

Topical delivery of selected female hormones from different formulations

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The best view comes after the hardest climb.

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ABSTRACT

Skin ageing is an unavoidable biological phenomenon inextricably linked to menopause. The rapid and drastic decline in oestrogen levels brought about by menopause has a profound effect on skin integrity and physiology, resulting in atrophic skin changes. Skin becomes wrinkled, thin and dry. Hormone replacement therapy (HRT) has become one of the cornerstones of anti-ageing practices, as it has been shown to improve skin thickness, hydration and elasticity. However, a positive correlation exists between the increased risk of breast cancer in postmenopausal women and conventional HRT. This is cause for concern, as breast cancer is the most common malignancy among women worldwide. Conventional HRT cannot however be used by women at risk due to genetic predisposition or those previously diagnosed with breast cancer. Idexis (compounding pharmacy) aimed to formulate complex systems that deliver female hormones topically, preventing systemic exposure, thereby reducing the risk of hormone-induced breast cancer. The female hormones used in the Idexis products were oestriol, oestradiol and progesterone.

For this study, three topical products were provided by Idexis, which included a face serum (**FS**), eye cream (**EC**) and male cream (**MC**). However, due to the classified nature of this study, limited information was made available. During this study, simple oil-in-water (o/w) emulsions were formulated containing the female hormones and selected natural oils (coconut, castor and emu oil), which was used as the oil phase and as penetration enhancer. The aim was to determine which formulation (o/w emulsion *versus* Idexis product) proved more advantageous for the topical delivery of the hormones, and in addition, to evaluate the efficacy of the polymers used in the Idexis products to inhibit systemic absorption.

Initially, three o/w emulsion formulas were characterised and compared; thereafter, the optimised formula was utilised to formulate three o/w emulsions containing the female hormones and the selected natural oils. Oestriol was the only female hormone that was quantifiable during the *in vitro* diffusion studies. Membrane release studies were performed to determine whether oestriol was released from the respective formulations. The flux values obtained indicated that oestriol was released from all six formulations. Skin diffusion studies were conducted to evaluate the extent of oestriol absorption through the skin, which is indicative of the transdermal delivery. Thereafter, tape stripping was performed to determine the amount of oestriol within the skin layers (topical delivery). Oestriol was delivered transdermally from the Idexis products, and to a lesser extent from the o/w emulsion containing castor oil (**CaOE**) and the o/w emulsion containing coconut oil (**CoOE**). It can be concluded that the aim of topical delivery was achieved only by the o/w emulsion containing emu oil (**EOE**).

Lastly, accelerated stability studies were performed on the Idexis products to determine whether any physicochemical changes occurred in the formulations due to elevated temperature and humidity variations, altering the stability and shelf-life of the products. The products were stored at three different conditions (25 °C/60% relative humidity (RH), 30 °C/65% RH and 40 °C/75% RH) for a period of 3 months. No product complied completely with the criteria of the International Conference on Harmonisation (ICH) for stability.

Keywords: Anti-ageing; postmenopausal; breast cancer; oestriol; topically

UITTREKSEL

Veroudering van die vel is 'n onvermydelike biologiese verskynsel wat onlosmaaklik aan menopause gekoppel is. Die vinnige en drastiese afname in estrogeenvlakke wat deur menopause teweeg gebring word, het 'n kenmerkende uitwerking op die velintegriteit en fisiologie, wat tot atrofiese velveranderings lei. Vel word geplooid, dun en droog. Hormoonvervangingsterapie (HVT) het een van die hoekstene van antiverouderingspraktyke geword, aangesien daar bewys is dat dit die veldikte, hidrasie en elasticiteit van die vel verbeter. Daar bestaan egter 'n positiewe korrelasie tussen die verhoogde risiko van borskanker in postmenopousale vroue en konvensionele HVT. Dit is kommerwekkend, aangesien borskanker die algemeenste kanker wêreldwyd onder vroue is. Konvensionele HVT kan dus nie gebruik word deur vroue met 'n genetiese predisposisie, of dié wat voorheen met borskanker gediagnoseer is, nie. Idexis (bereidingsapteek) het dit ten doel gestel om komplekse afleweringstelsens te formuleer wat vroulike hormone topikaal eerder as sistemies aflewering, om sodoende die risiko van hormoon-geïnduseerde borskanker te verminder. Idexis-produkte bevat die volgende vroulike hormone: oestriol, oestradiol en progesteron.

Vir hierdie studie is drie topikale produkte deur Idexis verskaf, wat 'n gesigserum (**GS**), oogroom (**OR**) en 'n gesigsroom vir mans (**GM**) insluit. Vanweë die geklassifiseerde aard van die studie is beperkte inligting egter beskikbaar gestel. Tydens hierdie studie is eenvoudige olie-in-water (o/w)-emulsies geformuleer wat die vroulike hormone en geselekteerde natuurlike olies (klapper-, kaster- en emoe-olie), wat as oliefase en penetrasie-bevorderaar gedien het, bevat. Die doel was om te bepaal watter formulering (o/w-emulsies teenoor Idexis-produkte) voordeliger is vir die topikale aflewering van die hormone, en ook om die effektiwiteit van die polimere, wat in die Idexis-produkte ingesluit is om sistemiese absorpsie te belemmer, te evalueer.

