-cell assembled and tightly clamped with circlips. The six assembled chambers, each with a donor and receptor cell, were mounted between the heating blocks of the apparatus (Figure 3-10). Each donor cell block was carefully filled with 7 ml pre-heated (32 °C) RE gel formulation until the gel made complete contact with the membrane surface (Figure 3-9). Thereafter, the receptor cell blocks were simultaneously filled with 7 ml pre-heated (32 °C) acetate buffer. Each chamber was supplied with gas, which flowed at a rate of 15 to 20 ml/min and consisted of 95% O_2 and 5% CO_2 (Legen *et al.*, 2005).

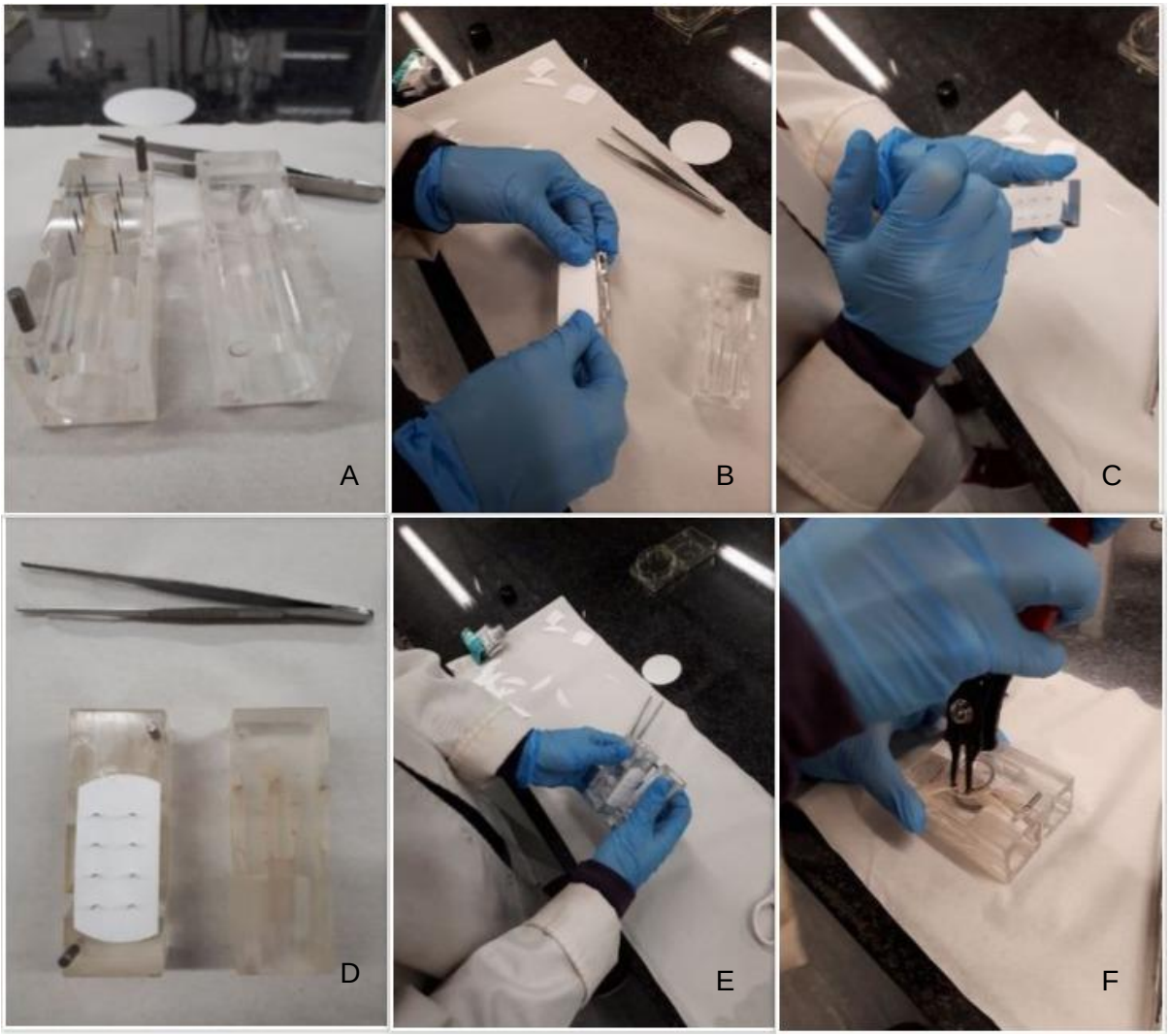

Figure 3-8: Photographs illustrating (A) the half cells with metal pins, (B-D) the process of mounting the synthetic membrane onto the half cells, (E) assembling the two half cells together and (F) adding circlips to hold the chamber together

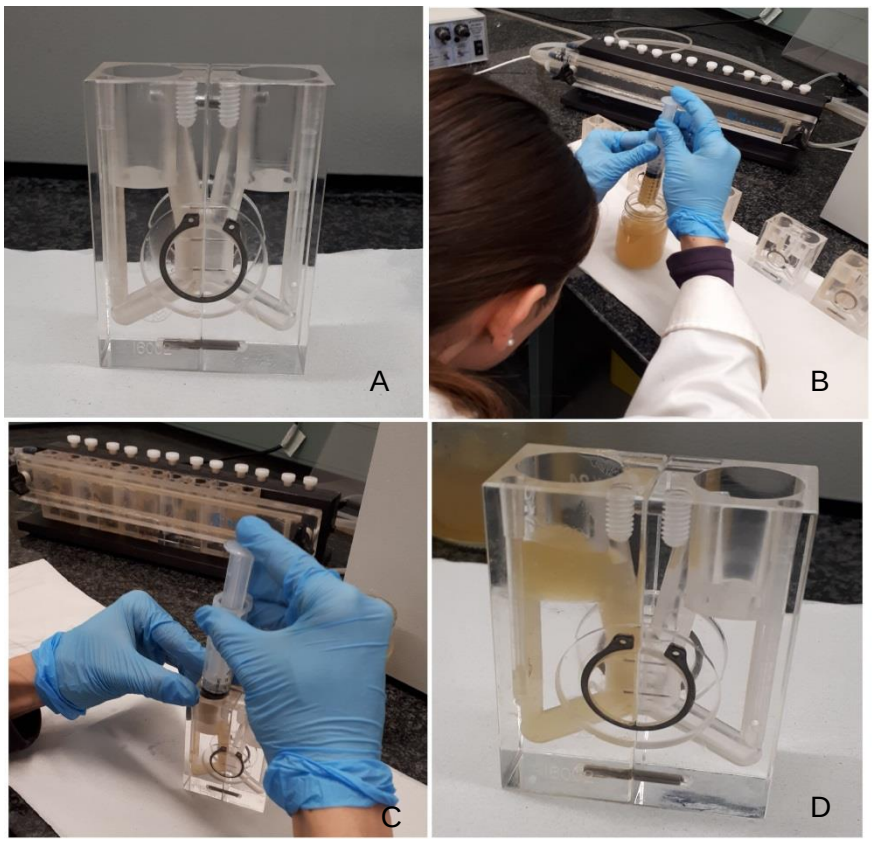

Figure 3-9: Photographs illustrating (A) assembled half-cells, (B) sample of gel being loaded, (C) gel injected into half-cell and (D) chamber loaded with gel

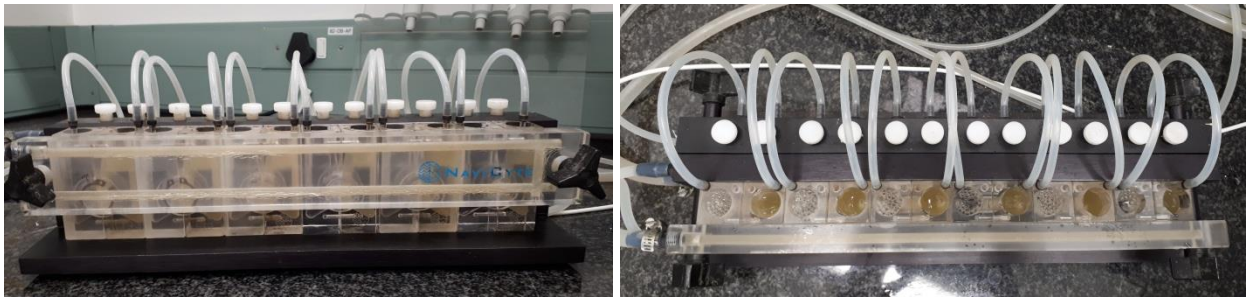

Figure 3-10: Photographs illustrating an assembled Sweetana-Grass diffusion apparatus loaded with gel and acetate buffer, connected to O_2/CO_2 supply and heating block

3.6.2 Analysis of samples

A volume of 1 ml of the acceptor chamber solution was sampled at 30 min and 1, 3, 5 and 6 hours (h). Immediately after sample withdrawal, 1 ml pre-heated (32 °C) acetate buffer was added to the acceptor cell to ensure that a constant volume and sink conditions was maintained (OECD, 2011). The collected samples were filtered (0.45 µm nylon syringe filter) and

transferred into HPLC auto-sampler vials and analysed with a validated HPLC method to determine the aspalathin content of the samples.

3.6.2.1 Data processing and statistical analysis

The aspalathin content of each experimental sample was determined with the validated HPLC method described earlier and then corrected for dilution and the average was calculated at each time interval for the six chambers. The resultant percentage aspalathin released from each gel formulation across the synthetic membrane was plotted as a function of time.

The apparent permeability coefficient (P_{app}) values for RE (aspalathin marker) were calculated to determine the release of aspalathin from each gel according to the following equation (Hellum & Nilsen, 2008):

$$P_{app} = \frac{dC}{dt}\left(\frac{1}{A.60.C_0}\right) \qquad \textbf{Equation 3.5}$$

Where P_{app} is the apparent permeability coefficient ($cm.s^{-1}$), $\frac{dC}{dt}$ is the permeability rate (amount of measured aspalathin permeated per min), A is the diffusion area of the membrane mounted between the half-cells (cm^2) and C_0 is the initial concentration (mg/ml) of aspalathin applied to the donor chamber.

Statistical analysis was performed on the experimental data obtained from the membrane release studies. An analysis of variance (ANOVA) test was performed to determine any statistical difference between the experimental data together with a 2-Way Table of Descriptive Statistics. A Brown-Forsythe Test of Homogeneity of Variances was performed to test the assumption of homogeneity of variances in the groups. A Tukey Honest Significant Difference (HSD) post hoc test was performed on the P_{app} values to identify statistical significance between the gel formulations. Data was deemed statistically significant when $p \leq 0.05$. All the statistical analyses data are depicted in Addendum I.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Introduction

Several cosmetic products are available on the market claiming to contain certain concentrations of RE, but the necessary quality control assays and scientific proof to confirm these claims are lacking. Aspalathin, the phytochemical marker found in RE, has been shown to be unstable in solutions, and yet there is limited research available regarding the chemical stability of aspalathin in RE topical products. In this study, different topical formulations (gels) were prepared containing aspalathin-enriched RE. A selection of anti-oxidants was incorporated in the formulations to aid in improving the stability of aspalathin in RE gel formulations. The gel formulations were then subjected to specific storage conditions to evaluate the stability of aspalathin in these formulations over time. Stability tests were performed at pre-determined time intervals and the assessment included aspalathin content assays and physical stability tests such as pH, viscosity (rheology) and visual inspection. Results from the stability studies were subsequently used to determine which formulation(s) performed the best in terms of chemical and physical stability.

Additionally, *in vitro* tests were also performed to determine the release of aspalathin from the experimental gel formulations. A Sweetana-Grass diffusion apparatus was used to evaluate the rate and extent of aspalathin release from the RE gel formulations across synthetic membranes.

4.2 High performance liquid chromatography validation

The chemical analysis of aspalathin concentration in the samples was performed by means of HPLC. The reproducibility and reliability of this analysis method was validated in terms of linearity, accuracy and precision, LOD, LOQ, and specificity.

4.2.1 Linearity

As described in chapter 3, section 3.3.3.1, a series of aspalathin reference standard concentrations was injected into the HPLC and analysed. The peak areas obtained for aspalathin from the resultant chromatograms are shown in Table 4-1.

Table 4-1: Mean chromatogram peak area values of aspalathin over a specified concentration range

Concentration aspalathin (µg/ml)	Mean peak area
7.2	127065
6.4	99731
4.0	50170
3.2	37628
2.4	17230

Figure 4-1 depicts the aspalathin standard curve of the measured mean peak area values as a function of aspalathin concentration with the straight line equation and coefficient of determination (R^2).

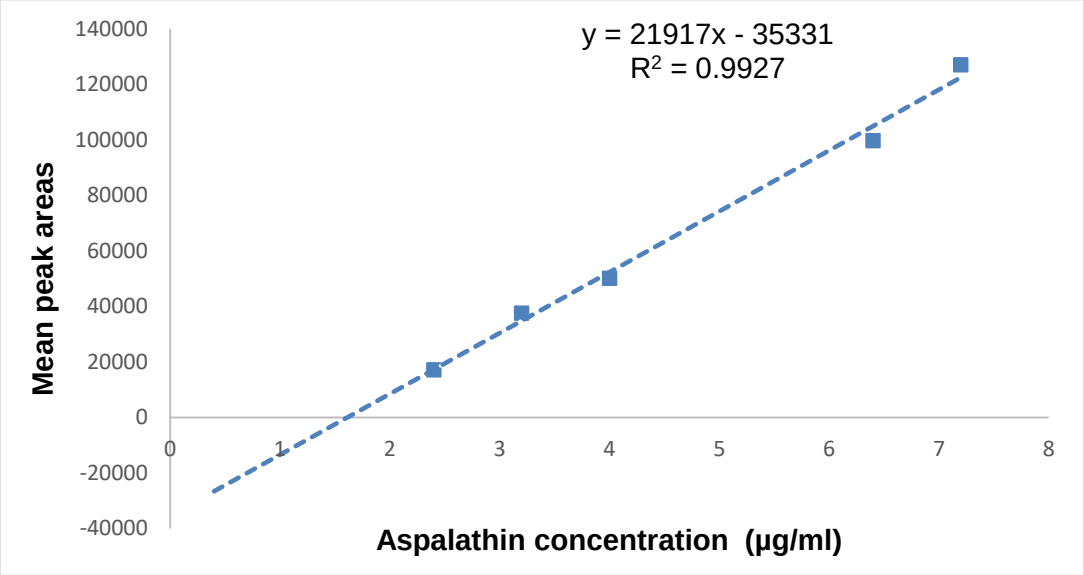

Figure 4-1: Standard curve of aspalathin with the straight line equation and coefficient of determination

From Figure 4-1 it can be seen that a linear relationship exists between aspalathin concentrations and peak area values, as measured with the HPLC. According to literature, a pharmaceutical product must have a $R^2 \geq 0.98$, and for samples from biological/natural origin, it must be ≥ 0.95 (Huber, 1999; Shabir, 2005). The aspalathin standard curve had an R^2-value of 0.9927 with a straight line equation of y = 21917x – 35331. From Table 4-1 and Figure 4-1, it is clear that the analytical method did meet the requirements in terms of linearity. The standard curve yielded an R^2-value ≥ 0.99, which is considered to be acceptable for a natural product (Shabir, 2005; Singh, 2013).

4.2.2 Accuracy

Table 4-2 provides information regarding the concentration range of RE and aspalathin reference standard with accuracy calculated as percentage recovery.

Table 4-2: Data obtained from sample analysis over a specified aspalathin concentration range in rooibos extract and aspalathin reference standard solutions to determine accuracy in terms of percentage recovery

	Rooibos extract (RE)			**Aspalathin**
Theoretical concentration RE (mg/ml)	4.525	4.522	4.526	N/A^
Theoretical concentration aspalathin (µg/ml)	834.41	833.86	834.59	835.00
Peak area values of four injections (10 µl)	14374278	14197144	13949542	12933689
	14128901	14159046	13890050	12930929
	14120650	14165926	13944483	12910173
	14130283	14160898	13936085	12925066
	14365804	14177696	13918753	12914598
Average peak area values	14188528	14170753	13930040	12924964
Experimental concentration aspalathin (µg/ml)	915.29	914.20	899.38	837.52
Accuracy (% recovery)	109.69	109.64	107.76	100.30
Mean % recovery	106.85			

N/A^, Not applicable

The analytical accuracy of the HPLC method regarding aspalathin detection over the specified concentration range delivered a percentage recovery value ranging between 100 and 109%. An analytical method is regarded to be accurate when the percentage recovery values are within the range of 100 ± 10% of the theoretical concentration. A mean percentage recovery of 106% was obtained for aspalathin, which confirms that the analytical method was accurate (Shabir, 2003).

4.2.3 Precision

To determine the precision of the analytical method, three solutions with different RE concentrations and an aspalathin reference standard solution were each injected four times. The average peak areas were used to calculate the SD and resultant %RSD values, which were used to determine the repeatability of the method. Data regarding the precision of the analytical method is presented in Table 4-3.

Table 4-3: Mean peak area values with standard deviation and percentage relative standard deviation (%RSD) values for a specified aspalathin concentration range

Aspalathin concentration (µg/ml)	Mean peak area	Standard deviation	%RSD
834.41	14188528	107306.04	0.76
833.86	14170753	15443.12	0.11
834.59	13930040	23583.20	0.17
835.00	12924964	9089.70	0.07
Average		38855.52	0.27

The analytical results presented in Table 4-3 confirm that the analytical method complied with the precision criteria based on the %RSD value of less than 2%. The average %RSD of 0.27 is a good indication that the method is precise and repeatable, as the %RSD should be ≤ 2% for intra-assay precision (Shabir, 2003).

4.2.4 Limit of quantification and limit of detection

The aspalathin reference standard solutions that were used for the linearity evaluations were diluted, as described in Chapter 3, section 3.3.3.3. The %RSD was calculated for the aspalathin concentration range, as shown in Table 4-4, to determine the LOD and LOQ of the combination of the instrument and analytical method (Singh, 2013).

Table 4-4: Percentage relative standard deviation (%RSD) for a specified aspalathin concentration range to determine the limit of detection (LOD) and limit of quantification (LOQ) for aspalathin

Aspalathin concentration (µg/ml)	%RSD
7.52	1.45
6.68	1.73
4.12	2.23
3.34	2.32
2.51	4.86
1.25	Not quantified
0.25	Not quantified

It is evident from the values in Table 4-4 that the %RSD increased as the aspalathin concentrations decreased. Aspalathin could not be quantified in the samples at concentrations of 0.25 and 1.25 µg/ml. The lowest concentration of aspalathin that could be analytically detected (LOD) was determined to be in a concentration range of 1.25 to 2.51 µg/ml. The LOQ was selected as the minimum concentration where the %RSD was still acceptable (< 3%) (Shabir, 2005), and thus the analytical LOQ of aspalathin was determined to be 3.34 µg/ml.

4.2.5 Specificity

Specificity evaluation of the analytical method with regards to the active ingredient (aspalathin) was conducted in accordance with the method in Chapter 3, section 3.3.3.4 and produced the chromatograms of RE and aspalathin reference standard depicted in Figures 4-2 and 4-3, respectively. To determine if the retention time of the aspalathin peak was the same in the RE sample and aspalathin reference standard, the RE sample was spiked with aspalathin reference standard (Figure 4-4). Sample solutions of other ingredients that were also used in the formulation and diffusion studies were also analysed separately. Another sample containing a placebo mixture of all the ingredients in the gel formulation was also analysed to inspect for any interfering peaks (Figure 4-5).