Aanvanklik is drie o/w-emulsieformules gekarakteriseer en vergelyk. Daarna is die geoptimaliseerde formule gebruik om drie o/w-emulsies te formuleer, wat die vroulike hormone en die geselekteerde natuurlike olies bevat. Oestriol was die enigste vroulike hormoon wat tydens *in vitro*-diffusiestudies kwantifiseerbaar was. Membraanvrystellingstudies is uitgevoer om te bepaal of die onderskeie formuleringe oestriol vrystel. Die vloedwaardes het aangedui dat al ses formuleringe oestriol vrygestel het. Veldiffusiestudies is uitgevoer om die omvang van oestriolabsorpsie deur die vel te evalueer, wat aanduiding gee dat transdermale aflewering plaasgevind het. Vervolgens is kleefbandstropping uitgevoer om die hoeveelheid oestriol wat in die verskeie vellae (topikale aflewering) teenwoordig is, te bepaal. Oestriol is transdermaal deur die Idexis-produkte, en in 'n mindere mate deur die o/w-emulsie wat kasterolie bevat (**KaOE**) en die o/w-emulsie wat klapperolie (**KOE**) bevat, afgelewer. Die gevolgtrekking kan

gemaak word dat slegs die o/w-emulsie wat emoe-olie bevat (**EmOE**), die doel vir topikale aflewering bereik het.

Ten slotte is versnelde stabiliteitsstudies op die Idexis-produkte uitgevoer om te bepaal of daar enige fisies-chemiese veranderinge in die formulerings plaasgevind het, as gevolg van verhoogde temperatuur en humiditeitsvariasies, wat die stabiliteit en rakleef tyd van die produkte kon beïnvloed. Die produkte is vir die tydperk van drie maande onder drie verskillende toestande (25 °C/60% relatiewe humiditeit (RH), 30 °C/65% RH en 40 °C/75% RH) geberg. Geen produk het volledig aan die stabiliteitskriteria van die International Conference of Harmonisation (ICH) voldoen nie.

Sleutelwoorde: Antiveroudering, postmenopousale, borskanker, oestriol, topikaal

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ABBREVIATIONS

%RSD	Percentage relative standard deviation
ALA	Alpha lipoic acid
ANOVA	Analysis of variance
ANVISA	Brazil National Health Surveillance Agency
API	Active pharmaceutical ingredient
APVMA	Australian Pesticides and Veterinary Medicines Authority
ATL	Analytical Technology Laboratory
CaOE	O/w emulsion containing castor oil
CBHRT	Custom-compounded bioidentical hormone replacement therapy
CEE	Conjugated equine oestrogens
CL	Caffeine liposomes
COLIPA	The European Cosmetic, Toiletry and Fragrance Association
CoOE	O/w emulsion containing coconut oil
CoQ10	Co-enzyme Q10
CPMP	Committee for Proprietary Medicinal Products
DNA	Deoxyribonucleic acid
EC	Eye cream
ED	Epidermis-dermis
EFSA	European Food Safety Authority
EMA	European Medicines Agency
EmOE	Olie-in-water-emulsie wat emoe-olie bevat
EOE	O/w emulsion containing emu oil
ER α	Oestrogen receptor α
ER β	Oestrogen receptor β
FDA	Food & Drug Administration
FS	Face serum

FSR	Full scale range
GM	Gesigsroom vir mans
GS	Gesigserum
GTE	Green tea extract
H ₂ O ₂	Hydrogen peroxide
H ₃ PO ₄	Orthophosphoric acid
HCl	Hydrochloric acid
HLB	Hydrophilic-lipophilic balance
HPLC	High performance liquid chromatography
HRT	Hormone replacement therapy
HSD	Honest significance difference
HVT	Hormoonvervangende terapie
ICH	International Conference on Harmonisation
IMS	The International Menopause Society
KaOE	Olie-in-water-emulsie wat kasterolie bevat
KH ₂ PO ₄	Potassium dihydrogen orthophosphate
KOE	Olie-in-water-emulsie wat klapperolie bevat
LD	Laser diffraction
LLOD	Lower limit of detection
LLOQ	Lower limit of quantification
LOD	Limit of detection
Log P	Octanol-water partition coefficient
LOQ	Limit of quantification
MC	Male cream
MMPs	Matrix metalloproteinases
MPA	Medroxyprogesterone acetate
MSDS	Material Safety Data Sheet
MUFA	Monounsaturated fatty acids

MVR	Minimum viscosity range
MW	Molecular weight
NAMS	The North American Menopause Society
NaOH	Sodium hydroxide
NMF	Natural moisturising factor
NOE	Natural oil emulsions
NWU-HREC	North-West University Health Research Ethics Committee
OECD	Organisation for Economic Co-operation and Development
OEW1	O/w emulsion formula 1
OEW2	O/w emulsion formula 2
OEW3	O/w emulsion formula 3
OR	Oogroom
o/w	Oil-in-water
PBS	Phosphate buffer solution
PCP	Potassium cetyl phosphate
PCS	Photon correlation spectroscopy
PdI	Polydispersity index
PEG	Polyethylene glycol
PR	Progesterone receptor
PR α	Progesterone receptor α
PR β	Progesterone receptor β
PTFE	Polytetrafluoroethylene
PUFA	Polyunsaturated fatty acids
PVDF	Polyvinylidene fluoride
R ²	Coefficient of determination
RH	Relative humidity
ROS	Reactive oxygen species
SCCP	Scientific Committee on Consumer Products

SCE	Stratum corneum-epidermis
SD	Standard deviation
SFA	Saturated fatty acids
SPF	Sun protection factor
TEWL	Transepidermal water loss
UFA	Unsaturated fatty acids
UNODC	United Nations Office on Drugs and Crime
UV	Ultraviolet
UVA	Ultraviolet A
UVB	Ultraviolet B
UVC	Ultraviolet C
UVR	Ultraviolet radiation
VEA	Vitamin E acetate
WHI	Women's Health Initiative
WHO	World Health Organization
w/o	Water-in-oil
w/v	Weight per volume

CHAPTER 1:

INTRODUCTION, RESEARCH PROBLEM AND AIMS

1.1 Introduction

Skin ageing is a degenerative process associated with the loss of physiological function and structural stability in the skin due to the cumulative effects of intrinsic (chronological) and extrinsic (environmental) mechanisms (Bocheva *et al.*, 2019:2798; Zhang & Duan, 2018:729). Intrinsic ageing is an unavoidable, genetically determined process, mainly caused by the accumulation of harmful by-products of cellular metabolism, along with an increase in cellular ageing. Extrinsic ageing is caused by external factors, such as ultraviolet radiation (UVR), tobacco smoking, air pollution, repetitive muscle movements (i.e. frowning or squinting) and sleeping position (Krutmann *et al.*, 2017:158; Piérard *et al.*, 2013); these factors are controllable to varying degrees (Farage *et al.*, 2008:88). Menopause is closely related to skin ageing (Dalal & Agarwal, 2015:S222). Oestrogen has a profound effect on skin physiology and integrity; therefore, the drastic decrease in circulating levels with the onset of menopause causes premature ageing in women when compared with men of similar age (Farage *et al.*, 2013:5). Oestrogen deficiency leads to atrophic skin changes; skin becomes dry, thin and wrinkled with a decrease in elasticity and reduced vascularity (Stevenson & Thornton, 2007:286; Thornton, 2013:264). Hormone replacement therapy (HRT) is one of the key elements of anti-ageing practices (Samaras *et al.*, 2014:1176), as it has been shown to increase skin thickness, elasticity and epidermal hydration (Stevenson & Thornton, 2007:283).

Breast cancer is the most commonly diagnosed malignancy in women worldwide (Bray *et al.*, 2018:394; Sun *et al.*, 2017:1387). The term hormone-dependant cancer is defined as one that turned cancerous under the same hormonal effects that control tissue growth (Dargent, 1971:307). Various studies have shown that the increased risk for breast cancer in postmenopausal women is linked to the elevated circulating concentrations of endogenous hormones, especially oestrogen and androgens (Kaaks *et al.*, 2005:1071; Key *et al.*, 2011:709; Missmer *et al.*, 2004:1862). Hence, women diagnosed (or previously diagnosed) with breast cancer or who are at risk due to genetic predisposition cannot use conventional HRT.

Topical delivery systems aim to retain the active pharmaceutical ingredient (API) in the skin, reducing systemic exposure, and in the case of female hormones, the related risk of breast cancer development (Garg *et al.*, 2015:969). Various studies have reported that the topical application of hormones, significantly reduced the clinical manifestations of facial ageing (i.e. wrinkling and thinness) (Kanda & Watanabe, 2005:6; Sator *et al.*, 2004:39; Silva *et al.*, 2017:845; Stevenson & Thornton, 2007:287; Thornton, 2013:265). Moreover, topical drug

delivery presents with numerous advantages, such as sustained and controlled drug delivery, circumvention of the hepatic first-pass metabolism and reduced systemic adverse effects (Leite-Silva *et al.*, 2012:384). The skin, however, poses a formidable barrier against the percutaneous absorption of APIs (Wiechers, 1989:185). The skin is an easily accessible site for drug administration (Benson, 2012:3; Deepika *et al.*, 2013:89), as it covers a surface area of roughly 2 m² in average adults (Hadgraft, 2001:1). It is composed of four distinctive layers: the stratum corneum, viable epidermis, viable dermis and hypodermis (Foldvari, 2000:417). The barrier function of the skin is primarily mediated by the stratum corneum due to its “brick and mortar” structure, in which 10 to 15 layers of protein-rich corneocytes serve as the “bricks” and the lipid bilayer as the “mortar” (Lee *et al.*, 2006:293; Ng, 2018:51; Ruela *et al.*, 2016:527; Zhou *et al.*, 2018:1713). The lipid bilayer, composed of ceramides, cholesterol and free fatty acids (Coderch *et al.*, 2003:107), is responsible for the selective permeability of the stratum corneum, regulating the movement of compounds across the skin (Williams, 2003:10).

A lipophilic API will easily permeate through the stratum corneum; however, the permeation rate will decrease once it reaches the hydrophilic viable epidermis (Holmgaard & Nielsen, 2009:20). Hence, certain physicochemical properties are required for successful topical drug delivery, which enables the API to penetrate through the stratum corneum to reach the target-site (Alkilani *et al.*, 2015:444). These physicochemical properties include molecular weight (MW), aqueous solubility, octanol-water partition coefficient (log P) and melting point, to name a few. Since most APIs do not possess the ideal physicochemical properties, selecting an appropriate vehicle as drug carrier is essential to overcome these limitations.