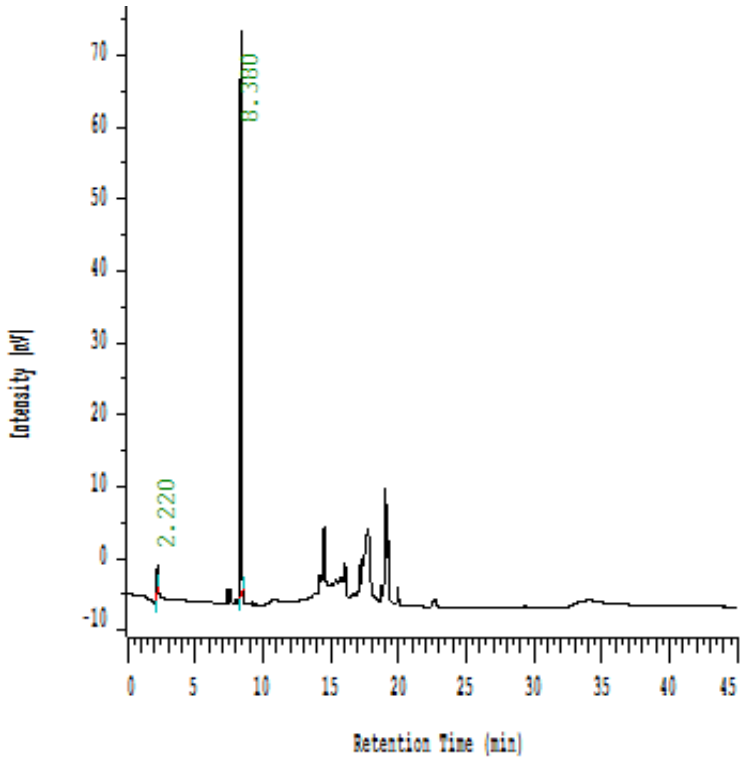

Figure 4-2: Chromatogram of rooibos extract sample with aspalathin peak at 8.3 min

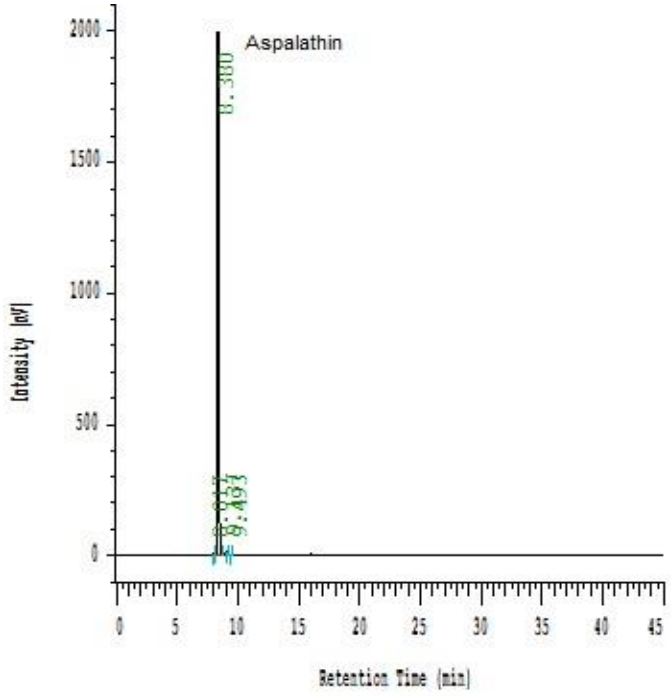

Figure 4-3: Chromatogram of aspalathin reference standard with a peak at 8.3 min

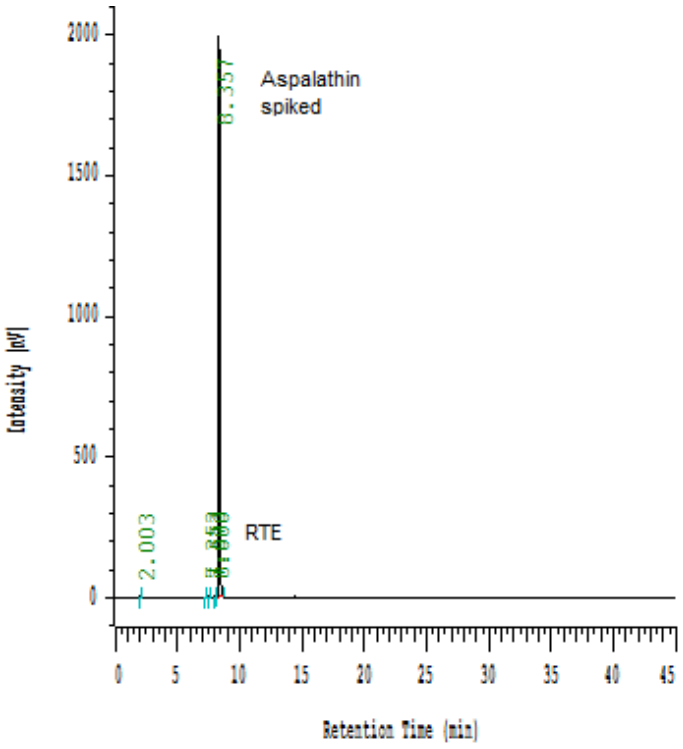

Figure 4-4: Rooibos extract spiked with aspalathin reference standard showing higher aspalathin peak intensity at the same retention time (8.3 min)

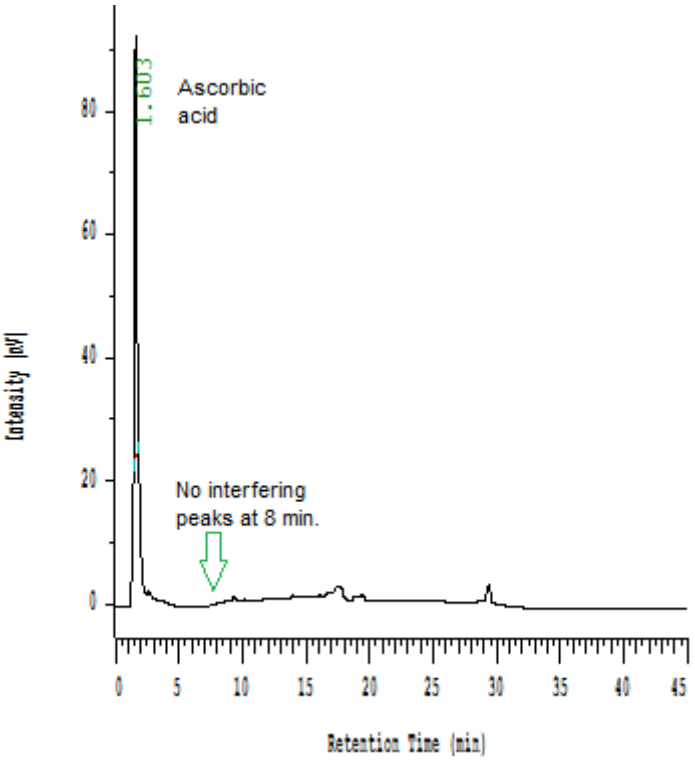

Figure 4-5: Chromatogram resulting from analysis of placebo mixture showing no interfering peaks at 8.3 min

The aspalathin reference standard was used to verify the aspalathin peak in the RE samples and as evident in Figures 4-2 and 4-3, it can be seen that both samples had clear discernible peaks at the same retention time (8.3 min). Even when RE was spiked with aspalathin, their peaks overlapped, causing a higher peak intensity compared to the RE sample that was not spiked with the aspalathin reference standard (Figure 4-4). Therefore, it can be confirmed that aspalathin yielded a peak at 8.3 min in the RE solution. In order to establish specificity of the analytical method, a mixture of all the ingredients in the gel formulation without the active ingredient (RE) was prepared and the chromatogram (Figure 4-5) was compared to that of aspalathin reference standard and RE solution. When chromatograms of each ingredient were inspected, it showed that the additional peaks were clearly separated from the aspalathin peak, and that no interference between other ingredient peaks and the aspalathin peak was generated (Figures C.1 – C.12 in Addendum C).

4.2.6 Validation summary

The HPLC analytical method complied with all the required validation criteria, including linearity, accuracy, precision, LOD, LOQ and specificity. Validation results confirmed that the analytical method was capable of producing accurate and reproducible results and that the method may be considered reliable to accurately quantify the aspalathin content in experimental samples of the RE gel formulations (assays) and diffusion studies.

4.3 Stability evaluation of gels

The gels were subjected to specific stability storage conditions as described in sections 2.2.6.1 and 3.5.1. Chemical and physical stability tests were performed on each gel formulation at pre-determined time intervals and experimental results are illustrated and discussed below.

4.3.1 Assay (quantification of aspalathin content)

In order to quantify the aspalathin content in the formulated RE containing gels, samples were analysed with HPLC and the percentage aspalathin content was calculated using Equation 3.3. The percentage aspalathin content for each preliminary pilot gel formulation, at the specific storage conditions and respective time intervals, is presented in Table 4-5. After T1, gel 2 appeared to be physically unstable and the gel had changed into a liquid and it was not feasible to continue with stability studies for this formulation (Figure D.1 in Addendum D).

Table 4-5: Percentage (%) aspalathin content for the different preliminary pilot gel formulations at each specific storage condition over the respective time intervals

		% Aspalathin content in pilot gel formulations over time						
Gel	**Storage***	**T0#**	**T1#**	**T2#**	**T3#**	**T4#**	**T5#**	**T6#**
1	**A**	132.31	119.14	110.13	95.87	88.61	43.85	30.94
1	**B**	132.31	99.66	82.01	100.28	78.04	35.94	26.74
1	**C**	132.31	87.73	61.84	58.59	31.91	17.25	13.30
2	**A**	124.21	127.31	N/A^				
2	**B**	124.21	99.68					
2	**C**	124.21	85.86					
3	**A**	102.26	102.89	111.83	107.74	93.80	42.74	35.22
3	**B**	102.26	99.96	152.90	99.48	76.97	30.85	0.00
3	**C**	102.26	92.84	71.55	66.19	37.21	18.17	15.87
4	**A**	79.75	111.04	87.87	69.34	65.60	25.49	18.17
4	**B**	79.75	84.40	73.58	67.16	59.43	20.44	0.00
4	**C**	79.75	105.36	70.00	42.52	21.11	17.67	0.00

*****Storage condition A = (25°C ± 2 °C/60 ± 5 % RH), B = (30°C ± 2°C/65 ± 5 % RH) and C = (40°C ± 2°C/75 ± 5 % RH). #T0 = initial time, T1 = after 1 month, T2 = after 2 months, T3 = after 3 months, T4 = after 4 months, T5 = after 5 months and T6 = after 6 months. N/A^, Not applicable

The assays of the pilot gel formulations showed a decline in aspalathin content over time for each gel formulation. The aspalathin content in all the gel formulations was also much lower when stored at higher temperature and humidity conditions (storage conditions B and C). However, some gel formulations yielded higher aspalathin content at T1 and/or T2, and it may possibly be attributed to upward migration of RE in the gel bottles (Figure D.2 in Addendum D) coupled with the sampling method. Samples were withdrawn from the gel surface where gel formulations appeared to be darker in colour and where the RE content may have been higher than in the underlying gel formulation, such as in the case of pilot gel 4 (Hashisaka *et al.*, 2004). To circumvent this scenario in the follow up studies, the gel formulations were stirred evenly before each sample collection.

At the end of storage time (T6), aspalathin could still be quantified in gel 1 (A, B and C), 3 (A and C) and 4A, with a maximum aspalathin content of 35% recorded in gel 3A. No aspalathin could be detected at the end of the stability studies in gel 4, which were stored at conditions B and C, or in gel 3 stored at conditions B. Aspalathin content was the highest in gel formulations

1 and 3 at the end of the stability studies. Both of these formulations contained Carbopol® 974P NF as gelling agent and based on the chemical stability and viscosity of gel formulations 1 and 3, it was decided to continue formulation of the experimental gels with the same polymer system.

The experimental gel formulations were prepared with the same quantity of gelling agent, but different selected anti-oxidants (either alone or in specific combinations) were also incorporated in the formulations. The percentage aspalathin content for the experimental gel formulations at the specific storage conditions and respective time intervals is presented in Table 4-6. The gel formulations were stored in stability chambers for three months and the percentage reduction in aspalathin content at each time interval, relative to T0, was calculated with Equation 3.4 and is depicted in Table 4-6.

Table 4-6: Percentage (%) aspalathin content for the different experimental gel formulations and specific storage conditions over the respective time intervals with the percentage reduction in aspalathin content at the time intervals, relative to the initial time (T0).

		% aspalathin content at specific time interval				% aspalathin reduction relative to T0		
Gel	**Storage***	**T0#**	**T1#**	**T2#**	**T3#**	**T0# – T1#**	**T0# – T2#**	**T0# – T3#**
1	**A**	107.92	95.37	35.67	22.27	11.63	66.95	79.36
1	**C**	107.92	80.14	23.68	14.23	25.74	78.06	86.81
2	**A**	112.82	105.14	44.33	24.27	6.81	60.71	78.49
2	**C**	112.82	81.52	34.95	15.10	27.74	69.02	86.62
3	**A**	93.74	68.83	40.67	22.93	26.57	56.61	75.54
3	**C**	93.74	47.48	20.06	11.47	49.35	78.60	87.76
4	**A**	102.71	60.36	40.93	25.55	41.23	60.15	75.12
4	**C**	102.71	23.94	23.47	13.32	76.69	77.15	87.03
5	**A**	110.25	57.11	34.25	25.57	48.20	68.93	76.81
5	**C**	110.25	52.24	30.53	14.09	52.62	72.31	87.22
6	**A**	107.70	52.47	29.96	21.96	51.28	72.18	79.61
6	**C**	107.70	47.34	25.93	18.41	56.04	75.92	82.91
7	**A**	108.20	57.34	35.14	26.77	47.01	67.52	75.26
7	**C**	108.20	47.79	33.61	22.15	55.83	68.94	79.53
Control	**A**	101.02	42.13	0.00	0.00	58.30	100.00	100.00
Control	**C**	101.02	31.66	18.61	0.00	68.66	81.58	100.00

*Storage condition A = (25°C ± 2 °C/60 ± 5 % RH) and C = (40°C ± 2°C/75 ± 5 % RH). #T1 = after 1 month, T2 = after 2 months and T3 = after 3 months

Gel (G) formulations stored under long-term conditions (storage A) that presented with the highest aspalathin content at the end of the stability study (T3) were in the following order: G7A (ascorbic acid, citric acid and sodium metabisulfite) > G5A (citric acid and sodium metabisulfite) > G4A (ascorbic acid and sodium metabisulfite) > G2A (citric acid) > G3A (sodium metabisulfite) > G1A (ascorbic acid) > G6A (ascorbic acid and citric acid) > Control GA (no anti-oxidants). These results demonstrated that the formulation containing a combination of all three anti-oxidants (G7) was the most effective system to ensure chemical stability of aspalathin in the gel formulations. Stability was also improved with the use of sodium metabisulfite as anti-oxidant in

the combination gel formulations (G7, 5, 4) and alone (G3). This is in line with a study performed by Franklin and Myrdal (2015), where greater improvement in the stability of a natural flavonoid was achieved when using sodium metabisulfite instead of ascorbic acid as anti-oxidant. The RE gel formulations containing ascorbic- and citric acid did not perform well, but aspalathin stability was still improved when citric acid was used solely as an anti-oxidant (G2), compared to a previous study (De Beer *et al.*, 2012). A pronounced reduction in aspalathin content was evident in the control gel, which contained no anti-oxidants and no aspalathin could be detected in the gel at the end of stability studies.

Gel formulations stored under accelerated conditions (storage C) performed in the following order: G7C (ascorbic acid, citric acid and sodium metabisulfite) > G6C (ascorbic acid and citric acid) > G2C (citric acid) > G1C (ascorbic acid) > G5C (citric acid and sodium metabisulfite) > G4C (ascorbic acid and sodium metabisulfite) > G3C (sodium metabisulfite) > Control GC (no anti-oxidants) in terms of aspalathin content. Both in long-term (A) and accelerated (C) storage conditions, the combination gel (G7) containing all of the anti-oxidants exhibited the best chemical stability of aspalathin. When comparing results from the specific storage conditions, gel formulations with sodium metabisulfite exhibited less chemical stability at higher temperature and humidity storage conditions (40 °C ± 2 °C/75 ± 5 % RH). Gel formulations containing ascorbic acid and citric acid (G6, 2, 1) yielded higher aspalathin stability, as opposed to the same formulations stored under long-term conditions (25 °C ± 2 °C/60 ± 5 % RH). The use of ascorbic- and citric acid has been reported to assist in the preservation of phenolic compounds, such as aspalathin, in RE solutions (De Beer *et al.*, 2012). Complete aspalathin degradation had occurred in control gel C and it can therefore be concluded that the addition of anti-oxidants had most likely improved the chemical stability of RE containing gel formulations.

According to Aulton (2018), an acceptable limit for active ingredient content in pharmaceutical products after stability evaluation is generally 90% of the labelled content. ICH stability testing guidelines (ICH, 2003) describe a significant change as a 5% loss in content of the active ingredient, but the WHO (2009) suggests that other values may be applied for herbal preparations. From the results in Tables 4-5 and 4-6, the content assays of aspalathin in all the gel formulations showed a pronounced reduction in aspalathin content over time, which were higher than the limit value suggested by the ICH guidelines.

The percentage aspalathin (% Asp) content was plotted as a function of time during stability testing, while subjected to specific storage conditions (Figures 4-6 and 4-7). The results demonstrated a decrease in aspalathin content for each gel formulation at the specific storage conditions. The aspalathin content had decreased in all RE containing gel formulations over time at both of the storage conditions at which gel formulations were tested (25 °C ± 2°C/60 ± 5 % RH and 40 °C ± 2 °C/75 ± 5 % RH). This was expected when taking into

consideration the instability of aspalathin at higher temperatures (De Beer *et al.*, 2012; Joubert *et al.*, 2009).

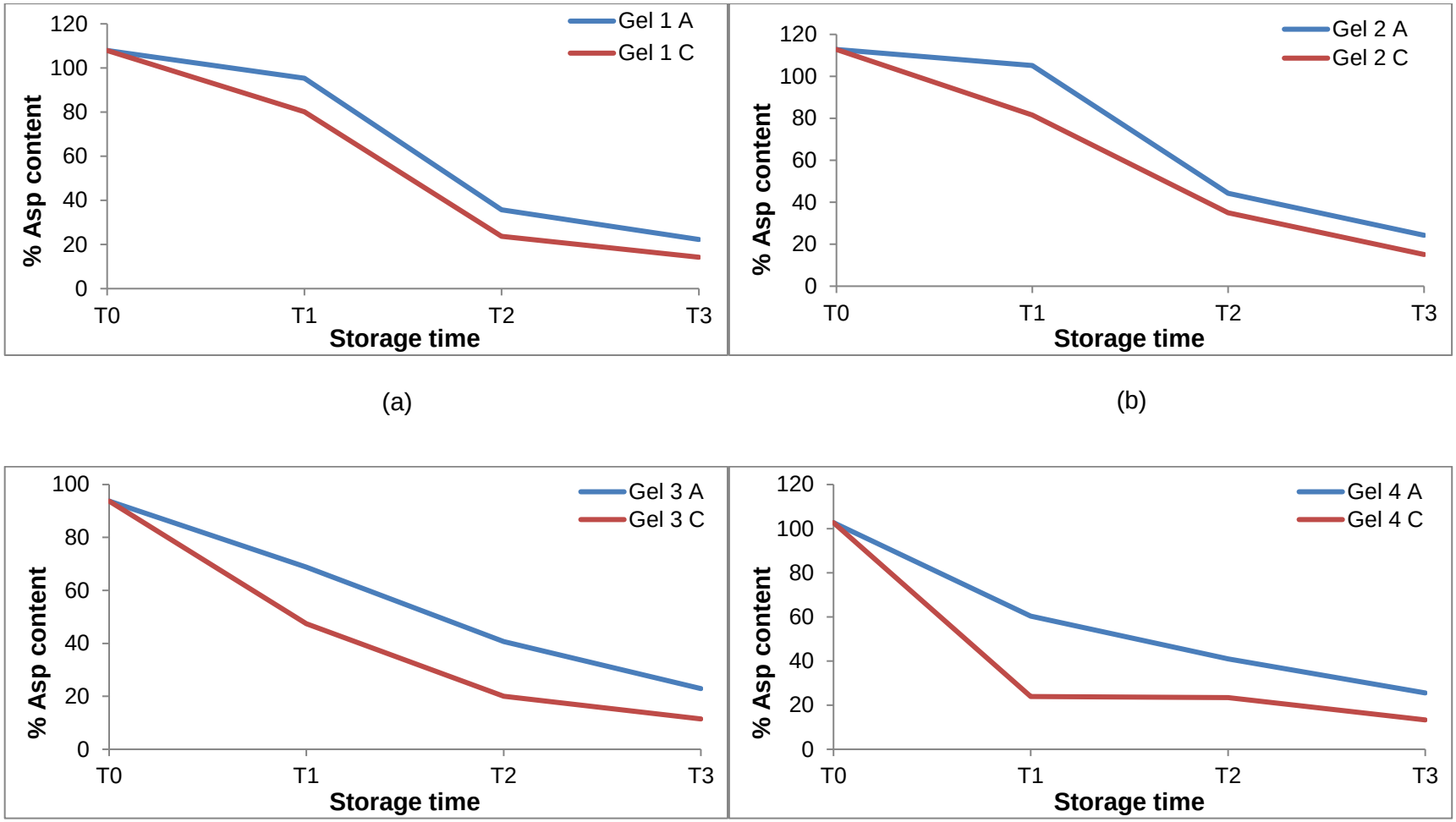

Figure 4-6: Decrease in the percentage aspalathin content over time for (a) gel 1, (b) gel 2, (c) gel 3, and (d) gel 4 at long-term (A) and accelerated (C) storage conditions

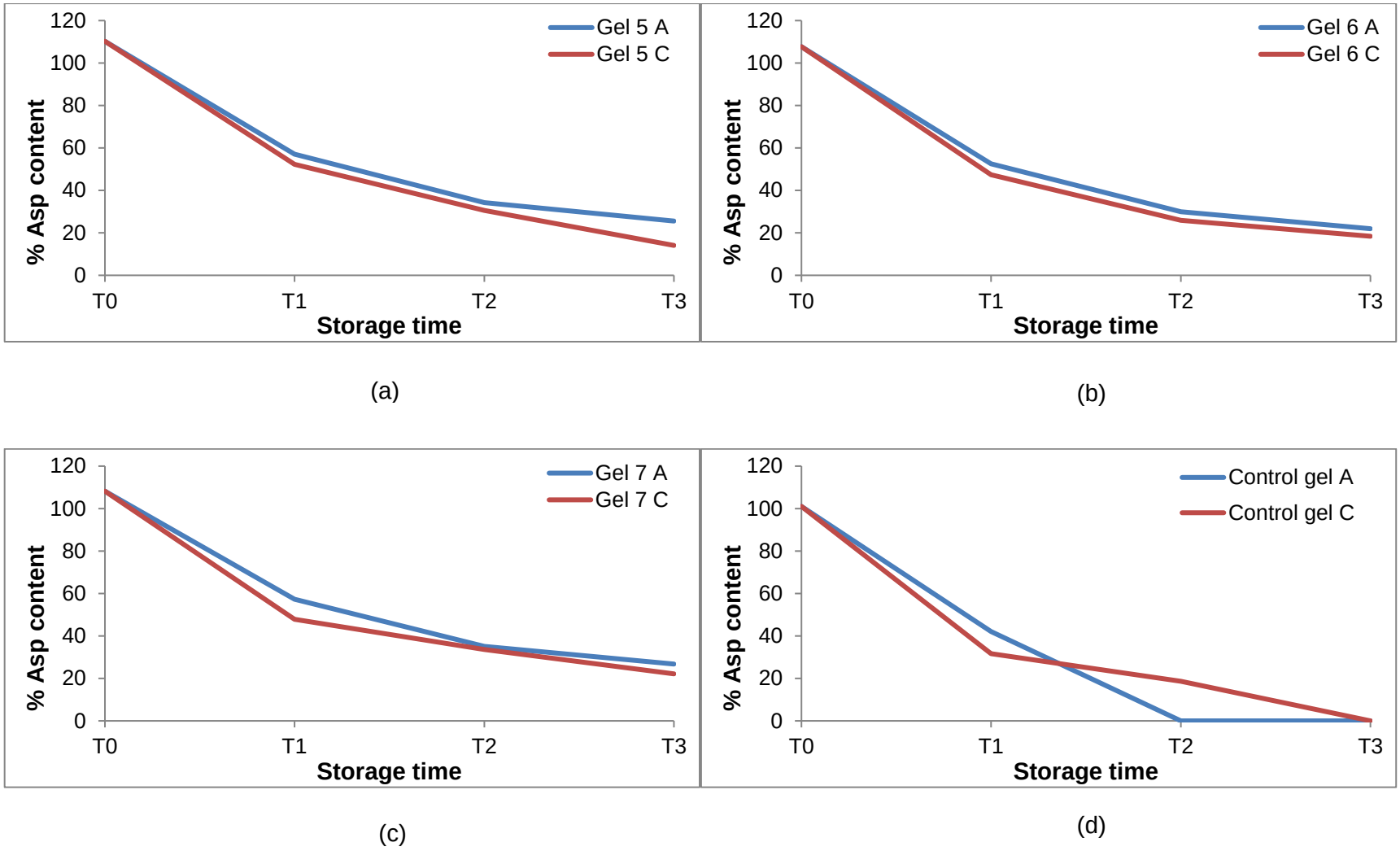

Figure 4-7: Decrease in the percentage aspalathin content over time for (a) gel 5, (b) gel 6, (c) gel 7, and (d) the control gel at long-term (A) and accelerated (C) storage conditions

From Figures 4-6 and 4-7 and Table 4-6, it can be observed that the aspalathin content had gradually declined in all of the experimental gel formulations at the specific storage conditions between time (T0) and month 3 (T3). The extent of the decrease in aspalathin content was more pronounced when the gel formulations were subjected to accelerated storage conditions (C). When considering the percentage reduction in aspalathin content (Table 4-6) for the different anti-oxidant gel formulations over time, it can be seen that there was a notable difference between the gel formulations when compared at time point T1, where the extent of aspalathin reduction ranged between 6.8 and 76.7%. At time point T2, the trend in aspalathin reduction continued and the percentage content change ranged between 56.6 and 78.1% in the different gel formulations. At the end of the stability study (T3), the difference in the gel formulations was small, with regards to the percentage reduction values of aspalathin which ranged between 75.1 and 87.8%. As expected, the control gel did not contain any aspalathin (concentration below LOD value) at T2A and T3C, due to the absence of anti-oxidants in the formulation (Human, 2019; Sheraz *et al.*, 2011).

The most likely cause of aspalathin degradation may be attributed to deterioration of phenolic compounds in RE, which in turn possibly leads to a reduction in anti-oxidative capacity of aspalathin (Deuschle *et al.*, 2015). Ascorbic acid is known to protect other compounds against oxidation by reacting with potential ROS. However, it is well known that chemical instability of ascorbic acid may be problematic when incorporated into aqueous solutions (Sheraz *et al.*, 2011). The chemical stability of ascorbic acid may further be compromised by other factors such as exposure to light, temperature fluctuations and exposure to oxygen and/or metal ions (Koop *et al.*, 2009). Previous studies have reported browning of formulations containing RE during storage, despite the addition of ascorbic acid to aid in aspalathin stability, and the browning may be attributed to oxidation of aspalathin and other constituents which are susceptible to oxidation (De Beer *et al.*, 2012). Brown polymers form during high temperatures as a result of oxidative changes in the phenolic composition in RE. From the visual inspection (Table 4-9), it is apparent that browning of the gel formulations had occurred after storage during stability testing, and the browning may be indicative of aspalathin oxidation (Human, 2019). It was also previously reported that aspalathin formed polymeric substances as a result of reacting with other flavonoids in extracts, such as orientin and iso-orientin, and that aspalathin may be converted to flavanones due to oxidation (Joubert *et al.*, 2009; Joubert *et al.*, 2010; Krafczyk & Glomb, 2008; Walters *et al.*, 2017).

Limited studies are available regarding the stability of aspalathin in cosmetic or topical products containing RE. In a study performed by Chuarienthong *et al.* (2010), decomposition of flavonoids in RE gel preparations occurred and it was attributed to a reduction in the anti-oxidant efficacy of flavonoids in the RE, even though an anti-oxidant (sodium metabisulfite) was

present in the formulations. However, their results lacked chemical stability data regarding aspalathin or other constituents, which may have been present in RE. Huang *et al.* (2008) determined the aspalathin content in two commercially available RE cream products by means of HPLC analysis, but the concentrations were below the LOD, which suggested that the aspalathin content was very low and would not have elicited any of the claimed effects on the skin. Therefore, further research regarding chemical stability improvement of RE, specifically aspalathin in topical formulations is of great importance for the consumer.

Given the known instability of aspalathin in topical preparations as described in the literature, the gel formulations prepared in this study containing aspalathin-enriched RE together with selected anti-oxidants delivered promising results. Even though the aspalathin content had decreased over time, a relatively fair amount of aspalathin was still present in the topical gel formulations at time T3 when stored under specific stability storage conditions. The results of the content assays of the experimental gel formulations demonstrated an improvement in aspalathin stability (based on aspalathin content at T3) when compared to the control gel formulation and also to previously published studies, which reported severe or even complete degradation of aspalathin in aqueous based formulations (De Beer *et al.*, 2012). In some of the aspalathin-enriched RE pilot gel formulations in this study, aspalathin was still present in quantifiable amounts after six months of storage (Table 4-5). Varying amounts of aspalathin could also be quantified in all experimental gel formulations containing anti-oxidants at the T3 time period (i.e. the end of the stability studies). These results therefore provided substantial evidence that the chemical stability of aspalathin could be improved in topical gel formulation systems with the use of anti-oxidants, especially when used together in combination (Jacobs *et al.*, 2016). This is in agreement with previously published studies, which reported that the addition of ascorbic- and citric acid to RE iced teas improved aspalathin stability up to 12 weeks (De Beer *et al.*, 2012; Joubert *et al.*, 2010).

4.3.2 pH

From the literature search it was also evident that aspalathin stability is very sensitive to pH and that its chemical stability may be compromised in formulations with pH values higher than 5. For example, aspalathin content decreased in RE iced tea formulations with an increase in pH (De Beer *et al.*, 2012; Joubert *et al.*, 2010). It has also recently been reported that the extent of aspalathin degradation (measured over a period of 120 min) was much lower in aqueous solutions with pH values of 2 (13.2% degradation) compared to solutions with pH values of 7 (58.6% degradation) (Human, 2019). Deprotonation and subsequent oxidation of flavonoids in RE occurs more easily at higher pH values (> 7) than at lower pH values (< 5) (Joubert *et al.*, 2009). Greater aspalathin stability may be achieved at lower pH values due to protonation of OH groups (Joubert *et al.*, 2010). At lower pH values the weakly acidic phenolic

compounds will be fully protonated and result in greater aspalathin stability (De Beer *et al.*, 2015). The pH of the aspalathin-enriched RE gel formulations were buffered and adjusted to a target pH of 4.7, which provided a more stable environment for aspalathin and is also more representative of the pH of the skin.

The pH of the experimental gel formulations prepared in this study was tested monthly as part of the physical stability evaluation. The average pH readings of each gel formulation at the respective time intervals and specific storage conditions (A and C), together with the SD and %RSD values calculated for each gel formulation over the entire stability study and specific storage conditions, are presented in Table 4-7. A comprehensive table of all the pH measurements, SD and %RSD values after each month and specific storage condition are provided in Table E.1 (Addendum E).

Table 4-7: Average pH values of the different gel formulations at the respective time intervals stored under specific storage conditions.

Storage*	Gel 1	Gel 2	Gel 3	Gel 4	Gel 5	Gel 6	Gel 7	Control
T0/ Initial time								
A / C	4.72	4.65	5.03	4.94	4.79	4.54	4.69	4.93
T1/ Month 1								
A	4.83	4.80	5.17	5.08	4.93	4.72	4.86	5.09
C	4.84	4.83	5.18	5.10	4.91	4.74	4.79	5.12
T2/ Month 2								
A	4.67	4.72	4.99	4.96	4.78	4.60	4.79	5.07
C	4.61	4.64	5.01	4.93	4.80	4.65	4.79	5.07
T3/ Month 3								
A	4.50	4.49	4.87	4.72	4.65	4.41	4.53	4.77
C	4.53	4.58	4.81	4.81	4.70	4.46	4.53	4.90
SD	0.12	0.11	0.13	0.13	0.09	0.12	0.12	0.12
%RSD	2.67	2.38	2.56	2.55	1.96	2.54	2.62	2.39

*Storage condition A = (25 °C ± 2 °C/60 ± 5 % RH) and C = (40 °C ± 2°C/75 ± 5 % RH).

The pH of all gel formulations remained in a pH range of 4.4 to 5.1 and is considered to be acceptable for topical application (Ansari; 2009; Lambers *et al.*, 2006). Guidelines from the Organisation for Economic Co-operation and Development (OECD) (2014) suggest that no skin corrosion will take place with the use of formulations having pH values ranging between 4 and

8. An acceptable range for a topical formulation to avoid skin irritation is between 4 and 6 (Lucero *et al*., 1994). The measured pH values of the RE gel formulations were within the acceptable range and will most likely not elicit skin irritation due to the pH (Xavier-Santos *et al.*, 2018).

All of the gel formulations exhibited a slight decrease in pH values at the final storage time (T3). It was evident that anti-oxidants played a role in the pH of the gel formulations, because the inclusion of ascorbic acid and citric acid produced gel formulations (G1, 2, 6) with slightly lower pH values than the other gel formulations.

When comparing the baseline pH measurements (T0) with pH readings over time, there was no pronounced change in the pH for all the test samples at the specific storage conditions. The average pH values of all the experimental gel formulations remained within the pre-determined acceptable range and did not fluctuate by more than ± 0.3 pH units. Although there was a small variation in pH over the test period, the buffer system that was included in the gel formulations was able to keep the pH stable at a pH of approximately 4.7 during the period of stability assessment. The small %RSD values (< 3%) as well as the small variation in pH of 4.7 ± 0.3 for each gel formulation at both storage conditions during the stability studies indicated that the pH of all the gel formulations remained stable over time.

4.3.3 Viscosity and rheology

Viscosity of the experimental gel formulations were determined at the minimum shear rate measurement (0.01 s^{-1}) that provides an indication of the gels' viscosity on the shelf (Shukr & Metwally, 2013). Table 4-8 provides the average viscosity values measured for each gel formulation while under the specific storage conditions at time T0 – T3. The percentage change in viscosity between T0 and T3 is presented in the column on the right in Table 4.8.

Table 4-8: Viscosity values (Pa.s) of the experimental gel formulations over time while under specific storage conditions with the percentage (%) change in viscosity between the initial (T0) and final month (T3).

Gels	T0[#]	T1[#]	T2[#]	T3[#]	% change (T0[#] − T3[#])
G1A*	628.49	351.86	245.82	259.36	-58,73
G1C*	628.49	132.94	234.65	273.11	-56,55
G2A*	646.87	667.99	618.04	650.74	0,60
G2C*	646.87	729.03	593.47	682.77	5,55
G3A*	680.68	683.32	679.18	660.66	-2,94
G3C*	680.68	714.41	686.48	704.89	3,56
G4A*	647.56	646.21	633.43	646.95	-0,09
G4C*	647.56	628.00	619.33	644.50	-0,47
G5A*	555.68	535.19	500.03	554.88	-0,14
G5C*	555.68	550.44	558.14	490.59	-11,71
G6A*	491.31	410.62	403.63	477.48	-2,81
G6C*	491.31	374.43	212.13	321.02	-34,66
G7A*	508.59	495.37	484.53	521.27	2,49
G7C*	508.59	545.42	535.51	488.80	-3,89
Control GA*	605.53	758.46	702.70	795.34	31,35
Control GC*	605.53	728.52	683.68	760.22	25,55

*****Storage condition A = (25°C ± 2 °C/60 ± 5 % RH) and C = (40°C ± 2°C/75 ± 5 % RH), with [#]T1 = after 1 month and T2 = after 2 months and T3 = after 3 months

Even though the gel formulations contained the same concentration of gelling agent, their initial viscosities varied and ranged between 491 and 680 Pa.s. The addition of plant extracts to topical formulations have previously been shown to decrease viscosity (Di Mambro & Fonseca, 2005). Results from a previously published study reported that the addition of anti-oxidants significantly affected the viscosity in food samples when compared to a control sample containing no anti-oxidants. It was suggested that the anti-oxidants had interfered with the protein network formation and caused a decrease in gel strength (Pereira *et al.*, 2016). This result is further supported by evidence from another study which demonstrated weaker gel rheology when ascorbic acid was added to a hydrogel, which was possibly caused by a reduction in intermolecular interactions (Koop *et al.*, 2009). Furthermore, a decrease in viscosity has also been reported with the incorporation of sodium sulfite as anti-oxidant into a

green tea extract gel (Saib, 2010). The most probable explanation for the small variation in the initial viscosities of the gel formulations could most likely be attributed to the different selected anti-oxidants that were used in this study.

The average viscosities (Visc.) measured as a function of shear rate were plotted for each gel formulation at the specific storage conditions and respective time intervals (Figures 4-8 and 4-9).

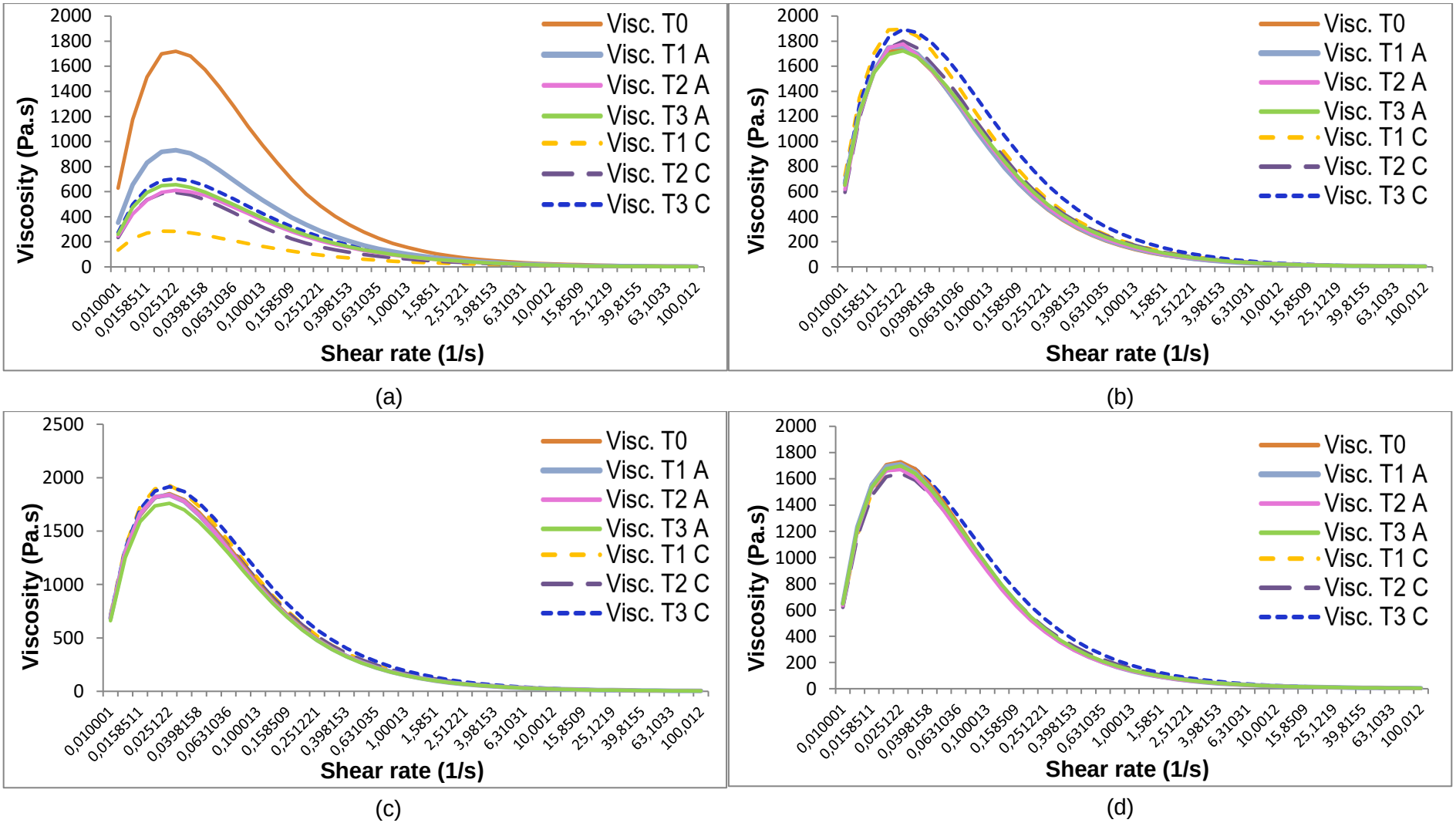

Figure 4-8: Viscosity (Pa.s) as a function of shear rate (s^{-1}) for the respective time intervals and specific storage conditions for (a) gel 1, (b) gel 2, (c) gel 3 and (d) gel 4

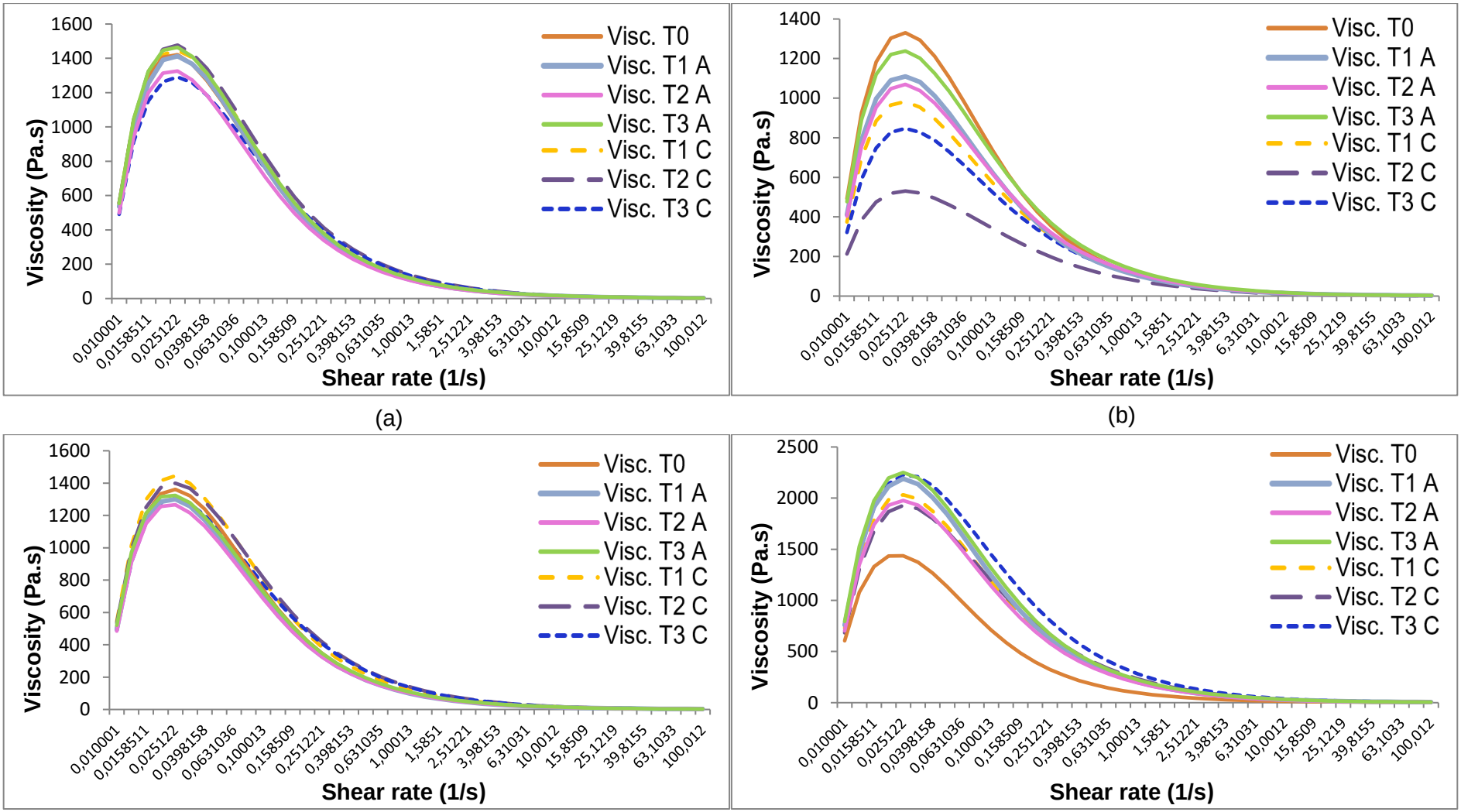

Figure 4-9: Viscosity (Pa.s) as a function of shear rate (s^{-1}) for the respective time intervals and specific storage conditions for (a) gel 5, (b) gel 6, (c) gel 7 and (d) the control gel

From the results presented in Table 4-8 and Figures 4-8 to 4-9, it can be seen that the viscosities of the RE gel formulations were similar for T0 and T3. Most of the gel formulations exhibited a relatively small decrease in viscosity when comparing the T3 values to the T0 values. A more pronounced change in the viscosity of both gel formulation 1 and the control gel was observed at T3 and these formulations were unstable in terms of rheology. The reduction in viscosity of gel 1 (A and C) and 6C may most likely be attributed to the formation of a fluid layer on the surface of the gels and sample collection included this fluid layer, which resulted in the recording of lower viscosities values. This phenomenon of fluid movement out of the gel is termed syneresis and is a common occurrence in formulations, which contain large amounts of fluids such as gels. The decrease in viscosity, which occurred in the experimental RE gel formulations may be attributed to syneresis (Chang *et al.*, 2013). However, if the fluid layer in the surface of gel 1 and 6C was evenly stirred into the rest of the gel, a gel formulation still suitable for topical application could probably be attained. The viscosity of the control gel had increased at both storage conditions and the more rigid gel structure may likely be attributed to the absence of anti-oxidants in the formulation, which could have caused a stronger gel network through the interlinking of gelling agent particles (Islam *et al.*, 2004).

When comparing the specific storage conditions, the change in viscosity was greater in the anti-oxidant containing gel formulations that were stored under accelerated storage conditions (C), as opposed to those formulations stored under long-term storage conditions (A). The decrease in viscosity at higher temperatures, as seen in Figure 4-8 and 4-9, has also been described in the literature (Di Mambro & Fonseca, 2005; Owen *et al.*, 2003). High temperatures are known to accelerate chemical reactions, which may have a negative effect on the strength of molecular bonds and may cause the polymer gel structure to weaken and this may explain why the gel formulations that were stored at 40°C exhibited lower viscosity values than the gel formulations that were stored at 25°C (Di Mambro & Fonseca, 2005).

Viscosity reflects the consistency of gel formulations and is an important factor in semisolid product development (Dantas *et al.*, 2016). The polymer used in the gel formulations in this study (Carbopol® 974P NF) is characterised by a medium cross-link density and is known to produce gels with medium to high viscosity, compared to other gelling agents (Lubrizol, 2011). Experimental gel formulations 2 to 7 (except 5C and 6C) did not show a pronounced change in viscosity over the entire stability testing period and these formulations therefore exhibited better physical stability. This is in accordance with information in the literature, which also reported that carbomer gels, consisting of cross-linked polymer structures, exhibit good physical stability during temperature fluctuations (Islam *et al.*, 2004). Furthermore, in comparison with other gelling agents, this high molecular weight polymer has previously been reported to aid in the manufacturing of more stable formulations in terms of rheology (Rozman *et al.*, 2009).