Emulsions are colloidal systems composed of two or more immiscible liquids, usually oil and water (Smith, 2003:262), in which one of the liquids is uniformly dispersed as droplets (0.1 – 5.0 µm) (dispersed phase) throughout the other (continuous phase). Depending on whether the dispersed phase is oil or water, emulsions are classified as oil-in-water (o/w) or water-in-oil (w/o) emulsions (Lu & Gao, 2010:86; Piacentini, 2016:679). These systems present with many advantages, including the ability to deliver either lipophilic or hydrophilic APIs in o/w or w/o emulsions respectively, and therefore, improve the percutaneous absorption of water and oil-insoluble compounds (Buchmann, 2001:149; Shaker *et al.*, 2019:20). However, emulsions are thermodynamically unstable and phase separation can occur over time through creaming, flocculation or coalescence (Goodarzi & Zendehboudi, 2018:284; Ngan *et al.*, 2014:1). Emulsion stability is greatly related to droplet size (Goodarzi & Zendehboudi, 2018:281). In general, smaller droplets can be achieved with the use of a high-energy input (Hu *et al.*, 2017:17). Hence, high-pressure homogenisation can be used to produce more elegant and kinetically stable dispersions, by decreasing the droplet size and improving the homogeneity in the emulsion (Ding & Kan, 2017:4502; Engman *et al.*, 1998; Hebishy *et al.*, 2013:166).

Numerous techniques have aimed to overcome the barrier function of the stratum corneum and improve drug permeation (Sugino *et al.*, 2009:1453). Penetration enhancers are compounds that have the ability to either disrupt the lipid bilayer or extract the component providing the barrier function, and in turn, increase the percutaneous absorption of APIs (Patel *et al.*, 2019). Fatty acids (in natural oils) have been used as chemical penetration enhancers, as they have the ability to alter the stratum corneum barrier function by means of fluidisation and perturbation, and therefore, increase the permeability of APIs through the skin (Correa *et al.*, 2014:39; Haque & Talukder, 2018:174; Kezutyte *et al.*, 2013:3). Fatty acids can be divided into two groups: unsaturated fatty acids (UFA) and saturated fatty acids (SFA). It has been shown that UFA present with higher penetration-enhancing effects compared to SFA (Ibrahim & Li, 2010:116). Natural oils are frequently used in skin care products, as they are considered non-hazardous and safe due to their natural origin (Dreger & Wielgus, 2013:150). In addition, the presence of fatty acids in the skin lowers the likelihood of skin irritation with the use of natural oils (Büyüktimkin *et al.*, 1997:433; Vermaak *et al.*, 2011:922). Simple o/w emulsions will be formulated using selected natural oils as the oil phase and penetration enhancers in an attempt to overcome the barrier function of the stratum corneum, and deliver the incorporated female hormones to the target-site (epidermis-dermis). The selected natural oils (coconut, castor and emu oil) will form the core component, together with the lipophilic hormones (oestriol, oestradiol and progesterone).

In addition to the simple o/w emulsions formulated during this study, complex topical formulations containing female hormones were also investigated. These formulations were provided by Idexis (compounding pharmacy), which included a face serum (**FS**), eye cream (**EC**) and male cream (**MC**). The female hormones include oestriol, oestradiol and progesterone.

1.2 Research problem

The profound anti-ageing effects that hormones have on human skin, influencing various skin functions (Holzer *et al.*, 2005:633; Kanda & Watanabe, 2005:6; Shin *et al.*, 2005:315), have led to the formulation of numerous skin care products to meet the ever-increasing demand for youthful-looking skin. However, transdermally delivered hormones pose an increased risk for cancer in postmenopausal women. Oestrogen increases the risk of endometrial cancer, but combination therapy with progesterone has shown to minimise this adverse effect. However, combination oestrogen-progesterone therapy causes a significant increase in the incidence of breast cancer, greater than any other hormone therapy (Persson *et al.*, 1999:253).

1.3 Aims and objectives

The main aim of this study was to investigate whether targeted delivery (epidermis-dermis) of the female hormones (oestriol, oestradiol and progesterone) was achieved with the **(FS)**, **(EC)** and **(MC)**. Three simple o/w emulsions were formulated using selected natural oils (coconut, castor and emu oil), each containing the respective female hormones. Subsequently, the topical delivery of the female hormones from the simple emulsions will be compared to that of the complex Idexis products to evaluate the efficacy of the vehicle and polymers used in the Idexis products to inhibit: 1) penetration beyond the dermis and, as a result, 2) reduce the risk of hormone-induced breast cancer in postmenopausal women.

The objectives of this study were:

- To develop a validated high performance liquid chromatography (HPLC) method to determine the concentrations of the selected female hormones.
- To formulate three simple o/w emulsions containing female hormones and selected natural oils (separately) as the oil phase and as penetration enhancer.
- To characterise the three simple o/w emulsions (pre-formulated and optimised) with regard to particle size and distribution, pH, zeta-potential and viscosity.
- To characterise and conduct stability studies on the respective Idexis products at predetermined time intervals (months 0, 1, 2 and 3), evaluating the concentration assay, visual appearance, mass loss, pH, particle size and distribution, zeta-potential and viscosity.
- To determine the release of the selected female hormones from both the Idexis products and simple o/w emulsions by means of membrane release studies.
- To determine the transdermal and topical delivery of the selected female hormones by means of Franz cell skin diffusion studies and tape stripping, respectively.

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CHAPTER 2:

TOPICAL DELIVERY OF FEMALE HORMONES AGAINST AGEING IN POSTMENOPAUSAL WOMEN

2.1 Introduction

Skin ageing is an unavoidable biological phenomenon caused by an age-related decline in hormone levels and cell function (intrinsic ageing), along with exposure to harmful environmental factors (extrinsic). The synergistic action of intrinsic and extrinsic factors induces skin changes, which ultimately leads to clinical manifestations, such as skin sagging, wrinkling and dryness (Mesa-Arango *et al.*, 2017:160; Zhang & Duan, 2018:729). Menopause causes a decrease in circulating oestrogen levels, accelerating the ageing process, which in turn, leads to a decrease in skin elasticity and firmness, and an increase in skin dryness and wrinkle formation (Stevenson & Thornton, 2007:283; Thornton, 2013:264).