The viscosity of the medium can also influence stability of ingredients in the formulation, as formulations with higher viscosity have revealed some degree of protection of ascorbic acid (Sheraz *et al.*, 2011). The stability of vitamin C and E has also been improved by formulating it into gels with higher viscosity (Rozman *et al.*, 2009). When considering the degradation of aspalathin in aqueous solutions, the viscous gel structure could most likely have reduced oxidation of RE in the gel formulations by impeding oxygen inclusion into the gel. This can be supported by previously published literature regarding high viscosity semisolid formulations, containing *Castanea satina,* where oxygen diffusion into the topical formulations had been prevented and the stability of the plant extract had been increased (Almeida *et al.*, 2015). Therefore, it can be suggested that stability of aspalathin may also have been enhanced by incorporating RE into gel formulations with higher viscosity values.

Rheograms illustrating shear stress (Pa) as a function of shear rate (s^{-1}) were constructed for each gel formulation at the respective time intervals (T0 – T3) and specific storage conditions and are presented in Figures 4-10 and 4-11.

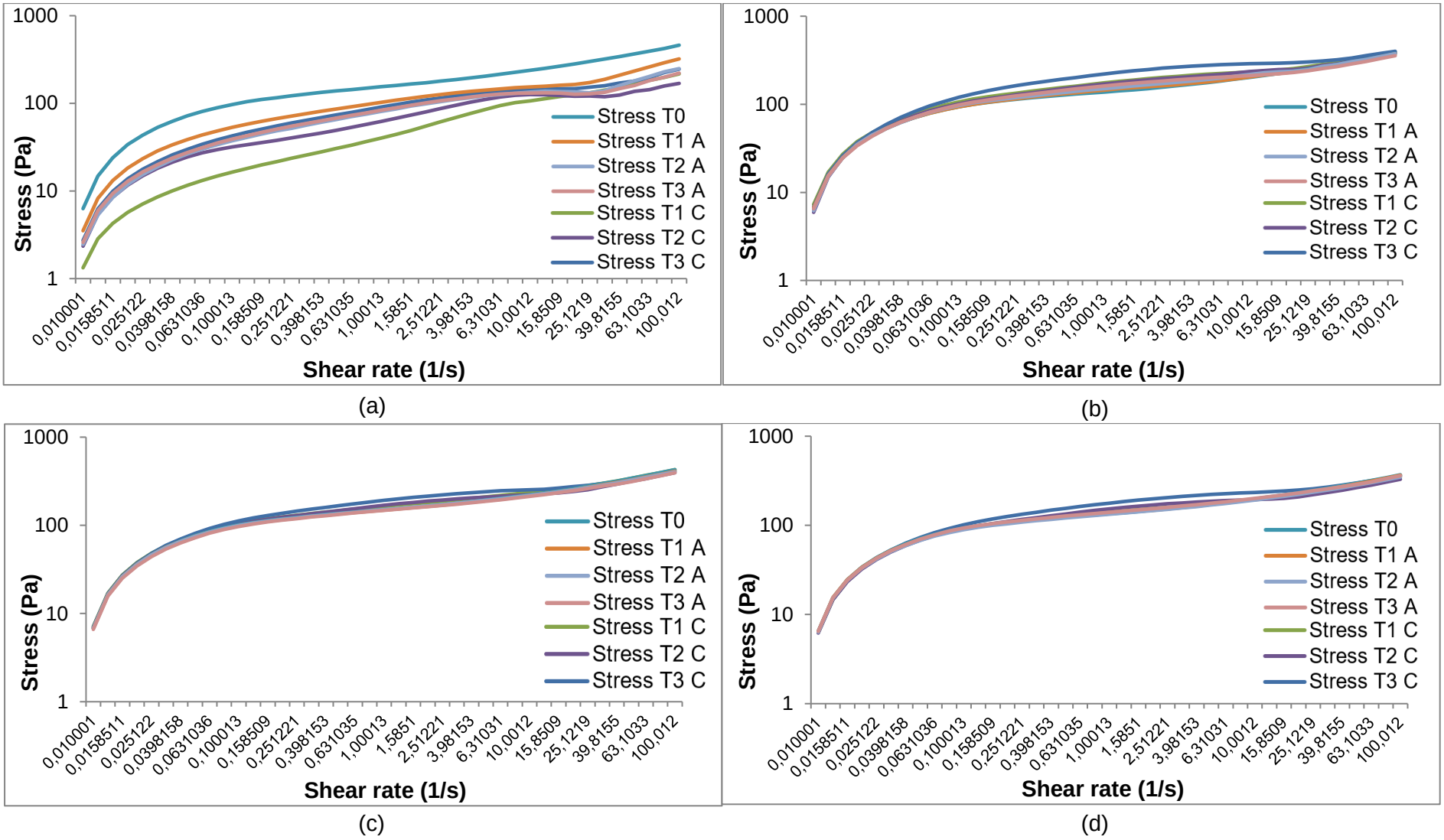

Figure 4-10: Rheograms depicting shear stress (Pa) as a function of shear rate (s^{-1}) for the specific storage conditions (A and C) and respective time intervals (T0 – T3) for (a) gel 1, (b) gel 2, (c) gel 3 and (d) gel 4

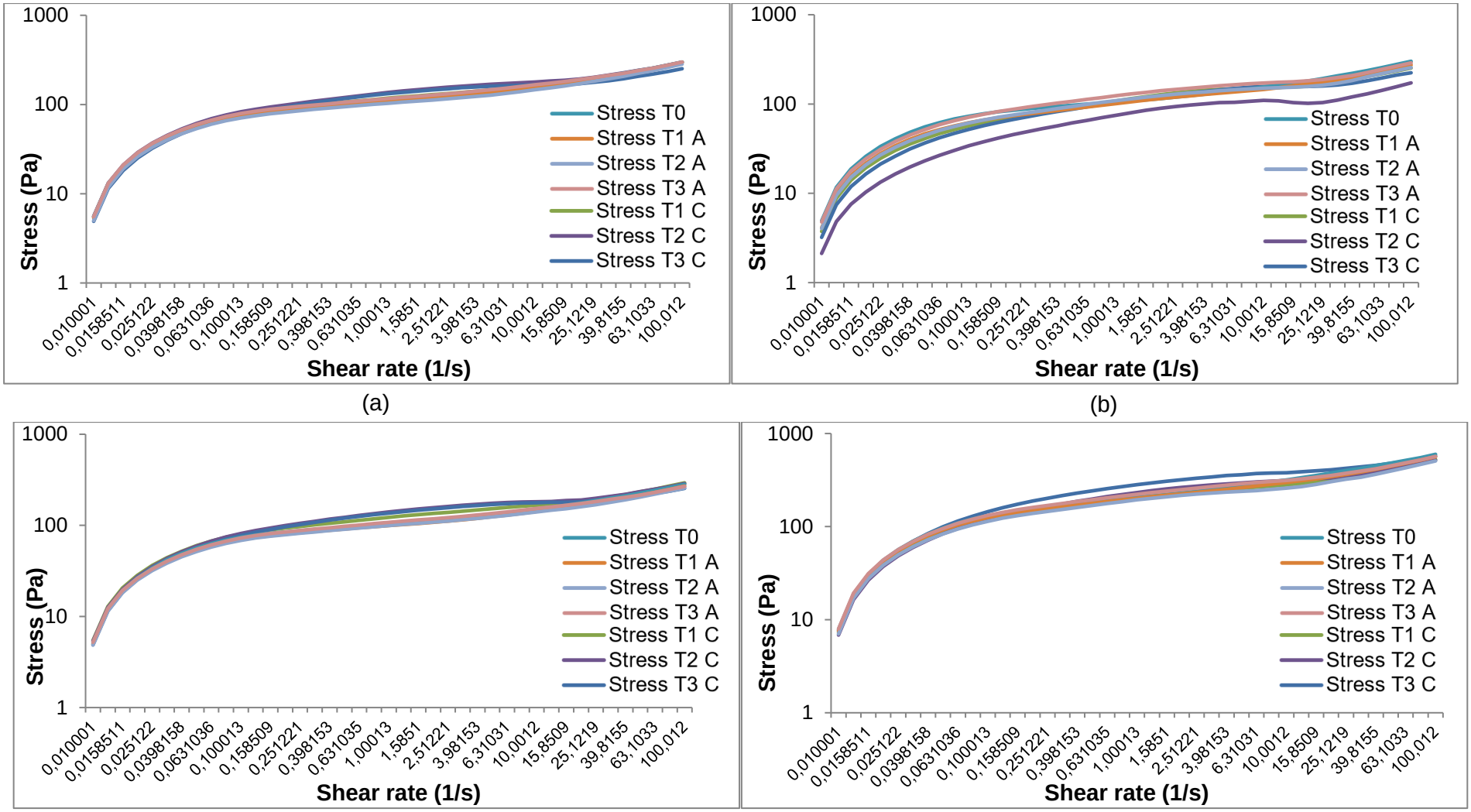

Figure 4-11: Rheograms depicting shear stress (Pa) as a function of shear rate (s^{-1}) for the specific storage conditions (A and C) and respective time intervals (T0 – T3) for (a) gel 5, (b) gel 6, (c) gel 7 and (d) the control gel

The rheograms illustrating shear stress as a function of shear rate demonstrated that all gel formulations presented non-Newtonian flow with pseudoplastic behaviour (Mohamed, 2004; Neves *et al.*, 2009; Rathod & Mehta, 2015). This rheological behaviour is known as shear-thinning and can be described as a decrease in viscosity when shear rate is increased (Brookfield, 2017). Pseudoplastic behaviour is characteristic of gel formulations and it can be indicative of good gel spreadability during topical application (Dantas *et al.*, 2016). When comparing the rheograms of each gel formulation between T0 – T3, for the specific storage conditions, no discernible changes were evident in the rheology, which suggests that the flow behaviour of all the gel formulations remained unchanged throughout the study.

4.3.4 Visual inspection

Photographic images were taken of each gel at the specific storage conditions between T0 – T3. The change in texture and colour that occurred in each gel between T0 – T3 can be seen in the photographs in Tables 4-9 and Table F.1 (Addendum F). The gel bottles were also photographed next to each other to compare the difference in the colour of the gel formulations (Table 4-10). Colour transitions between T0 – T3 were compared with paint colour charts (Addendum G).

Table 4-9: Photographic images of the top view of each gel at the specific storage conditions (A and C) at the respective time intervals

	Initial (T0)	Month 1 (T1)	Month 2 (T2)	Month 3 (T3)
Gel 1 A*				
C*				
Gel 2 A*				
C*				
Gel 3 A*				
C*				

*Storage condition A = (25°C ± 2 °C/60 ± 5 % RH) and C = (40°C ± 2°C/75 ± 5 % RH).

Table 4-9 (continued)

	Initial (T0)	Month 1 (T1)	Month 2 (T2)	Month 3 (T3)
Gel 4 A*				
C*				
Gel 5 A*				
C*				
Gel 6 A*				
C*				

*Storage condition A = (25°C ± 2 °C/60 ± 5 % RH) and C = (40°C ± 2°C/75 ± 5 % RH).

Table 4-9 (continued)

	Initial (T0)	Month 1 (T1)	Month 2 (T2)	Month 3 (T3)
Gel 7 **A***				
C*				
Control gel **A***				
C*				

*Storage condition A = (25°C ± 2 °C/60 ± 5 % RH) and C = (40°C ± 2°C/75 ± 5 % RH).

The gel formulations remained homogenous and no phase separation, texture changes or contaminants could be detected in any of the gel formulations after storage stability testing. The gels also appeared smoother, with less air bubbles at T3. Only gel 1 (A and C) and 6 (C) formed a fluid-like layer at the top surface of the gel and syneresis can be seen in these gel formulations at T2 and T3 (Table 4-9). The overall texture of the gels was acceptable for consumer use, because the formulations still remained as gel preparations and topical application would have been possible.

Table 4-10: Photographic images taken of the experimental gel formulations showing colour changes at the specific storage conditions and respective time intervals. Gel 1 to 7 can be seen in the order from left to right with the control gel on the far right.

T0[#]	
T0[#]	
T1[#] **A***	
A*	

*****Storage condition A = (25°C ± 2 °C/60 ± 5 % RH), B = (30°C ± 2°C/65 ± 5 % RH) and C = (40°C ± 2°C/75 ± 5 % RH). [#]T0 = initial time, T1 = after 1 month, T2 = after 2 months, T3 = after 3 months.

Table 4-10 (continued)

T1# **C***	
C*	
T2# **A***	
A*	

***Storage condition A = (25°C ± 2 °C/60 ± 5 % RH), B = (30°C ± 2°C/65 ± 5 % RH) and C = (40°C ± 2°C/75 ± 5 % RH). #T0 = initial time, T1 = after 1 month, T2 = after 2 months, T3 = after 3 months.

Table 4-10 (continued)

T2# **C***	
C*	
T3# **A***	
A*	

*****Storage condition A = (25°C ± 2 °C/60 ± 5 % RH), B = (30°C ± 2°C/65 ± 5 % RH) and C = (40°C ± 2°C/75 ± 5 % RH). #T0 = initial time, T1 = after 1 month, T2 = after 2 months, T3 = after 3 months.

Table 4-10 (continued)

T3# **C***	
C*	

*Storage condition A = (25°C ± 2 °C/60 ± 5 % RH) and C = (40°C ± 2°C/75 ± 5 % RH). #T1 = after 1 month, T2 = after 2 months and T3 = after 3 months

Paint colour charts (Addendum G) were used to determine the colour transition of the gel formulations by comparing the colour of the gel formulations at the end of storage stability (T3) with the initial colour directly after formulation (T0). Gel 3 was an aureoline colour and there were no pronounced colour changes throughout the stability study for this formulation. Gels 4, 5 and 7 were also an aureoline colour at the beginning of the study. After T3, gel 4 and 5 were raw sienna colours at both storage conditions, but G4C turned to a gamboge colour. Gel 7 changed from aureoline to an ocre amarillo (storage A) and copper colour (storage C) at the specific storage conditions. Gels 1 and 6 were an auerolina colour at the beginning of the study (T0) and both changed to a burnt sienna colour at T3A. However, gel 1 changed to a chestnut colour and gel 6 to reddish brown at T3C. A light brown colour was observed at T0 for the control and gel 2, but gel 2 also changed to a burnt sienna and reddish-brown colour at the specific storage conditions. The control gel exhibited the most pronounced colour transition, changing from light brown to venetian red and antique red, at the specific storage conditions.

According to the photographs in Table 4-10, gels 1, 2, 6 and the control gel showed a radical change in colour over time. Gel formulations 3, 4, 5 and 7 did not present with extreme colour changes between T0 – T3. Based on visual inspection gel 3 appeared to be the most physically stable gel, as was evident from the lack of colour change between T0 – T3. Gel formulations containing sodium metabisulfite as anti-oxidant (G3, 4, 5, 7) also remained virtually unchanged in colour, and these findings are in accordance with a previous study that showed no colour

change in a green tea extract gel containing sodium sulfite as anti-oxidant (Saib, 2010). Interestingly, a cosmetic preparation containing RE turned darker in colour, which contained a low concentration of sodium metabisulfite and it was attributed to oxidation of flavonoids in the RE formulation (Chuarienthong *et al.*, 2010). However, in this study, the aspalathin-enriched RE gel formulations containing sodium metabisulfite at a concentration of 1% (w/v) were the most stable in terms of colour stability. Furthermore, it appeared as if gels, which contained ascorbic- and citric acid (G1 and 2) turned much darker in colour, with the most discolouration observed in the combination gel (G6). Previous studies reported a colour change in gels due to the oxidation of ascorbic acid in the formulation and it may also explain the colour transformation in RE gel formulations containing ascorbic acid in this instance (De Beer *et al.*, 2012; Koop *et al.*, 2009).

In a recent study it was reported that GRE powders turned darker in colour due to the formation of brown by-products, which had occurred due to oxidation of aspalathin. Colour changes in the GRE was more pronounced in powders stored at higher temperature and moisture conditions, and the colour change was a result of oxidation in the extract (Human, 2019). Experimental RE gel formulations that were subjected to accelerated storage conditions (C) were also darker in colour, as opposed to gel formulations stored at lower temperature and humidity conditions (A). From the colour changes observed in some of the gel formulations, it is evident that oxidation in the gel formulations had increased over time and also at accelerated storage conditions.

The discoloration may indicate the oxidation of ingredients in the gel formulations. The darker colour observed can be caused by oxidation of flavonoids, such as aspalathin, in the RE gel formulations (Saib, 2010). As mentioned earlier, browning was present in RE iced teas after heat treatment and it could be indicative of degradation of aspalathin (De Beer *et al.*, 2012). Human (2019) suggested that the addition of anti-oxidants could slow down the oxidation process in GRE. The seven gel formulations in this study differed in colour, and the control gel, which contained no anti-oxidants, turned much darker in colour than the other formulations. The discoloration in the control gel is in line with findings from Human (2019), and it may suggest that more oxidation had occurred in the RE control gel formulation than in the other experimental gel formulations.

4.4 Diffusion studies

In vitro release experiments are important when developing novel formulations and it can be used for screening purposes prior to *in vivo* product testing (Olejnik *et al.*, 2018). The use of synthetic membranes for active ingredient release from semisolid formulations (e.g. gel formulations) is beneficial as it is easily sourced and may be used as a quality control measure to determine active ingredient release (Musa *et al.*, 2017). Membranes are chosen based on

the extent of their diffusional resistance and usually do not have a rate controlling function (Ueda *et al.*, 2009). Based on information available in the literature, PVDF membrane filters were selected and used in this study to assess the extent of aspalathin release from the experimental gel formulations (Olejnik *et al.*, 2018).

Diffusion studies were performed with each gel formulation on a Sweetana-Grass diffusion apparatus with synthetic PVDF membranes to determine the rate and extent of aspalathin release from the RE gel formulations. Release studies were performed in six-fold on all experimental gel formulations and samples were withdrawn from the receiver chamber at pre-determined time intervals over a period of 360 min. The collected samples were filtered and 100 µl was injected and analysed with a validated HPLC method.

Aspalathin is classified as a weakly acid compound with low permeability and a molecular weight of 452.41 g/mole, which may result in low bioavailability. Previous studies reported low lipophilicity for aspalathin and poor absorption through biological membranes such as intestinal tissue and the lipid bilayers of the skin. The permeation of GRE and pure aspalathin solution through human abdominal skin was as low as 0.07 and 0.08% of the applied doses, respectively (Bowles *et al.*, 2017; Huang *et al.*, 2008). Limited data is available on the topical application of RE formulations, and since there are numerous commercially available RE containing cosmetic products available, it was imperative to determine if aspalathin could be kept chemically stable and be released from topical RE gel formulations to be pharmaceutically available.

4.4.1 *In vitro* assessment for aspalathin release

All transport samples were corrected for dilution and the calculated average percentage aspalathin release from each gel formulation, at the pre-determined time intervals, is shown in Table 4-11. The calculated P_{app} values of each gel and SD values obtained from the release studies are also presented in Table 4-11. The percentage aspalathin release from each experimental gel formulation across synthetic PVDF membranes in a Sweetana-Grass diffusion apparatus is depicted in Figures H.1 to H.8 in Addendum H. A complete set of transport data regarding the extent of aspalathin release from each gel formulation, with the statistical analysis, is provided in Addendum H and I, respectively.

4.4.2 Comparing aspalathin release from the different aspalathin-enriched rooibos extract gel formulations

Table 4-11: Percentage aspalathin released from the experimental gel formulations at the pre-determined time intervals with standard deviation (SD) values and average (Avg) apparent permeability coefficient (P_{app}) values

	% Aspalathin released from the different rooibos extract (RE) gel formulations							
Time (min)	**Gel 1**	**Gel 2**	**Gel 3**	**Gel 4**	**Gel 5**	**Gel 6**	**Gel 7**	**Control gel**
0	0	0	0	0	0	0	0	0
30	0.30 ± 0.0	0	0	0	0	0	0.31 ± 0.4	0.22 ± 0.1
60	1.44 ± 0.2	1.09 ± 0.4	0.01 ± 0.0	0.61 ± 0.1	1.10 ± 0.3	1.07 ± 0.5	1.25 ± 0.6	1.09 ± 0.1
180	3.42 ± 0.5	2.85 ± 0.5	1.54 ± 0.4	2.47 ± 0.5	5.92 ± 1.4	4.91 ± 0.7	4.99 ± 0.8	5.00 ± 0.7
300	5.76 ± 1.5	3.74 ± 1.2	3.15 ± 0.7	2.46 ± 1.2	10.38 ± 0.4	7.28 ± 1.1	7.17 ± 1.3	7.41 ± 0.8
360	6.01 ± 2.5	3.90 ± 0.7	3.38 ± 0.7	2.94 ± 1.1	11.40 ± 2.3	6.70 ± 1.9	6.20 ± 1.0	7.94 ± 0.9
Avg P_{app}	1.68E-06 ±2.0E-07	1.09E-06 ±1.3E-07	0.97E-06 ±2.0E-07	0.80E-06 ±3.1E-07	3.24E-06 ±3.7E-07	2.04E-06 ±4.3E-07	1.90E-06 ±3.1E-07	2.23E-06 ±1.9E-07

Avg, average.

The *in vitro* release of aspalathin from the different gel formulations was compared with each other and the control gel and the rate and extent of aspalathin release from the experimental gel formulations are presented in Figure 4-12, and Table 4-11. Resultant P_{app} values obtained from each gel release study are depicted in Figure 4-13. The viscosity of each gel formulation was measured and the viscosity value at minimum shear rate is illustrated for each gel formulation in Figure 4-14.