HRT is one of the cornerstones of anti-ageing treatments (Samaras *et al.*, 2014:1176) as it has been shown to improve various symptoms of skin ageing (Stevenson & Thornton, 2007:283). Numerous studies have, however, associated the use of HRT with an increased risk of breast cancer in postmenopausal women (Sood *et al.*, 2014:47). Therefore, treatment of skin ageing in: 1) postmenopausal women, 2) women diagnosed with breast cancer, or 3) those who are at risk due to genetic predisposition, should commence through a topical delivery system, which aims to retain the active in the skin, decreasing systemic absorption, and in regard to female hormones, reduce the risk of hormone-induced breast cancer (Garg *et al.*, 2015:969; Williams, 2013:675). Other advantages of topical drug delivery include avoidance of hepatic first-pass metabolism, reduced systemic adverse effects and improved patient compliance (Brown *et al.*, 2006:177; Leite-Silva *et al.*, 2012:384; Singh *et al.*, 2016:213).

The skin is an easily accessible and prevalent area for drug delivery (Schoellhammer *et al.*, 2014:394); however, the selectively permeable barrier formed by the stratum corneum provides a rate-limiting step during topical drug delivery (Ali *et al.*, 2015:103; Lippold, 1992:294). Due to the lipophilic nature of the stratum corneum, a lipophilic API will easily cross this barrier; however, crossing the hydrophilic viable epidermis will be more challenging, often resulting in decreased permeation rates (Holmgaard & Nielsen, 2009:20). Consequently, effective topical delivery requires an API to possess both lipophilic and hydrophilic properties. Naik *et al.* (2000:319) suggest that an API should have a log P value between 1 and 3 and an aqueous solubility higher than 1 mg/ml. Most APIs, however, do not possess the ideal physicochemical characteristics; therefore, selecting an appropriate vehicle as a drug carrier is essential to overcome these drawbacks and promote effective drug delivery (Foldvari, 2000:417; Zsikó *et*

al., 2019:22). Emulsions present with numerous advantages, which include the ability to solubilise and deliver both hydrophilic and lipophilic APIs, and consequently, improve bioavailability and percutaneous absorption (Buchmann, 2001:151; Shaker *et al.*, 2019:20). For the purpose of this study, an o/w emulsion was selected as the drug delivery system. Due to the barrier function of the skin, various techniques have been explored and developed to enhance skin penetration (Alkilani *et al.*, 2015:446). Fatty acids (in natural oils) have been shown to enhance the percutaneous absorption of APIs by altering the barrier property of the stratum corneum (Viljoen *et al.*, 2015:2045). Therefore, natural oils served as the oil phase and as penetration enhancers in the o/w emulsions, which included coconut, castor and emu oil.

2.2 Skin ageing

Skin ageing is a multifaceted deteriorating process that affects the skin and skin support structures, i.e. the subcutaneous compartments, cartilage and bone (Sjerobabski-Masnec & Šitum, 2010:515). Human skin is subject to two types of ageing that collectively leads to the loss of physiological functions and structural integrity (Farage *et al.*, 2008:88). Intrinsic (chronological) ageing is the consequence of inevitable physiological changes that transpire over time at a genetically determined rate (Avcil *et al.*, 2019:2; Farage *et al.*, 2008:88; Sjerobabski-Masnec & Šitum, 2010:515). Extrinsic ageing is induced by environmental and behavioural factors that include solar radiation, air pollution, tobacco smoke, facial expressions, sleeping position and gravity (Krutmann *et al.*, 2017:158; Piérard *et al.*, 2013). Both ageing processes are associated with phenotypic changes in skin cells together with functional and structural changes in extracellular matrix components, which include elastin, collagens and proteoglycans that are essential to provide elasticity, hydration and tensile strength to the skin, respectively (Zhang & Duan, 2018:729). The onset and clinical signs of intrinsic and extrinsic ageing are shown in Table 2.1.

Table 2.1: Onset and clinical signs of intrinsic and extrinsic ageing

Feature	Intrinsic ageing	Extrinsic ageing
Onset	Normally 50 – 60 years ^a (hereditary predisposition; onset of menopause) ^{b, i}	Around 30 years ^j (severity is dependent on individual's behaviour and exposure to environmental factors) ^d
Clinical signs	Fine wrinkling ^e Thinning ^e Slight loss of elasticity ^c Unblemished ^c Sagging skin ^f Smooth texture ^f	Coarse wrinkles ^g Laxity ^g Major loss of elasticity ^g Sallow colour ^h Rough texture (leather-like) ^c Furrows ^c

a) Jia & Nash, 2017:283; b) Stevenson & Thornton, 2007:283; c) Singh, 2009:447; d) Vierkötter & Krutmann, 2012:227; e) Rittié & Fisher, 2015; f) Piérard, 2014:237; g) Zhang & Duan, 2018:729; h) Papanagioutou, 2009:57; i) Rodríguez-Rodero *et al.*, 2011:186; j) Silva *et al.*, 2017:367.