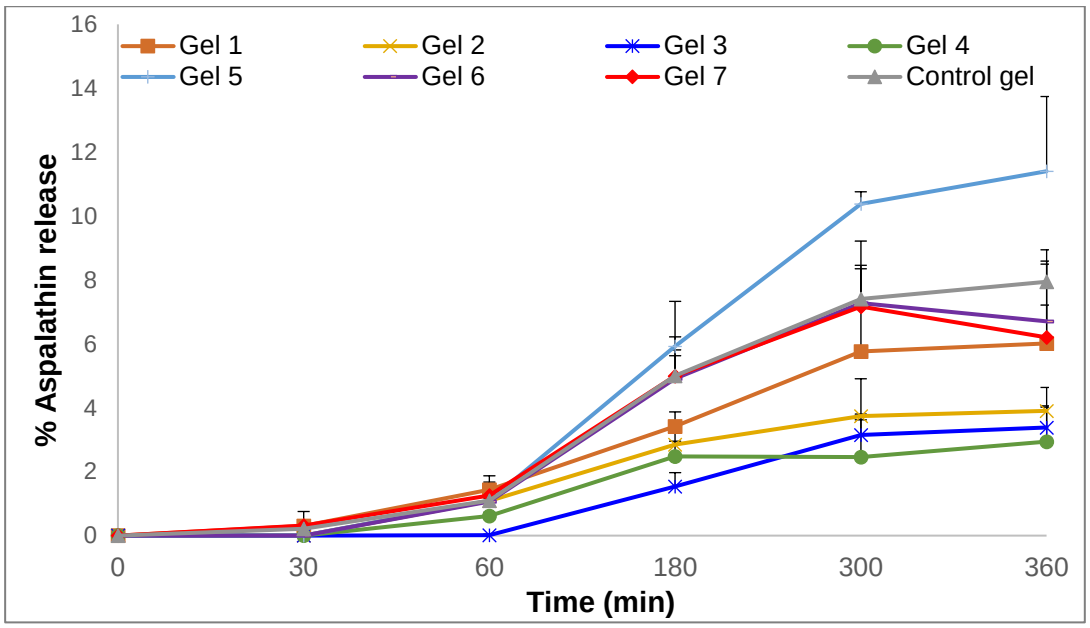

Figure 4-12: Percentage aspalathin released across synthetic membranes from the different experimental gel formulations at pre-determined time intervals

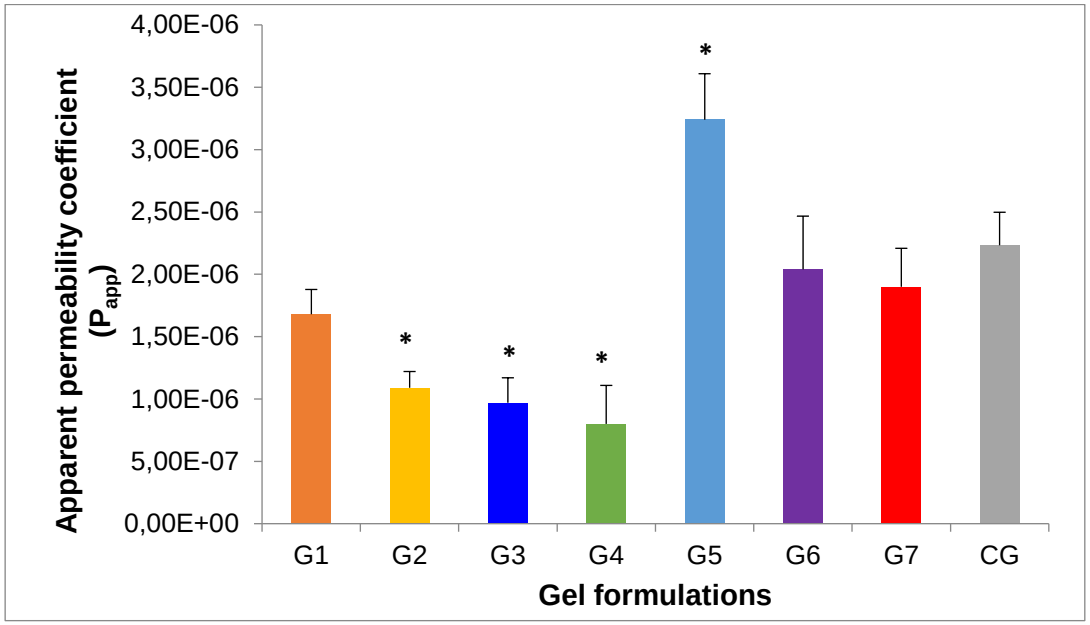

Figure 4-13: Average apparent permeability coefficient (P_{app}) values for aspalathin released from the different gel formulations (*statistically significant difference when compared to the control gel, $p \leq 0.05$)

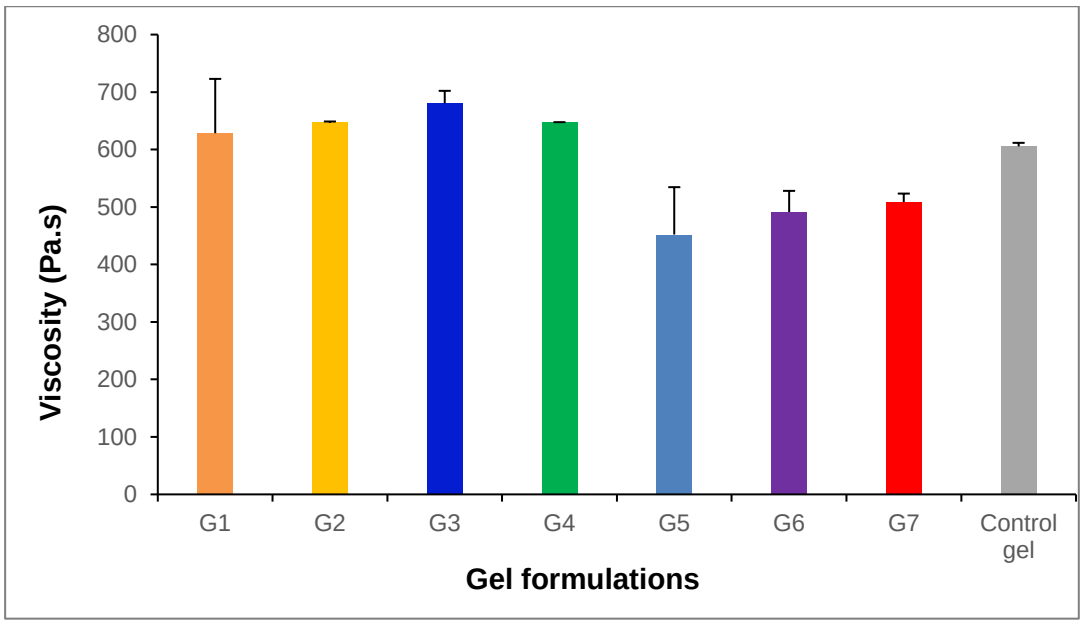

Figure 4-14: Viscosity (Pa.s) values of the different experimental gel formulations

Aspalathin release was achieved from all experimental gel formulations and the extent of release was in the following order (arranged from highest to lowest): G5 > Control G > G6 > G7 > G1 > G2 > G3 > G4. *In vitro* release studies revealed that aspalathin release could be achieved in all gel formulations with a maximum cumulative amount of aspalathin release of 11.4%. Most of the gel formulations had a lag period of approximately 60 min, with only gel formulations 1, 7 and the control gel showing initial release after 30 min. During the lag phase, aspalathin molecules diffused through the gel matrix to the contact surface of the membrane before release from the gel could be achieved. In general, the release rate increased from 180 to 300 min for all gel formulations as seen by the increase in gradient from the release curves (Figure 4-12). In most of the gel formulations, the maximum cumulative amount of aspalathin release that was measured at the acceptor site of the PVDF membrane was between 300 and 360 min.

Several factors can affect the mechanism of active ingredient release from dosage forms including the internal structure of the formulation, anti-oxidant solubility, pH, viscosity and rheological properties (Rozman *et al.*, 2009). Usually, the type and concentration of polymer used to produce a gel influences the release rate of active ingredients from the formulation (Ajazuddin *et al.*, 2013; Dragicevic-Curic & Maibach, 2015). However, in this study, the type of polymer (Carbopol® 974P NF) and its concentration was kept constant for all gel formulations. The difference in the extent of aspalathin release from the gel formulations may most likely be related to differences in the viscosity of the gels. It is a well-known fact that an increase in viscosity results in a decrease in the release of an active ingredient from the formulation (Dong *et al.*, 2018; Gallagher & Heard, 2005; Ghorpade *et al.*, 2012; Laxmi *et al.*, 2013; Tas *et al.*,

2003; Ueda *et al.*, 2009). The rheological properties of a gel matrix have a direct influence on the ability of a hydrogel to effectively deliver an active ingredient. Thus, viscosity influences the extent of diffusion of active ingredients through diffusion barriers and an increase in viscosity will impede the release of active ingredients from the formulation (Tas *et al.*, 2003).

The relatively high viscosity of the RE gel formulations may be attributed to the specific polymer gelling agent that was used. The intricate polymer structure had most likely caused an increase in diffusional resistance for the active ingredient and the density of the chain structure in the gel may have limited the diffusion of RE (Tas *et al.*, 2003). Physical properties of topical Carbopol® gels, the time these formulations remain on the applied area and release rates of active ingredients from Carbopol® gels are extremely sensitive to their rheological flow behaviour (Islam *et al.*, 2004). A previous study has reported that Carbopol® 974P NF, with a pKa value of 6, will ionise at a pH of 4.5 and that the swollen gel matrix layer, which is formed, most probably governs the release of active ingredients (Perez-Marcos *et al.*, 1996).

The viscosity values of the experimental gels (Figure 4-14) are in accordance with the available literature, and it is evident from the results that gel formulations with lower viscosity resulted in greater release (Figure 4-12). The release in the more viscous gels may have been impeded due to the aspalathin-enriched RE molecules being entrapped in the network structure of the highly viscous gels, causing a lower availability of aspalathin on the surface of the membrane (Al-Saidan *et al.*, 2004; Ueda *et al.*, 2009). It was previously reported that an interaction occurred between the bioactive compounds in GRE and polymers as swelling caused aspalathin-rich nanoparticles to be trapped in the polymer matrix and it resulted in a lower extent of aspalathin release (Human, 2019), however the aspalathin molecules in this study was in solution in the gel formulations.

Gels 3 and 4 achieved less aspalathin release than the other experimental gel formulations (P_{app}: 0.97-E06 and 0.80E-06, respectively). The results from this study are in accordance with literature, where formulations with the highest viscosities (gels 3 and 4, Visc. 680 and 647 Pa.s) exhibited statistically significantly less aspalathin release ($p \leq 0.05$) when compared to the control gel. It can be seen in Figure 4-13 and Table 4-11 that the extent of aspalathin release had increased as the viscosities of the gels had decreased. Gel 5 had the lowest viscosity (452 Pa.s) of all the formulations, and the extent of aspalathin release was statistically significantly higher for this gel (P_{app}: 3.24E-06, $p \leq 0.05$), compared to the control and all the other gel formulations. Both gels 6 and 7 also had low viscosities and the formulations were able to release a fair amount of aspalathin (P_{app}: 2.04E-06 and 1.90E-06, respectively). Thus, the extent of aspalathin release from the different gels was inversely related to the viscosities of the formulations (Tas *et al*., 2003). Although the viscosity of the control gel was slightly higher

than gels 6 and 7, it presented with similar release (P_{app}: 2.23E-06) and the difference was not statistically significantly different from gels 6 and 7.

The results from the *in vitro* diffusion studies demonstrated that the Sweetana-Grass diffusion apparatus can be used to determine release of the biomarker (aspalathin) in RE from gel formulations as proof of concept to membrane release claims in topical formulations. The concentration of aspalathin available in the RE gel formulations promotes the existence of a concentration gradient in between the diffusion chambers. A concentration gradient drives an active ingredient from the topical formulation to the application site and may have possibly enhanced the extent of aspalathin release from the gel formulations through the synthetic membranes (Chang *et al*., 2013; Williams, 2018). It may be suggested that sufficient aspalathin was released from the gel formulations as opposed to previous studies where aspalathin was absorbed poorly (Bowles *et al.*, 2017; Huang *et al.*, 2008). Given the absence of *in vitro* release studies for aspalathin from RE topical formulations, this novel research reporting aspalathin release from RE gel formulations will contribute to RE cosmeceutical product development. Further studies will, however, be necessary to determine if aspalathin can permeate skin tissues from topical RE gel formulations.

CHAPTER 5: FINAL CONCLUSIONS AND RECOMMENDATIONS

5.1 Final conclusions

Herbs have been used for many years as an alternative treatment option for many diseases. *Asplathus linearis* (rooibos tea) is an indigenous plant that is commonly used as a beverage and various health benefits have been associated with the intake of rooibos tea. Rooibos tea has sparked interest in the pharmaceutical industry due to several health promoting effects, but mostly for its role as an anti-oxidant. Aspalathin is the major flavonoid uniquely found in RE and its anti-oxidant potential has led to the development of aspalathin-enriched RE. Cosmetic industries typically include RE in topical formulations to reduce skin wrinkling and assist in anti-ageing. Even though there are many cosmetic products available claiming to contain RE, scientific studies dealing with the topical application of RE are very limited (Joubert & De Beer, 2011).

Information regarding the chemical stability of aspalathin, when incorporated into aqueous mixtures, is limited to RE infused iced teas only and limited information is available regarding the chemical stability of aspalathin when incorporated into cosmeceutical formulations (De Beer *et al.*, 2012). Therefore, the main aim of this study was to prepare experimental gel formulations containing aspalathin-enriched RE for topical application that would remain stable for an extended period of time. Results from the preliminary study showed that it was possible to manufacture aspalathin-enriched RE polymer-based gel formulations, which exhibited improved chemical stability of aspalathin. Based on the results of the preliminary study, experimental gels were prepared with the addition of selected anti-oxidants as variable. The experimental gel formulations were then subjected to specific stability storage conditions for three months. The gel formulations were evaluated at specific time points during the period of stability testing to determine the extent of aspalathin degradation in each formulation and to visually inspect the gels for signs of discolouration or any other physical changes.

The results showed that aspalathin did experience chemical degradation in all of the RE gel formulations but to a lesser extent than in the control gel formulation. During both long-term and accelerated storage conditions, the gel formulation that contained a combination of all three anti-oxidants delivered the best results in terms of chemical stability of aspalathin. Gel formulations, which contained sodium metabisulfite as anti-oxidant ensured better chemical stability of aspalathin in general when subjected to long-term storage conditions. However, gel formulations, which contained ascorbic- and citric acid had retained more aspalathin when subjected to accelerated stability conditions. The RE control gel, which contained no anti-oxidants, resulted in complete aspalathin degradation at the end of the stability testing at both

storage conditions. The experimental results prove that the chemical stability of aspalathin may be improved when formulated in RE topical gel formulations in combination with selected anti-oxidants. Based on the literature review it is likely that these experimental results are the first to be reported regarding the proven chemical stability enhancement of aspalathin in RE topical gel formulations.

The gel formulations were buffered at a pH of 4.7, which is representative of the pH of the skin, and it has been shown to help retain chemical stability of aspalathin when the experimental gels were buffered at this pH value (Human, 2019; Lambers *et al*., 2006). Furthermore, the experimental results showed that the pH values of the experimental gel formulations did not change much during the stability assessment when stored at specific storage conditions. The average pH values of all the gel formulations remained stable over time and within the pre-determined pH range (4.7 ± 0.3).

Rheological characteristics of the gel formulations were evaluated and showed that most of the gel formulations did not exhibit any pronounced changes in viscosity during stability storage testing. The viscosity reduction in gel 1 and 6 may be explained by the occurrence of syneresis of the gel formulations. There was an increase in viscosity of the control gel when stored at both storage conditions and it may have been due to the absence of anti-oxidants, causing an interlinking in the gel network and a stronger structure. It was also observed that higher temperature and humidity played a role in rheology, since the viscosity change was greater in most of the gel formulations stored at accelerated conditions as opposed to long-term storage conditions. The rheograms showed no change in the pseudoplastic flow behaviour in any of the gel formulations after specific storage conditions and it can be concluded that the gelling agent (Carbopol® 974P NF) was able to maintain RE gel formulations with stable rheology.

Visual inspection of all gel formulations indicated smooth gels with acceptable consistency for topical application after being subjected to specific storage conditions. Gel formulations which contained sodium metabisulfite as anti-oxidant showed the least change in colour over time. However, a pronounced colour change was observed in gel formulations which contained ascorbic- and citric acid as anti-oxidants. This is in accordance with a study that reported the presence of 5-hydroxymethylfurfural (HMF), a degradation product of ascorbic acid, that was responsible for the browning of RE iced teas containing ascorbic- and citric acid (De Beer *et al.*, 2012). The control gel appeared much darker in colour and the pronounced colour change may be attributed to discoloration (browning) which occurred in RE due to oxidation (Human, 2019). Apart from the formation of flavone dimers, the non-enzymatic oxidation of aspalathin is usually also accompanied by brown pigmented products that are formed upon heat exposure (Joubert *et al*., 2010). The colour changes revealed in the RE gel formulations may have been caused by oxidation and possible instability of aspalathin or other flavonoids present in the aspalathin-

enriched RE. This could be supported by a previous study reporting a darker colour change in a RE topical formulation which may possibly be attributed to flavonoid oxidation (Chuarienthong *et al*., 2010).

This study also evaluated the *in vitro* release potential of aspalathin from different topical gel formulations. In general, the release rates increased from 180 min and all of the gel formulations exhibited the ability to release aspalathin, albeit to different extents. Maximum cumulative amount of aspalathin release (11.4%) occurred in all gel formulations between 300 and 360 min. The results generated from this study confirmed that the extent of aspalathin release from the RE gel formulations increased as their viscosities decreased. The difference in the viscosities of the gel formulations was a result of the various selected anti-oxidants used. The lower extent of aspalathin release from gel formulations with higher viscosities may possibly be attributed to aspalathin that may have been entrapped in the polymer gel network structure of the viscous gel formulations, causing resistance in diffusion and less migration of aspalathin molecules (Dragicevic-Curic & Maibach, 2015; Mohamed, 2004). Human (2019) investigated the interaction of bioactive compounds in RE with polymers and found that the release of RE was hindered as the polymer matrix swelled. The P_{app} value (3.24E-06) of gel 5 indicated that this formulation exhibited the most statistically significant aspalathin release, and it was also the gel formulation with the lowest viscosity. This study was the first where the *in vitro* membrane release potential of aspalathin from RE topical formulations was assessed and the results warrant further studies regarding the topical delivery of aspalathin-enriched RE gel formulations.

5.2 Future recommendations

Different types of aspalathin-enriched RE topical gel formulations were successfully prepared and the gel formulations were evaluated with regards to chemical and physical stability, and *in vitro* release. It is recommended that future studies should investigate the following aspects:

- Optimisation of the stability of aspalathin-enriched RE topical formulations by incorporating other anti-oxidants and chelating agents. Permeation enhancers could also be included to aid in the absorption of aspalathin from the formulation.
- It would be beneficial to identify other phenolic compounds in RE using HPLC to identify any possible degradation products which were formed as a result of by-products from aspalathin oxidation.
- Alternative RE topical formulations could be explored such as lotions, liniments, emulgels or single use sachets that may provide better stability of aspalathin over time.
- To determine the shelf life of the product, the RE gel formulations could be subjected to long-term, intermediate or accelerated storage conditions where stability tests are

carried out at a more frequent time period, e.g. every week, in order to fully understand aspalathin degradation over a regular time period.

- Additional assessments could be performed on the experimental gel formulations including sensory analysis, skin irritation studies and anti-oxidant capacity of aspalathin.
- With regards to the *in vitro* release studies, further topical delivery of RE gel formulations could be performed to assess the extent of aspalathin absorption in tissues (e.g. pig ear or dermatomed human skin).
- Different types of synthetic membranes can be evaluated to determine if differences in membrane characteristics (lipophilic versus hydrophilic) have any effect on the release potential of aspalathin from the RE gel formulations.
- To increase the extent of aspalathin release, further modifications can be made to formulate RE topical gel formulations with even lower viscosity.

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ADDENDUM A

ETHICAL CLEARANCE

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South Africa 2520

Tel: 018 299-1111/2222
Web: http://www.nwu.ac.za

Health Sciences Ethics Office for Research, Training and Support

North-West University Health Research Ethics Committee (NWU-HREC)
Tel: 018-285 2291
Email: Wayne.Towers@nwu.ac.za

Prof JD Steyn
Pharmaceutics
Pharmacen

26 August 2019

Dear Prof Steyn

PROOF THAT THE FOLLOWING STUDY DOES NOT REQUIRE ETHICAL APPROVAL

Study title: ***In vitro*** **release and stability of rooibos tea extract in topical formulations**

Study leader: Prof JD Steyn

Student: I Peters - 24953733

Following review of a notification received from Higher Degrees Administration by the North-West University Health Research Ethics Committee (NWU-HREC), it was determined that no ethical approval was required as this study does not:

- Involve any human participants or their data/information
- Involve any human samples
- Involve any animals
- Involve any animal samples
- Have any possible environmental impact

The study involves laboratory work and the possible risks to researchers are covered by NWU-HREC approved Standard Operating Procedures for:

- Good laboratory practice
- Appropriate waste management
- Appropriate safety measures and protection of laboratory workers

Following review of this notification, the NWU-HREC is in agreement that the aforementioned study does not require ethical approval.

Yours sincerely

Digitally signed by Wayne Towers
Date: 2019.08.26 10:25:40 +02'00'

Prof Wayne Towers

NWU-HREC Chairperson

Digitally signed by Prof Minrie Greeff
Date: 2019.08.26 16:01:32 +02'00'

Prof Minrie Greeff

Head of Health Sciences Ethics Office for Research, Training and Support

Current details: (23239522) G:\My Drive\9. Research and Postgraduate Education\9.1.5.3 Letters Templates\9.1.5.4.1_NEA_NWU-HREC_Template.docm
11 April 2019

File reference: 9.1.5.4.1

ADDENDUM B

CERTIFICATE OF ANALYSIS FOR ASPALATHIN REFERENCE STANDARD

Certificate of Analysis
Primary reference standard

HWI group

Substance:	**Aspalathin**	Batch:	**HWI00319**
Article no:	**0352-05-85**		
Sample size:	**10 mg in an amber glass vial**		
Expiry date[1)]:	**08/2019**		

Parameter	Method	Requirement	Result
Characters			
Appearance	organoleptic	light yellow to yellowish powder	complies
Identity			
^{1}H-Nuclear magnetic resonance spectroscopy	M-0352-E, 2.2	^{1}H-NMR spectrum corresponds to structure	complies
Assay			
Liquid chromatography (Method 1)	M-0352-E, 3.1, normalisation procedure	> 90.0 %[2)]	97.52%
Quantitative NMR spectroscopy	M-0352-E, 3.2	> 85.0 %[2)]	94.84%

Storage and handling: The standard should be stored in the original amber glass vial at <-15°C under inert gas and protected from light.
Bring the amber glass vial to room temperature and shake before use.
Do not dry, use on as is basis.

Intented use: Our HWI primary reference standard can be used e.g. for identification, assay and purity testing, method development and validation

[1)] storage in unopened, original container according to specified conditions.

[2)] **Values for CP and qNMR may vary due to residual solvents and water content which can not be detected by chromatographic methods. For quantification purposes only qNMR value is valid.**

The definition for Primary reference standard to the WHO Technical Report 943 (2007) is:
„A designated primary chemical reference substance is one that is widely acknowledged to have the appropriate qualities within a specified context and whose assigned content when used as an assay standard is accepted without requiring comparison to another chemical substance."

checked and approved 23. APR. 2018
Katharina Hilsch
Project leader
Reference Standard Services

HWI pharma servicesGmbH Rheinzaberner Straße 8 | 76761 Rueizheim Germany | Phone: +49 7272 7767-0 Telefax: +49 7272 7767 -11 | Email: info@hwi-group.de Web: www.hwi-group.de

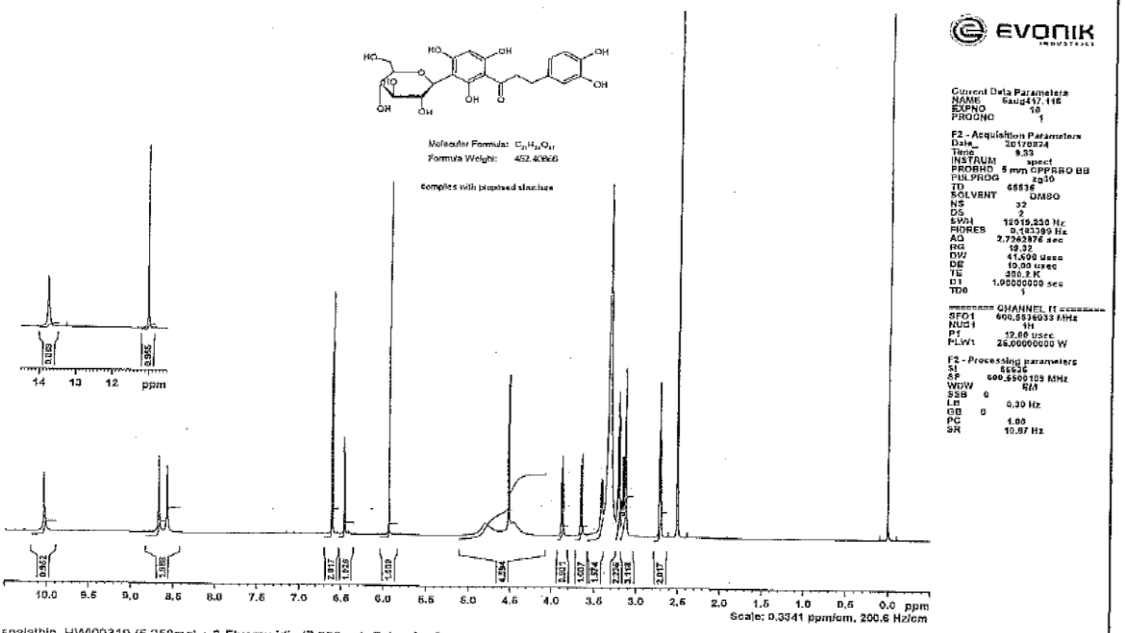
Certificate of Analysis
Primary reference standard

HWI group

Substance:	**Aspalathin**	Batch:	**HWI00319**
Article no:	**0352-05-85**		
Sample size:	**10 mg in an amber glass vial**		
Expiry date[1]:	**08/2019**		

NMR spectra

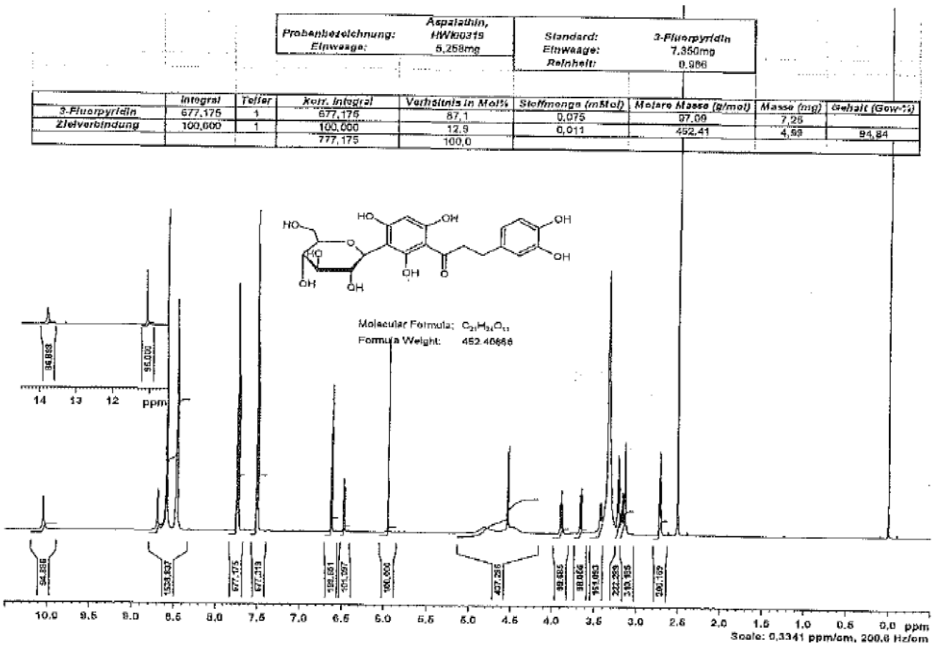
Aspalathin, HWI00319 (4,317mg) Schaefer,Fr. A170011806/1 H1/DMSO ml 180298



Aspalathin, HWI00319 (5,258mg) + 3-Fluorpyridin (7,350mg) Schaefer,Fr. A170011806/1 H1/DMSO ml 180298-a

Probenbezeichnung:	Aspalathin, HWI00319	Standard:	3-Fluorpyridin
Einwaage:	5,258mg	Einwaage:	7,350mg
		Reinheit:	0,986

	Integral	Teiler	Korr. Integral	Verhältnis in Mol%	Stoffmenge (mMol)	Molare Masse (g/mol)	Masse (mg)	Gehalt (Gew-%)
3-Fluorpyridin	677,175	1	677,175	87,1	0,075	97,09	7,25	
Zielverbindung	100,000	1	100,000	12,9	0,011	492,41	4,58	94,84
			777,175	100,0				



ADDENDUM C

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY CHROMATOGRAMS FOR VALIDATION: SPECIFICITY

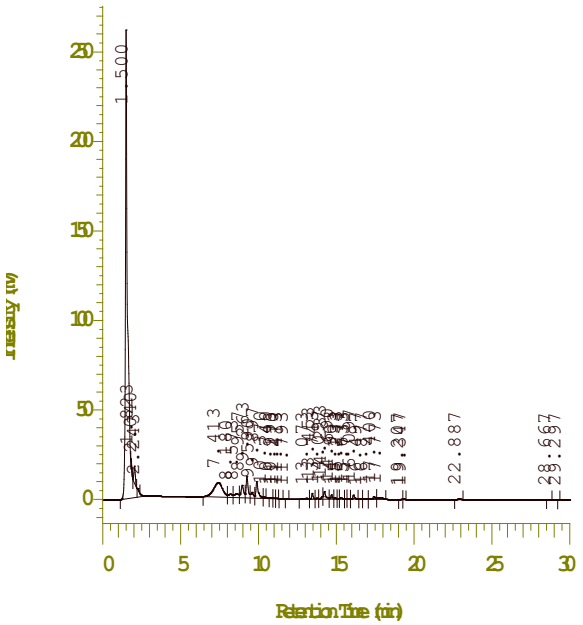


Figure C.1: Chromatogram of propylene glycol

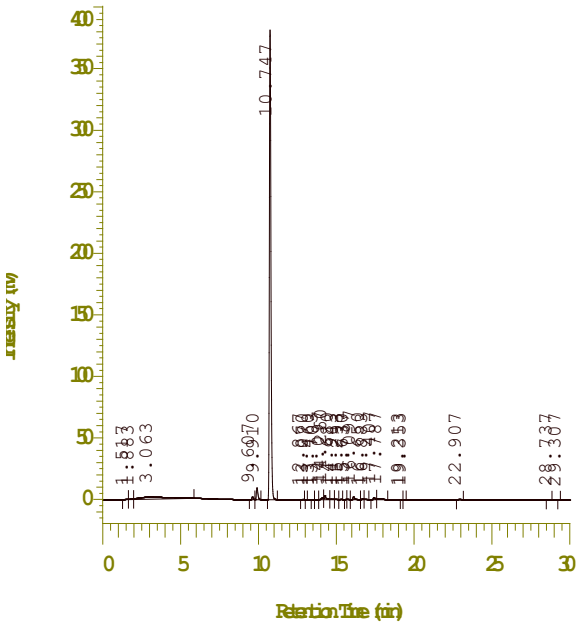


Figure C.2: Chromatogram of methylparaben

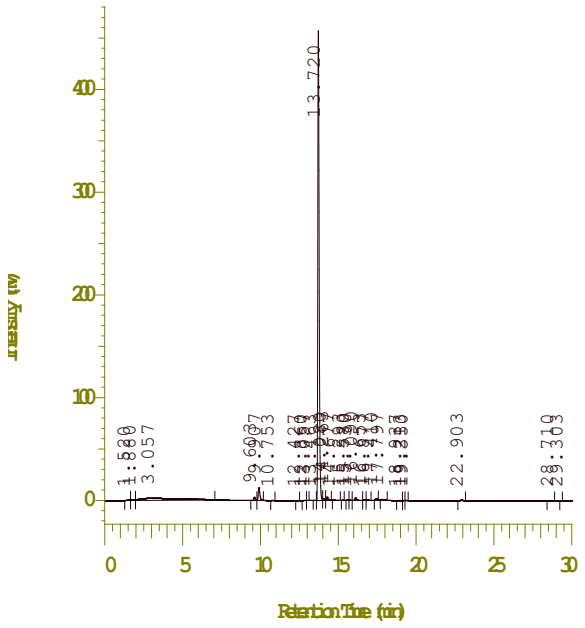


Figure C.3: Chromatogram of propylparaben

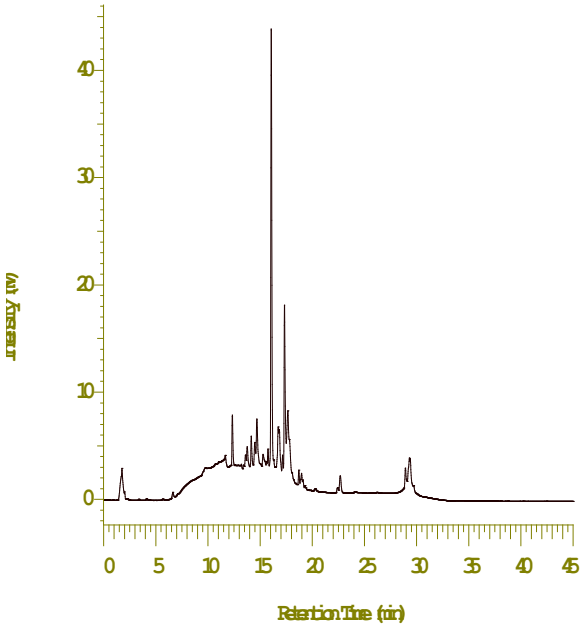


Figure C.4: Chromatogram of citric acid

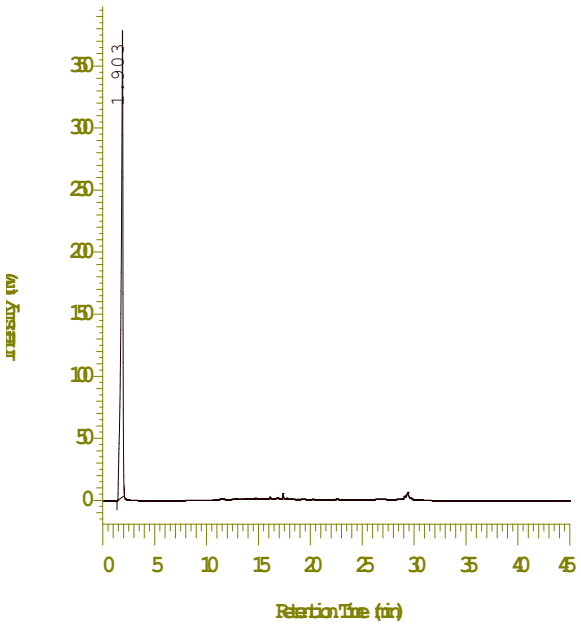


Figure C.5: Chromatogram of sodium metabisulfite

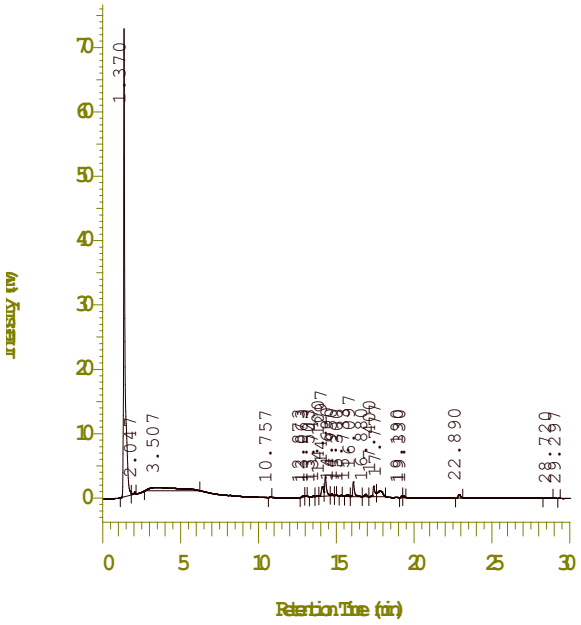


Figure C.6: Chromatogram of l-ascorbic acid

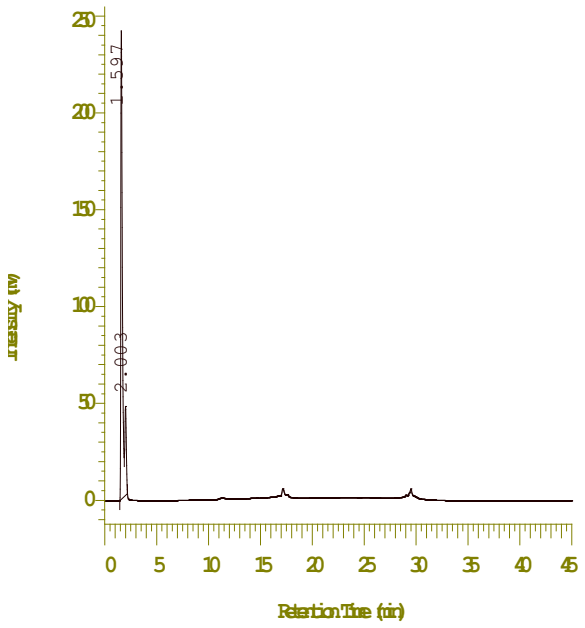


Figure C.7: Chromatogram of ascorbic acid used in gel preparations

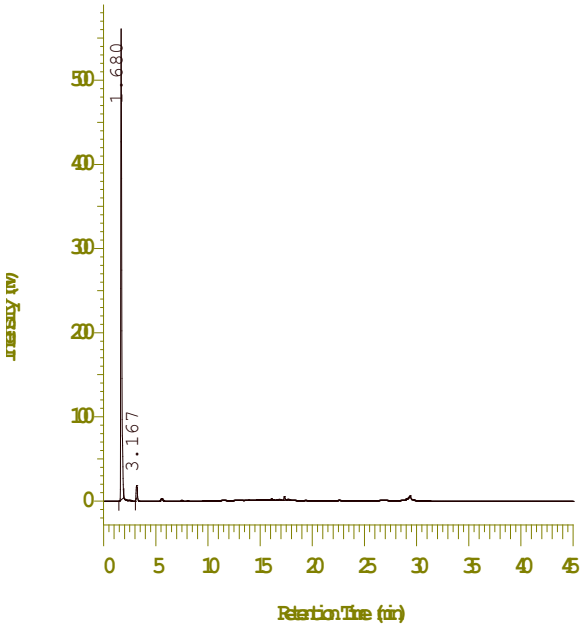


Figure C.8: Chromatogram of ascorbic acid and water (solvent for assays)

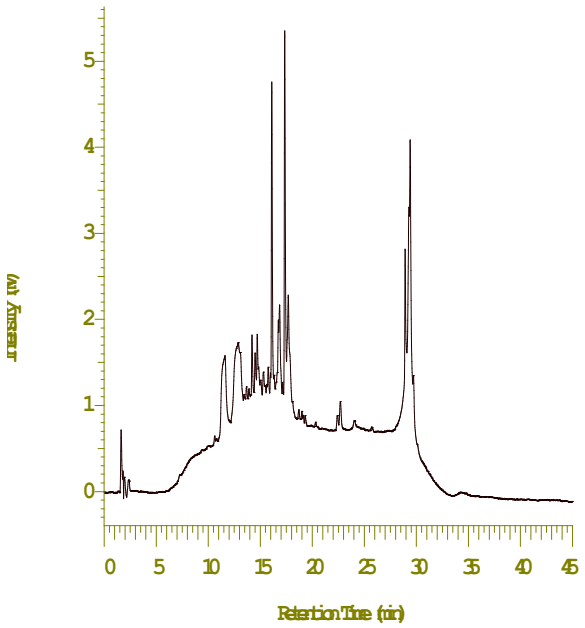


Figure C.9: Chromatogram of triethanolamine

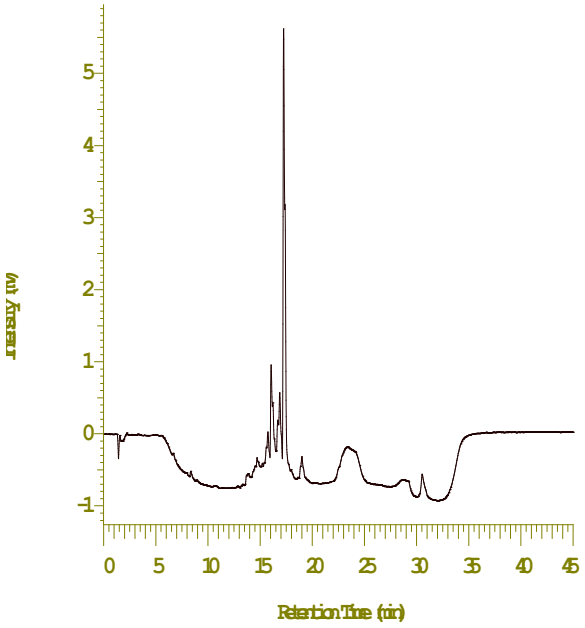


Figure C.10: Chromatogram of acetate buffer solution

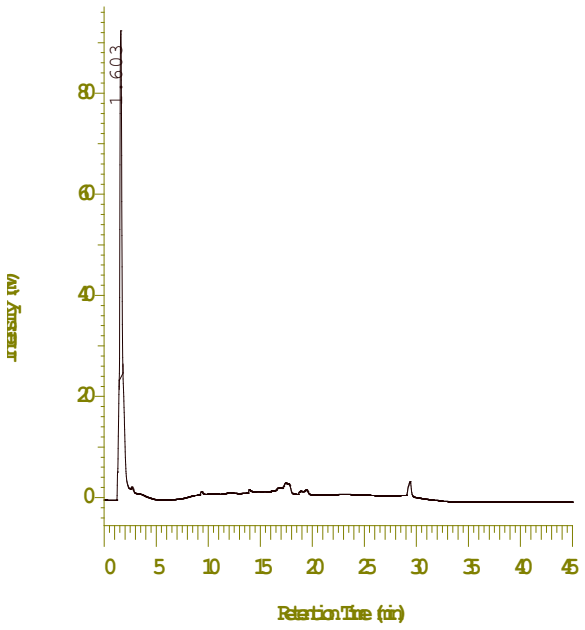


Figure C.11: Chromatogram of placebo gel mixture

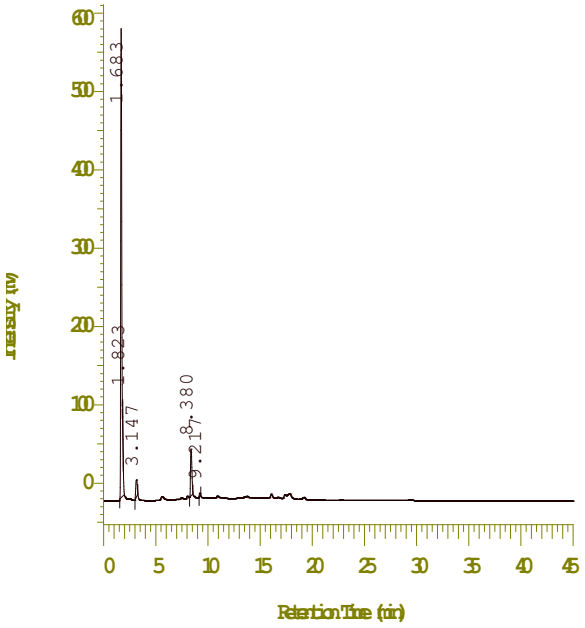


Figure C.12: Chromatogram of aspalathin peak at 8.3 min in a gel assay

ADDENDUM D

PILOT GEL DISCREPANCIES

Figure D.1: Photographs illustrating phase separation in pilot gel 2

Figure D.2: Photograph illustrating active migration of rooibos extract in pilot gel 4

ADDENDUM E

pH

Table E.1: pH values of the different gels stored at the specific storage conditions at pre-determined time intervals, with the average (AVG), SD and %RSD of each gel after each month and stability conditions

	Gel 1	Gel 2	Gel 3	Gel 4	Gel 5	Gel 6	Gel 7	Control gel
Initial/ T0								
A/C*	4.755	4.641	5.008	4.930	4.779	4.547	4.681	4.824
A/C*	4.734	4.639	5.036	4.932	4.787	4.535	4.689	4.991
A/C*	4.678	4.661	5.060	4.944	4.802	4.540	4.701	4.989
AVG	4.722	4.647	5.035	4.935	4.789	4.541	4.690	4.935
SD	0.032	0.010	0.021	0.006	0.010	0.005	0.008	0.078
%RSD	0.688	0.214	0.422	0.125	0.199	0.108	0.175	1.586
Month 1/ T1								
A*	4.825	4.794	5.126	5.032	4.945	4.712	4.814	5.062
A*	4.833	4.795	5.155	5.088	4.929	4.717	4.872	5.106
A*	4.824	4.802	5.223	5.116	4.927	4.716	4.886	5.106
AVG	4.827	4.797	5.168	5.079	4.934	4.715	4.857	5.091
SD	0.004	0.004	0.041	0.035	0.008	0.002	0.031	0.021
%RSD	0.083	0.074	0.787	0.688	0.163	0.046	0.642	0.407
C*	4.834	4.800	5.158	5.065	4.902	4.727	4.819	5.123
C*	4.848	4.835	5.173	5.114	4.907	4.738	4.767	5.109
C*	4.850	4.856	5.194	5.120	4.910	4.759	4.791	5.142
AVG	4.844	4.830	5.175	5.100	4.906	4.741	4.792	5.125
SD	0.007	0.023	0.015	0.025	0.003	0.013	0.021	0.014
%RSD	0.147	0.478	0.285	0.483	0.067	0.280	0.443	0.264

*Storage condition A = (25 ± 2 °C/60 ± 5 % RH) and C = (40°C ± 2°C/75 ± 5 % RH).