2.2.1 Intrinsic ageing

The process of intrinsic skin ageing is similar to that observed in multiple internal organs, resulting in cellular senescence and reformed anabolic activity (Jenkins, 2002:801). The structure of the stratum corneum remains relatively unaffected; however, epidermal and dermal thinning occurs with flattening of the dermal-epidermal junctions (Farage *et al.*, 2013:7; Jenkins, 2002:802; Langton *et al.*, 2016:14). The decrease in surface contact between the epidermis and dermis compromises the transfer of oxygen and nutrients between these two layers, increasing the risk of separation – a process which may be responsible for wrinkle formation (Farage *et al.*, 2013:7; Plahuta & Hamrick-King, 2006:231; Südel *et al.*, 2005:586). Moreover, the biosynthesis capacity and number of fibroblasts decreases along with the progressive loss of elastic tissue in the papillary dermis (Eidt, 2018:694; Jenkins, 2002:802). The water-holding capacity of chronologically aged skin is influenced by the age-related decrease in extracellular matrix components, which include fibrillin, collagen and elastin (Zhang & Duan, 2018:730). It is universally accepted that intrinsic ageing results from: 1) accumulated oxidative damage caused by free radicals and 2) macromolecule damage mediated by reactive oxygen species (ROS) (Poljšak *et al.*, 2012:33).

2.2.1.1 Intrinsic factors

Factors that may influence intrinsic ageing include ethnicity, anatomical variations and hormonal changes (Clarys & Barel, 2009:292).

2.2.1.1.1 Ethnicity

The primary effect of ethnicity on skin ageing is related to the varied melanin concentration present in the epidermis of African and Caucasian skin (Singh, 2009:448). In general, darker skin types contain double the amount of melanin compared to lightly pigmented skin. The increased melanin content provides protection against accelerated ageing induced by solar exposure (Vashi *et al.*, 2016:32). The natural photoprotection of African skin was measured by Kaidbey *et al.* (1997:257), which found that black epidermis provides an average sun protection factor (SPF) of 13.4, a possible mechanism responsible for the small differences observed between exposed and unexposed skin in African Americans. Lastly, African skin is compacted with a greater lipid content, which may attribute to the increased resistance against ageing (Singh, 2009:448).

2.2.1.1.2 Anatomical variations

Skin thickness varies significantly between different areas of the body, which plays an integral role in the visible manifestation of cutaneous ageing. Skin ageing is more apparent in areas of thinner skin, for instance the eyelids (< 0.5 mm), compared to thicker skin areas like the soles of

the feet (> 6 mm) (Farage *et al.*, 2008:89). The stratum corneum displays regional variation in both lipid content and distribution, which influences the permeability of the specific skin region (Lampe *et al.*, 1983:120). Another variation between skin sites includes the age-related decrease in epidermal thickness, which was found to be greater at the volar forearm than at the temple (Farage *et al.*, 2008:89; Pouradier *et al.*, 2013:384).

2.2.1.1.3 Hormonal changes

Skin ageing is inextricably linked to menopause. The rapid and dramatic change in endogenous hormone levels brought about by menopause alters skin characteristics and functions, ultimately contributing to fine wrinkling, epidermal atrophy and dryness (Dalal & Agarwal, 2015:S222; Papakonstantinou *et al.*, 2012:253; Piérard *et al.*, 2013). Men are also affected by the age-related decrease in circulating hormone levels (Samaras *et al.*, 2014:1175). Although the reproductive changes in women are considerably larger compared to men (Wespes & Schulman, 2002:S93), the decrease in testosterone production by the adrenals, gonads and pituitary glands are associated with decreased epidermal moisture, elasticity and thickness (Bernard *et al.*, 2012:352; Zouboulis & Makrantonaki, 2012:302). A study conducted by Sator *et al.* (2001:43) showed that the use of HRT is able to significantly improve the parameters of cutaneous ageing in men.

2.2.2 Extrinsic ageing

Degenerative changes that occur in the skin with age are more related to the skin's interaction with external elements as opposed to genetic predisposition. Therefore, in humans, personal lifestyle plays a primary role in skin ageing (Silva *et al.*, 2017:367). Sun exposure and smoking are well-documented environmental factors that contribute significantly to the accelerated decline in the skin's structural integrity and physiological functions (Vierkötter & Krutmann, 2012:227). Exposure to UVR and smoking has been shown to induce deoxyribonucleic acid (DNA) damage, causing structural changes in the genetic material of skin cells due to mutations, which can accumulate and promote malignant transformation (i.e. cancer development). ROS accumulation can also cause: 1) immunosuppression or inflammation by activating different interleukins, or the 2) overexpression of matrix metalloproteinases (MMPs); both mechanisms are responsible for dermal matrix breakdown (Mesa-Arango *et al.*, 2017:163).

2.2.2.1 Extrinsic factors

Factors that may influence extrinsic ageing include tobacco smoke and solar radiation (Vierkötter & Krutmann, 2012:227).

2.2.2.1.1 Tobacco smoke

Tobacco smoke has been proven to be one of the main behavioural factors that contribute to skin ageing (Krutmann *et al.*, 2017:158). Compared to non-smokers, smokers have amplified skin wrinkling, which is mostly concentrated around the mouth and eyes (Demierre *et al.*, 1999:27; Koh *et al.*, 2002:24; Model, 1985:1761). The puckered position of smokers' lips and knitted brows could add to the prominent formation of lines in the perioral and crow's-feet area (Francès, 1998:567; Just-Sarobé, 2008:178). Tobacco smoke affects the epidermis directly by decreasing the moisture in the stratum corneum, resulting in dry skin (Just-Sarobé, 2008:178). Exposure to side stream smoke and heat from the burning cigarette may cause skin irritation (Kennedy *et al.*, 2003:553). Nicotine constricts blood vessels in the skin, leading to a grey complexion (Benowitz & Burbank, 2016:519; Francès, 1998:566; Jensen *et al.*, 1991:1132). Cutaneous vasoconstriction reduces blood flow to the skin, resulting in the loss of collagen and elastic fibres (Jensen *et al.*, 1991:1131; Marwah & Tandon, 2017:698). Smoking has also been linked to a range of anti-estrogenic effects, such as skin atrophy and reduced collagen synthesis, which promotes the development of wrinkles (Francès, 1998:568).