Table E.1 (continued)

	Gel 1	Gel 2	Gel 3	Gel 4	Gel 5	Gel 6	Gel 7	Control gel
Month 2/ T2								
A *	4.662	4.716	4.955	4.955	4.776	4.594	4.787	5.070
A*	4.666	4.716	4.971	4.960	4.780	4.603	4.783	5.069
A*	4.671	4.715	5.040	4.963	4.786	4.609	4.785	5.069
AVG	4.666	4.716	4.989	4.959	4.781	4.602	4.785	5.069
SD	0.004	0.000	0.037	0.003	0.004	0.006	0.002	0.000
%RSD	0.079	0.010	0.739	0.067	0.086	0.134	0.034	0.009
C*	4.612	4.630	4.997	4.918	4.794	4.644	4.800	5.069
C*	4.615	4.637	5.012	4.927	4.799	4.650	4.788	5.070
C*	4.600	4.638	5.015	4.944	4.806	4.649	4.781	5.068
AVG	4.609	4.635	5.008	4.930	4.800	4.648	4.790	5.069
SD	0.006	0.004	0.008	0.011	0.005	0.003	0.008	0.001
%RSD	0.141	0.077	0.157	0.219	0.103	0.056	0.164	0.016
Month 3/ T3								
A*	4.506	4.496	4.865	4.737	4.649	4.408	4.514	4.757
A*	4.498	4.495	4.874	4.705	4.647	4.418	4.519	4.778
A*	4.492	4.487	4.874	4.733	4.649	4.415	4.544	4.786
AVG	4.499	4.493	4.871	4.725	4.648	4.414	4.526	4.774
SD	0.006	0.004	0.004	0.014	0.001	0.004	0.013	0.012
%RSD	0.127	0.090	0.087	0.301	0.020	0.095	0.290	0.256
C*	4.538	4.679	4.792	4.805	4.737	4.464	4.522	4.978
C*	4.529	4.539	4.817	4.823	4.733	4.454	4.526	4.858
C*	4.523	4.531	4.826	4.787	4.628	4.447	4.534	4.848
AVG	4.530	4.583	4.812	4.805	4.699	4.455	4.527	4.895
SD	0.006	0.068	0.014	0.015	0.050	0.007	0.005	0.059
%RSD	0.136	1.483	0.299	0.306	1.074	0.157	0.110	1.207

*Storage condition A = (25 ± 2 °C/60 ± 5 % RH) and C = (40°C ± 2°C/75 ± 5 % RH).

ADDENDUM F

Table F.1: Photographic images of the front and back view of each gel at the specific storage conditions, with photographs from the respective time intervals in the columns.

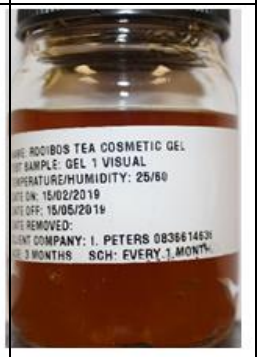
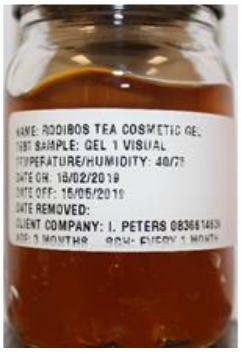
Gel 1	T0#	T1#	T2#	T3#
A*				
C*				
A*				
C*				

Table F.1: (continued)

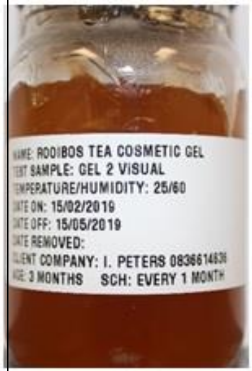
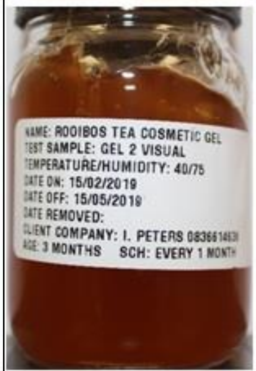
Gel 2	T0[#]	T1[#]	T2[#]	T3[#]
A*				
C*				
A*				
C*				

Table F.1: (continued)

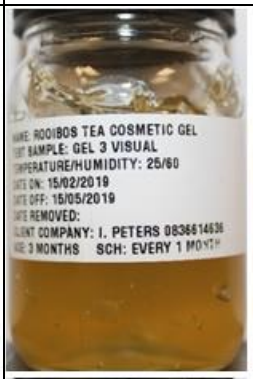
Gel 3	T0#	T1#	T2#	T3#
A*				
C*				
A*				
C*				

Table F.1: (continued)

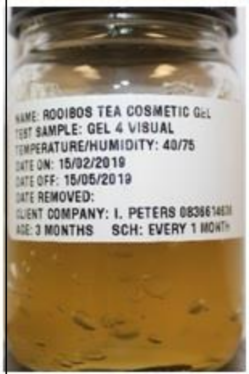
<table>
<tr><th>Gel 4</th><th>T0#</th><th>T1#</th><th>T2#</th><th>T3#</th></tr>
<tr><td>A*</td><td> 
</td><td> 
</td><td> 
</td><td> 
</td></tr>
<tr><td>C*</td><td></td><td> 
</td><td> 
</td><td> 
</td></tr>
<tr><td>A*</td><td>  </td><td>  </td><td>  </td><td>  </td></tr>
<tr><td>C*</td><td></td><td>  </td><td>  </td><td>  </td></tr>
</table>

Table F.1: (continued)

<table>
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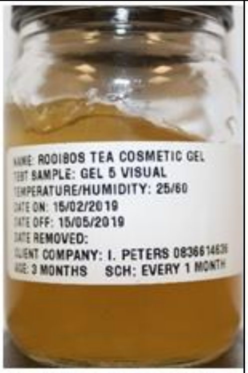
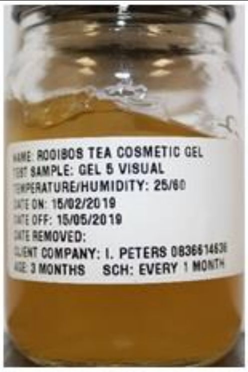
</td><td> 
</td><td> 
</td></tr>
<tr><td>C*</td><td></td><td> 
</td><td> 
</td><td> 
</td></tr>
<tr><td>A*</td><td>  </td><td>  </td><td>  </td><td>  </td></tr>
<tr><td>C*</td><td></td><td>  </td><td>  </td><td>  </td></tr>
</table>

Table F.1: (continued)

Gel 6	T0#	T1#	T2#	T3#
A*				
C*				
A*				
C*				

Table F.1: (continued)

<table>
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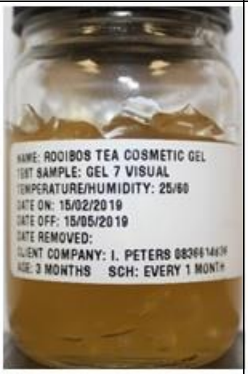
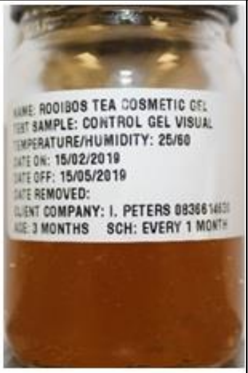
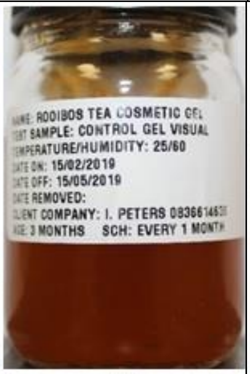
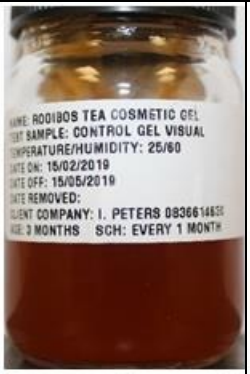
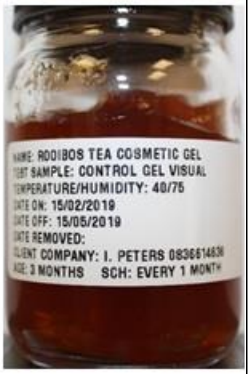
</td><td> 
</td><td> 
</td></tr>
<tr><td>C*</td><td> 
</td><td> 
</td><td> 
</td></tr>
<tr><td>A*</td><td rowspan="2">  </td><td>  </td><td>  </td><td>  </td></tr>
<tr><td>C*</td><td>  </td><td>  </td><td>  </td></tr>
</table>

Table F.1: (continued)

Control gel	T0#	T1#	T2#	T3#
A*				
C*				
A*				
C*				

*Storage condition A = (25 ± 2 °C/60 ± 5 % RH) and C = (40°C ± 2°C/75 ± 5 % RH). #T1 = after 1 month, T2 = after 2 months and T3 = after 3 months

ADDENDUM G

PAINT COLOUR CHARTS

Figure G.1: Paint colour charts from Royal Talens used to describe colour changes in gel formulations

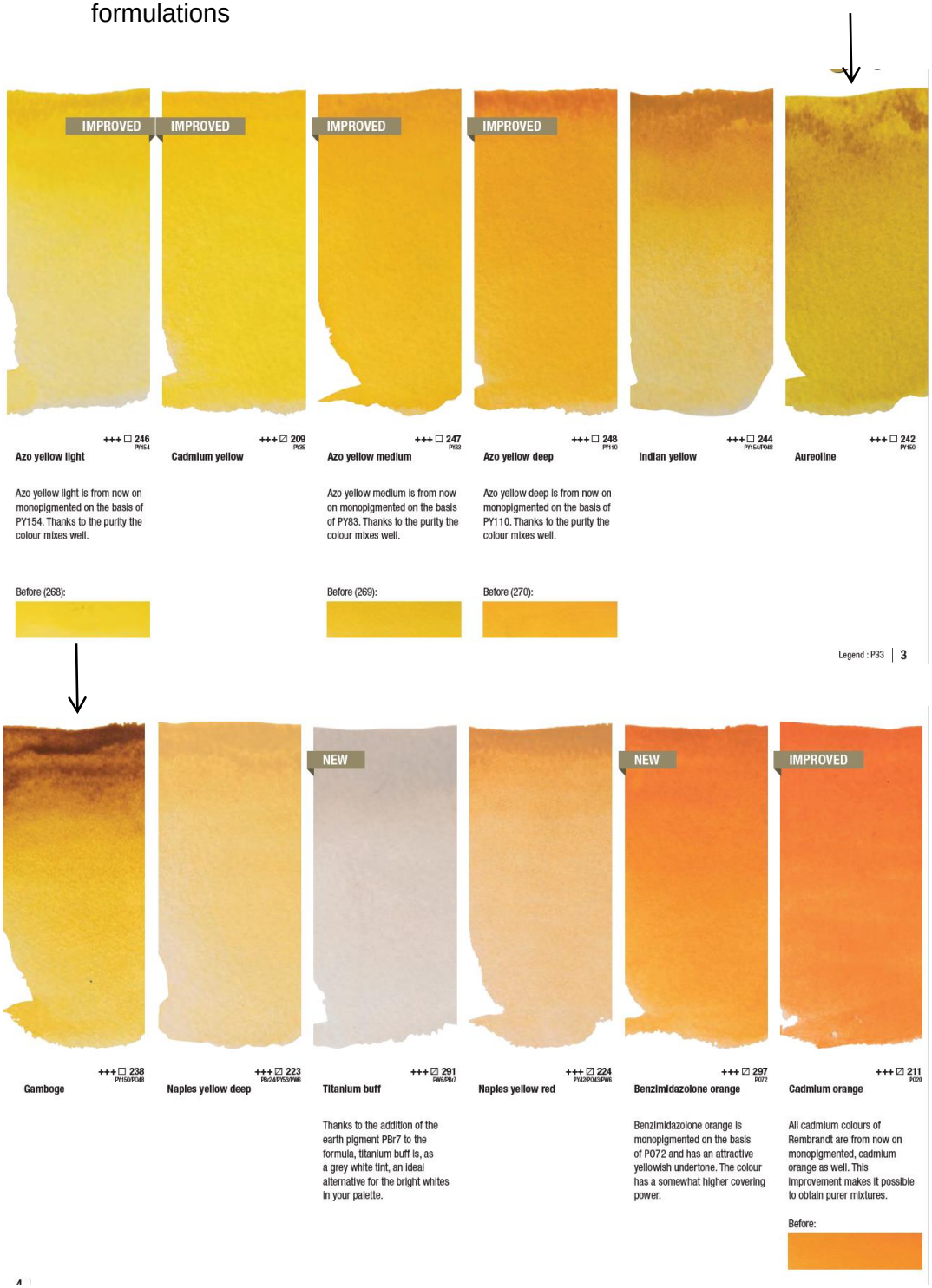


Figure G.1 (continued)

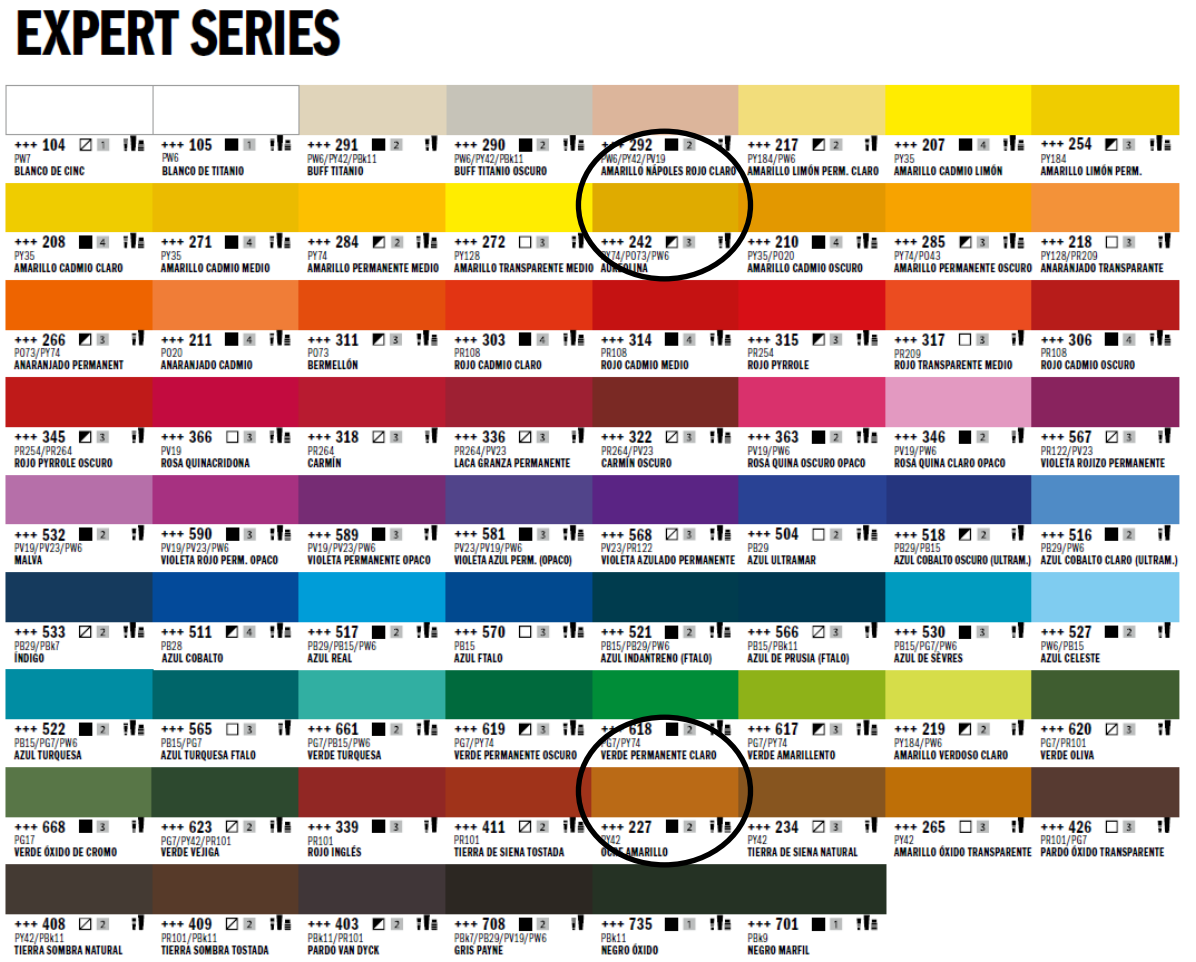
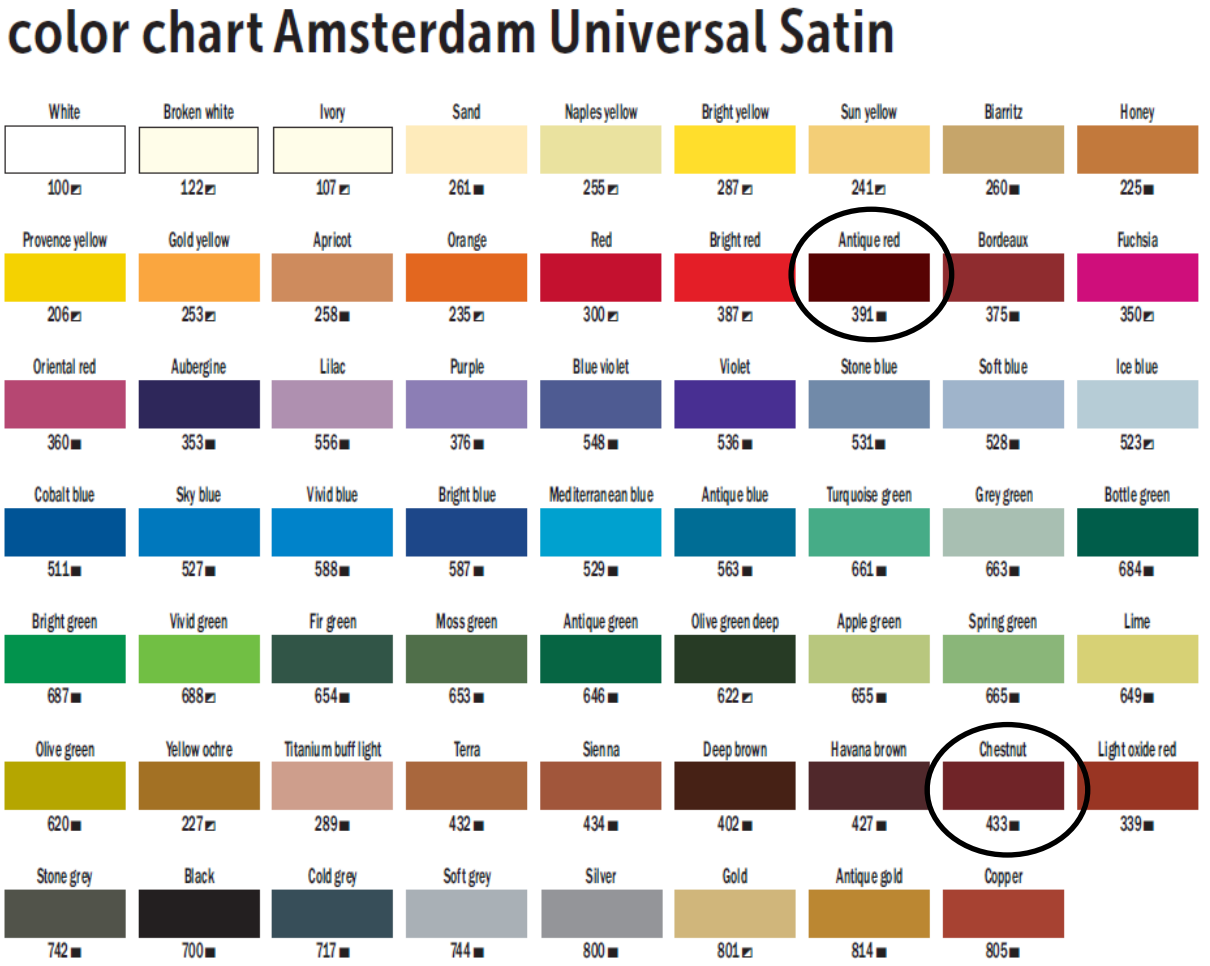


Figure G.1 (continued)



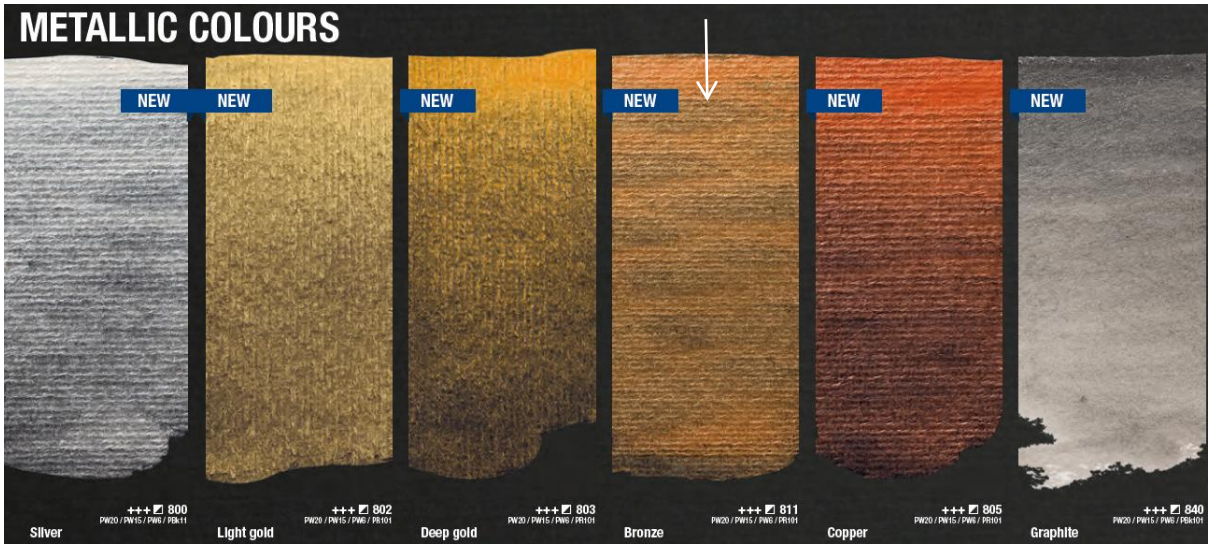


Figure G.1 (continued)

ADDENDUM H

IN VITRO RELEASE DATA

Table H.1: Percentage aspalathin released from gel 1 at the pre-determined time intervals and different chambers with apparent permeability coefficient (P_{app}), standard deviation (SD) and average values

Gel 1	**Percentage release**							
Time (min)	**Chamber 1**	**Chamber 2**	**Chamber 3**	**Chamber 4**	**Chamber 5**	**Chamber 6**	**SD**	**Average**
0	0.000	0.000	0.000	0.000	0.000	0.000	0.0	0.00
30	0.000	0.493	0.582	0.713	0.594	0.671	0.0	0.31
60	1.484	0.924	0.752	1.694	1.962	1.809	0.2	1.44
180	3.804	3.263	4.269	4.303	0.268	4.614	0.5	3.42
300	0.513	7.048	8.298	6.806	5.661	6.249	1.5	5.76
360	8.092	5.972	5.451	3.811	6.561	6.198	2.5	6.01
P_{app} (E-06)	1.434	1.856	1.959	1.444	1.625	1.778	0.2	1.68

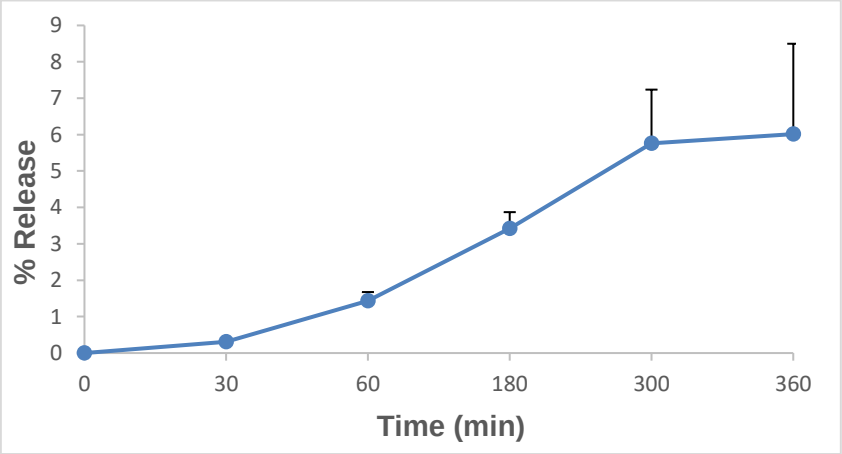


Figure H.1: Percentage aspalathin released from gel 1 at pre-determined time intervals

Table H.2: Percentage aspalathin released from gel 2 at the pre-determined time intervals and different chambers with apparent permeability coefficient (P_{app}), standard deviation (SD) and average values

Gel 2	Percentage release							
Time (min)	**Chamber 1**	**Chamber 2**	**Chamber 3**	**Chamber 4**	**Chamber 5**	**Chamber 6**	**SD**	**Average**
0	0.000	0.000	0.000	0.000	0.000	0.000	0.0	0.00
30	0.000	0.000	0.000	0.000	0.000	0.000	0.0	0.00
60	0.634	1.172	1.510	1.406	1.244	0.551	0.4	1.09
180	2.326	2.125	3.019	3.327	3.304	2.992	0.5	2.85
300	3.404	3.493	1.580	3.857	5.030	5.053	1.2	3.74
360	3.927	4.703	4.274	3.994	4.164	2.366	0.7	3.90
P_{app} (E-06)	1.080	1.177	0.864	1.106	1.290	1.0335	0.1	1.09

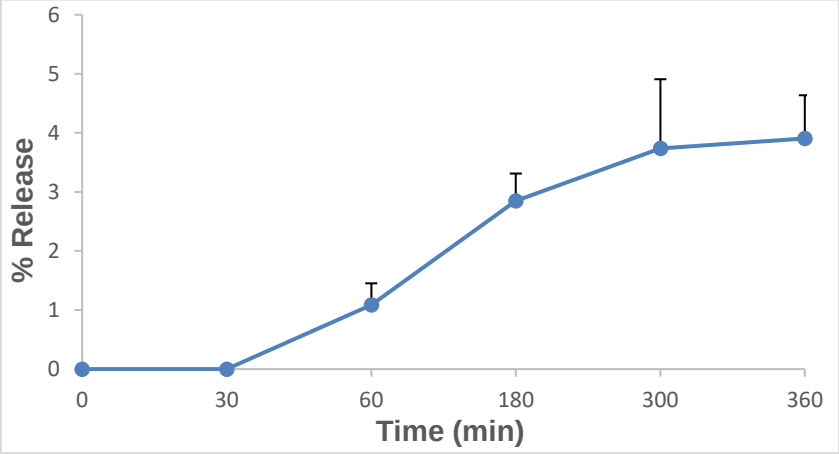


Figure H.2: Percentage aspalathin released from gel 2 at pre-determined time intervals

Table H.3: Percentage aspalathin released from gel 3 at the pre-determined time intervals and different chambers with apparent permeability coefficient (P_{app}), standard deviation (SD) and average values

Gel 3	Percentage release							
Time (min)	**Chamber 1**	**Chamber 2**	**Chamber 3**	**Chamber 4**	**Chamber 5**	**Chamber 6**	**SD**	**Average**
0	0.000	0.000	0.000	0.000	0.000	0.000	0.0	0.00
30	0.593	0.000	0.000	0.000	0.000	0.000	0.2	0.00
60	0.085	0.000	0.000	0.000	0.000	0.000	0.0	0.01
180	1.096	1.034	1.188	1.987	1.892	2.021	0.4	1.54
300	2.368	2.613	3.320	2.649	4.191	3.734	0.7	3.15
360	3.019	3.364	3.529	2.157	4.180	4.050	0.7	3.38
P_{app} (E-06)	0.756	0.912	1.029	0.730	1.260	1.185	0.2	0.97

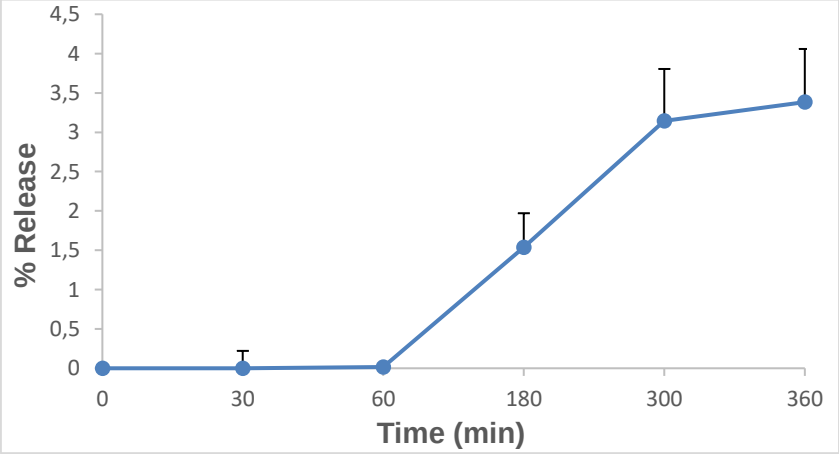


Figure H.3: Percentage aspalathin released from gel 3 at pre-determined time intervals

Table H.4: Percentage aspalathin released from gel 4 at the pre-determined time intervals and different chambers with apparent permeability coefficient (P_{app}), standard deviation (SD) and average values

Gel 4	Percentage release							
Time (min)	**Chamber 1**	**Chamber 2**	**Chamber 3**	**Chamber 4**	**Chamber 5**	**Chamber 6**	**SD**	**Average**
0	0.000	0.000	0.000	0.000	0.000	0.000	0.0	0.00
30	0.000	0.000	0.000	0.000	0.000	0.000	0.0	0.00
60	0.687	0.390	0.609	0.599	0.611	0.777	0.1	0.61
180	2.984	2.118	3.116	1.725	2.347	2.557	0.5	2.47
300	2.123	2.100	0.433	2.866	4.310	2.915	1.2	2.46
360	2.035	3.678	1.378	2.920	4.727	2.910	1.1	2.94
P_{app} (E-06)	0.612	0.895	0.304	0.834	1.329	0.841	0.3	0.80

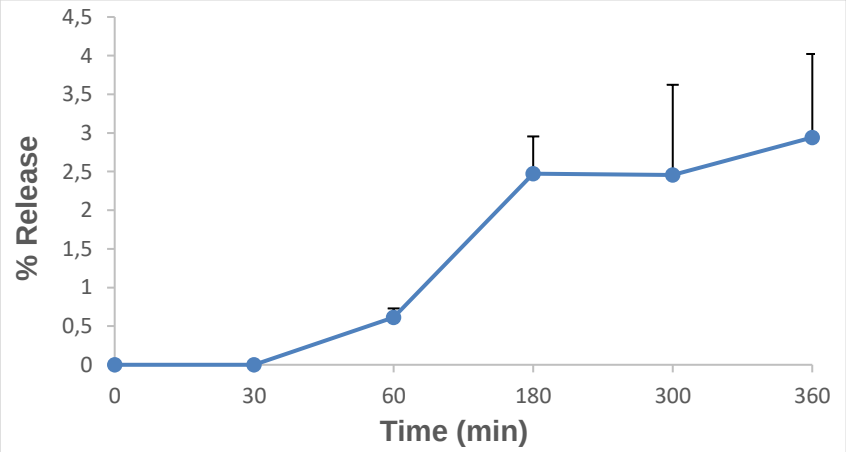


Figure H.4: Percentage aspalathin released from gel 4 at pre-determined time intervals

Table H.5: Percentage aspalathin released from gel 5 at the pre-determined time intervals and different chambers with apparent permeability coefficient (P_{app}), standard deviation (SD) and average values

Gel 5	Percentage release							
Time (min)	**Chamber 1**	**Chamber 2**	**Chamber 3**	**Chamber 4**	**Chamber 5**	**Chamber 6**	**SD**	**Average**
0	0.000	0.000	0.000	0.000	0.000	0.000	0.0	0.00
30	0.000	0.000	0.000	0.000	0.000	0.000	0.0	0.00
60	0.863	1.336	1.342	0.866	1.433	0.732	0.3	1.10
180	7.206	5.615	5.839	3.145	7.465	6.246	1.4	5.92
300	10.541	10.848	10.554	9.648	10.234	10.481	0.4	10.38
360	10.965	6.731	13.439	13.550	12.758	10.977	2.3	11.40
P_{app} (E-06)	3.223	3.587	3.459	2.472	3.478	3.214	0.4	3.24

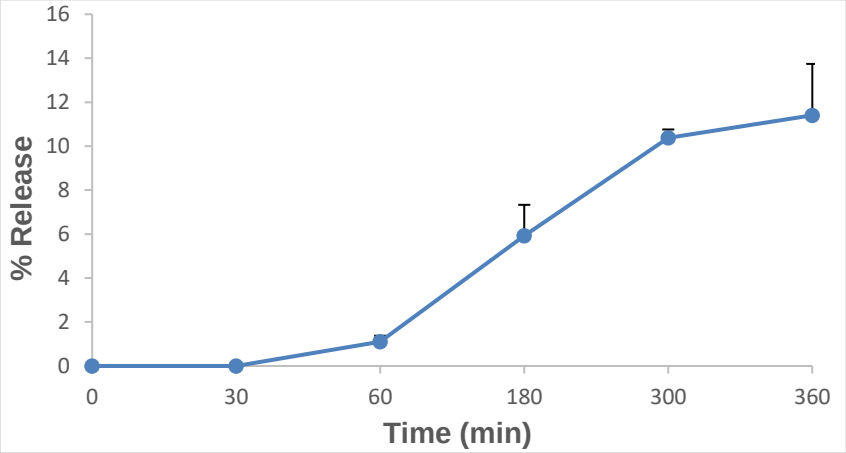


Figure H.5: Percentage aspalathin released from gel 5 at pre-determined time intervals

Table H.6: Percentage aspalathin released from gel 6 at the pre-determined time intervals and different chambers with apparent permeability coefficient (P_{app}), standard deviation (SD) and average values

Gel 6	Percentage release							
Time (min)	**Chamber 1**	**Chamber 2**	**Chamber 3**	**Chamber 4**	**Chamber 5**	**Chamber 6**	**SD**	**Average**
0	0.000	0.000	0.000	0.000	0.000	0.000	0.0	0.00
30	0.000	0.000	0.000	0.000	0.000	0.000	0.0	0.00
60	0.964	0.000	1.730	1.268	1.079	1.378	0.5	1.07
180	4.135	4.026	4.744	5.269	5.120	6.152	0.7	4.91
300	5.686	6.515	6.718	8.199	7.687	8.851	1.1	7.28
360	4.495	3.779	7.050	8.502	7.960	8.440	1.9	6.70
P_{app} (E-06)	1.465	1.978	2.310	1.517	2.453	2.531	0.4	2.04

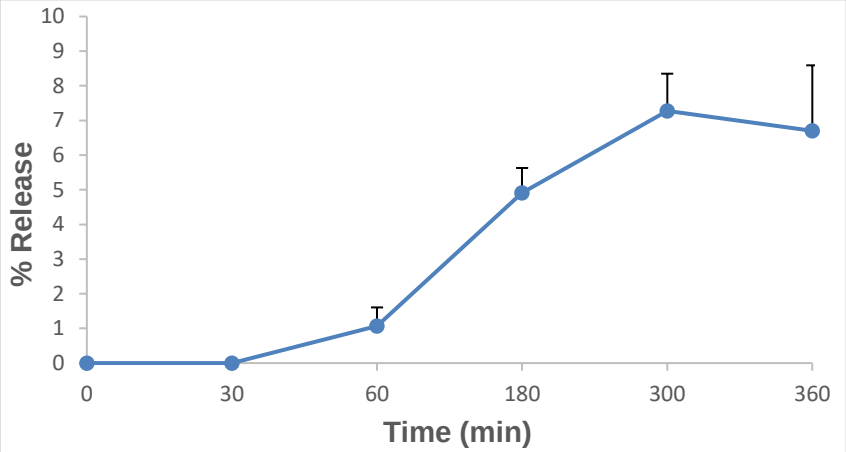


Figure H.6: Percentage aspalathin released from gel 6 at pre-determined time intervals

Table H.7: Percentage aspalathin released from gel 7 at the pre-determined time intervals and different chambers with apparent permeability coefficient (P_{app}), standard deviation (SD) and average values

Gel 7	Percentage release							
Time (min)	**Chamber 1**	**Chamber 2**	**Chamber 3**	**Chamber 4**	**Chamber 5**	**Chamber 6**	**SD**	**Average**
0	0.000	0.000	0.000	0.000	0.000	0.000	0.0	0.00
30	0.000	0.916	0.000	0.000	0.959	0.000	0.4	0.31
60	0.000	1.885	1.190	1.275	1.796	1.381	0.6	1.25
180	4.772	5.862	4.893	3.803	4.438	6.198	0.8	4.99
300	6.574	6.299	9.863	7.244	5.886	7.134	1.3	7.17
360	5.112	6.496	7.494	4.763	6.081	7.267	1.0	6.20
P_{app} (E-06)	1.767	2.480	1.601	1.748	1.667	2.124	0.3	1.90

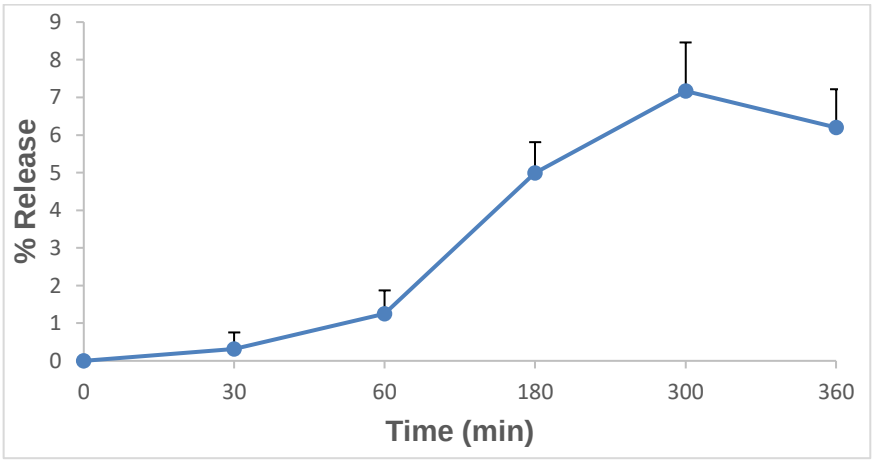


Figure H.7: Percentage aspalathin released from gel 7 at pre-determined time intervals

Table H.8: Percentage aspalathin released from the control gel at the pre-determined time intervals and different chambers with apparent permeability coefficient (P_{app}), standard deviation (SD) and average values

Control gel	Percentage release							
Time (min)	**Chamber 1**	**Chamber 2**	**Chamber 3**	**Chamber 4**	**Chamber 5**	**Chamber 6**	**SD**	**Average**
0	0.000	0.000	0.000	0.000	0.000	0.000	0.0	0.00
30	0.313	0.200	0.429	0.304	0.457	0.405	0.1	0.22
60	1.262	0.798	1.068	1.093	1.138	1.210	0.1	1.09
180	5.072	4.266	5.510	5.130	4.076	5.941	0.7	5.00
300	7.398	6.871	6.071	7.639	7.898	8.580	0.8	7.41
360	9.544	6.647	7.813	8.070	7.489	8.098	0.9	7.94
P_{app} (E-06)	2.497	1.971	2.054	2.291	2.181	2.406	1.9	2.23

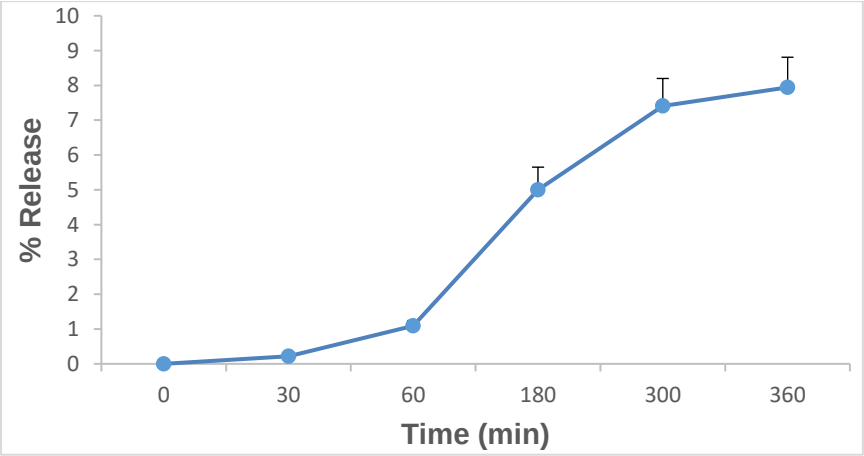


Figure H.8: Percentage aspalathin released from the control gel at pre-determined time intervals

ADDENDUM I

STATISTICAL ANALYSIS (RELEASE DATA)

Table I.1: The 2-Way Table of Descriptive Statistics for aspalathin across synthetic membranes

Name	2-Way Tables of Descriptive Statistics N=54 (No missing data in dep. var. list)		
	P_{app} (transport) Means	P_{app} (transport) N	P_{app} (transport) Standard Deviation.
Gel 1	0.000002	6	0.000000
Gel 2	0.000001	6	0.000000
Gel 3	0.000001	6	0.000000
Gel 4	0.000001	6	0.000000
Gel 5	0.000003	6	0.000000
Gel 6	0.000002	6	0.000000
Gel 7	0.000002	6	0.000000
Control gel	0.000002	6	0.000000
All Groups	0.000002	48	0.000001

Table I.2: The Brown-Forsythe Test of Homogeneity of Variances for aspalathin across synthetic membranes

Variable	Brown-Forsythe Test of Homog. of Variances Marked effects are significant at p < .05000						
	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F
P_{app} (transport)	0.000000	8	0.000000	0.000000	45	0.000000	1.006654

Variable	Brown-Forsythe Test of Homog. of Variances Marked effects are significant at p < .05000
	p
P_{app} (transport)	0.444581

Table I.3: Analysis of Variance test for aspalathin across synthetic membranes

Variable	Analysis of Variance Marked effects are significant at p < .05000						
	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F
P_{app} (transport)	0.000000	8	0.000000	0.000000	45	0.000000	41.77623

Variable	Analysis of Variance. Marked effects are significant at p < .05000
	p
P_{app} (transport)	0.000000

Table I.4: The Tukey Honest Significant Difference post hoc test for aspalathin release from the different gel formulations across synthetic membranes

Name	Tukey HSD test; Variable: P_{app} (transport) Marked differences are significant at p < .05000							
	1 M=.00000	2 M=.00000	3 M=.00000	4 M=.00000	5 M=.00000	6 M=.00000	7 M=.00000	C M=.00000
Gel 1		0.041183	0.007217	0.000453	0.000143	0.539332	0.951247	0.074273
Gel 2	0.041183		0.999300	0.783530	0.000143	0.000226	0.001389	0.000144
Gel 3	0.007217	0.999300		0.984752	0.000143	0.000150	0.000292	0.000143
Gel 4	0.000453	0.783530	0.984752		0.000143	0.000143	0.000146	0.000143
Gel 5	0.000143	0.000143	0.000143	0.000143		0.000143	0.000143	0.000167
Gel 6	0.539332	0.000226	0.000150	0.000143	0.000143		0.995918	0.974814
Gel 7	0.951247	0.001389	0.000292	0.000146	0.000143	0.995918		0.623940
Control gel	0.074273	0.000144	0.000143	0.000143	0.000167	0.974814	0.623940	