2.2.2.1.2 Solar radiation

The first association between solar exposure and skin ageing was made by Albert Kligman in 1969 (Kligman, 1969:2377). From then, numerous studies have provided significant evidence of the damaging effects that UVR has on skin (Flament *et al.*, 2013:221; Kimlin *et al.*, 2012:35). UVR is divided into three subtypes: ultraviolet A (UVA; 315 – 400 nm), ultraviolet B (UVB; 280 – 315 nm) and ultraviolet C (UVC; 100 – 280 nm). UVA and UVB are largely responsible for the deleterious effects of UVR on skin, since UVC is filtered by the atmospheric ozone before it can reach the skin's surface (Akhalaya *et al.*, 2014:2; Krutmann *et al.*, 2017:154; Matthews *et al.*, 2010:192). Both UVA and UVB are absorbed by chromophores in the skin, such as lipids, protein and DNA, which leads to photochemical reactions that cause damaging effects (Roy *et al.*, 2013:97; Sarkany, 2011:470). Moreover, UVR increases the production of ROS, which in turn, increases oxidative damage (Liebel *et al.*, 2012:1901). Excessive ROS production leads to the degeneration of collagen and other matrix components (Sarkany, 2011:472), which contributes to the formation of solar elastosis (Sellheyer, 2003:125). Exposure to UVR increases the expression of inflammatory cytokine receptors in the skin; it has been reported that UVR-mediated skin inflammation contributes significantly to the cumulative effect of solar radiation on skin ageing (Choi *et al.*, 2019:566; D'Orazio *et al.*, 2013:12228).

2.2.3 Changes in ageing skin

The processes of intrinsic and extrinsic ageing are superimposed (Assaf *et al.*, 2017:129), hence, skin ageing is the cumulative effect of both genetic and environmental factors, which manifest clinically as skin wrinkling, uneven skin tone, volume loss and roughness (Addor,

2018:437; Piérard *et al.*, 2014:237; Vashi *et al.*, 2016:13). It has been proposed that extrinsic factors are responsible for approximately 80% of the visible manifestations of cutaneous ageing (Avcil *et al.*, 2019:2). Structural and functional changes noted in ageing skin are summarised in Tables 2.2 and 2.3.

Table 2.2: Changes in the structural integrity of ageing skin

Skin layer	Structural changes
Stratum corneum	Lipid content reduces ^a
Epidermis	Turnover rate of keratinocytes decreases ^b Number of active melanocytes decreases ^c Number of Langerhans cells decreases ^d Number of pores increases ^e Delayed epithelialisation ^f Flattening of the dermo-epidermal junction ^g
Dermis	Reduced thickness ^g Decrease in collagen synthesis ^h Reduced vascularity and cutaneous perfusion ⁱ Number of elastin fibres decreases ^j
Hypodermis	Overall decrease in volume ^g

a) Léger *et al.*, 1988:161; b) Asgari & Leffell, 2001:868; c) Jablonski & Chaplin, 2010:8966; d) Farage *et al.*, 2017:20; e) Rawlings, 2006:87; f) Holt *et al.*, 1992:293; g) Farage *et al.*, 2013:7; h) Varani *et al.*, 2006:1861; i) Veysey & Finlay, 2016:135; j) Shin *et al.*, 2019:2126.

Table 2.3: Changes in the physiological function of ageing skin

Function	Physiological changes
Barrier function	Turnover rate of stratum corneum decreases ^a Decreased baseline transepidermal water loss (TEWL) ^b
Permeability	Sebum production decreases ^c Percutaneous absorption decreases ^a Vascularisation decreases ^a Reduced chemical clearance ^d
Miscellaneous	Reduced elasticity ^e Reduced previtamin D3 production ^f

a) Farage *et al.*, 2017:23; b) Farage *et al.*, 2013:7; c) Khavkin & Ellis, 2011:231; d) Quiroga, 2005:4; e) Coleman *et al.*, 2006:S4; f) Gallagher, 2013:321.

2.3 Menopause and hormone replacement therapy

Menopause is an unavoidable phenomenon for all women recognised after 12 months of consecutive amenorrhea resulting from the permanent exhaustion of functional ovarian follicles (Burger, 2006:27; Dalal & Agarwal, 2015:S222; Johnson, 1998:297). The age of onset is genetically determined and not influenced by socio-economic status, race or the age of

menarche (Dalal & Agarwal, 2015:S222). However, according to a study, the average age of onset is 51 (Greendale *et al.*, 1999:571). The phase following the final menses is described as the postmenopausal phase (Dalal & Agarwal, 2015:S222; Soules *et al.*, 2001:875), marking the end of fertility (Kok *et al.*, 2003:644; Voorhuis *et al.*, 2010:364). A decrease in hormone levels leads to the development of multiple symptoms, affecting various systems in the body, including the urogenital, muscular and vascular systems (Bachmann, 2005:155; Graziottin & Leiblum, 2005:133). There are three main forms of circulating endogenous oestrogen in women, namely oestrone, oestriol and oestradiol; the former being the most prevalent form of oestrogen in postmenopausal women (as summarised in Table 2.4) (Cui *et al.*, 2013:198; Ku *et al.*, 2009:2; Lipovka & Konhilas, 2016). However, oestrone is not present in adequate concentrations for the effective relief and inhibition of climacteric symptoms (Bosarge & Freeman, 2009:422).

Table 2.4: Serum oestrone, oestriol and oestradiol concentrations (pg/ml) in pre- and postmenopausal women

Serum concentration	Oestrone	Oestriol	Oestradiol	Progesterone
Premenopausal (pg/ml)	12.5 – 144.4 ^a	186.0 – 2408.0 ^a	30.0 – 400.0 ^b	150.0 – 15 000.0 ^c
Postmenopausal (pg/ml)	7.0 – 40.0 ^d	< 10.0 ^g	< 20.0 ^e	170.0 ^f

a) Wright *et al.*, 1999:268; b) Loftsson, 2015:108; c) Carmina & Lobo, 2009:831; d) Mayo Medical Laboratories, 2019; e) Choudhury *et al.*, 2011:1080; f) Burry *et al.*, 1999:1056; g) Donders *et al.*, 2014:376.

For decades, postmenopausal women have used HRT to not only alleviate symptoms, but also prevent chronic conditions associated with oestrogen deficiency, such as Alzheimer's disease, cardiovascular disease and osteoporosis (Gambacciani *et al.*, 2019:303; Janicki & Schupf, 2010:365; Palacios, 2008:22). HRT has also become one of the foundations of anti-ageing practices (Samaras *et al.*, 2014:1176). A broad spectrum of HRT regimens is commercially available that can be divided into two groups: conventional HRT and custom-compounded bioidentical hormone replacement therapy (CBHRT) (Files *et al.*, 2011:674).

2.3.1 Conventional hormone replacement therapy

Conventional HRT refers to products that have been approved by the United States Food & Drug Administration (FDA) and other regulatory authorities for the treatment of menopausal symptoms (Sood *et al.*, 2011:205). These products are marketed under various trade names and available in different dosage forms containing standardised concentrations of (Files *et al.*, 2011:674):

- only oestrogen (natural, non-human conjugated oestrogens, synthetic conjugated oestrogens, or plant-derived bioidentical oestrogens);
- only progesterone (bioidentical progesterone or synthetic progestin), or
- a combination of oestrogen and synthetic progestin.

Depending on the dosage form, HRT can be administered orally, topically, transdermally, intravaginally or through intramuscular injections (Bosarge & Freeman, 2009:423). The various hormones used in HRT are categorised into four groups for simplicity and to record possible pharmacological differences (Bhavnani & Stanczyk, 2012:756). Class A steroids are naturally occurring hormones that are processed without any chemical modifications, e.g. conjugated equine oestrogens (CEE) isolated from the urine of pregnant mares, which contains 50 – 60% oestrone sulphate along with equilin metabolites that are specific to horses (Agarwal *et al.*, 2018:3165; Lippert *et al.*, 2000:489). Class B steroids are classified as semi-synthetic hormones as they are chemically synthesised from natural steroidal precursors derived from plants, such as soy or Mexican yams (Bhavnani & Stanczyk, 2012:757). Common plant sterols include β -sitosterol, stigmasterol and diosgenin, which can be converted into steroid hormones via chemical processes (Heinrich *et al.*, 2018:86; Pattimakiel & Thacker, 2011:831). The lack of enzymes required for the conversion of sterols into steroids prevents the formation of any hormones in the human body following the ingestion of soybeans or Mexican yams (Calabrese *et al.*, 2014:373). In contrast to Class B steroids, Class C steroids are synthesised from non-steroidal precursors during a process termed total synthesis (Bhavnani & Stanczyk, 2012:757). One significant limitation of this process is the production of various isomers of which only one is structurally identical to the endogenous hormone produced in the human body, while the remaining isomers have different physicochemical properties or are completely inactive (Bhavnani & Stanczyk, 2012:757). Finally, Class D steroids include synthetic (man-made) steroidal compounds that are synthesised either from non-steroidal precursors through total synthesis or steroidal plant precursors through semi-synthesis, examples include ethinyl oestradiol, norgestrel and medroxyprogesterone acetate (MPA) (Bhavnani & Stanczyk, 2012:757).

Oestrogen was first isolated from urine in 1929 by Adolf Butenandt and Edward Doisy independently, leading to the introduction of HRT in the early 1930s. In 1942, the FDA approved and registered Premarin® (CEE) as a safe treatment for menopausal symptoms (Cuzick, 2008:2344; Stefanick, 2005:65S). However, early reports in the 1970s demonstrated an increased prevalence of endometrial cancer associated with the use of exogenous oestrogen therapy (Smith *et al.*, 1975:1166; Weiss *et al.*, 1976:1260), which incited the addition of progesterone. In present day, HRT is usually administered as oestrogen only therapy to women who have had a hysterectomy or oestrogen-progestin combination therapy to women with an intact uterus (Marsden, 2003:123; Sassarini & Lumsden, 2015:554). The function of synthetic progestin in combined regimens is to oppose the proliferative action of oestrogen on uterine epithelium cells (Otto *et al.*, 2008:3952; Valéra *et al.*, 2017:e0177043). The preference to use progestin is attributed to its rapid absorption, higher bioavailability and longer half-life compared to progesterone (Burke, 2012:607; Stanczyk *et al.*, 2013:177).

Misinterpretations and unjustified generalisations of the results from the Women's Health Initiative (WHI) clinical trial investigating the use of combined HRT (CEE and MPA) in postmenopausal women, led to the uncontrolled use of CBHRT products (L'Hermite, 2017:205).

2.3.2 Custom-compounded bioidentical hormone replacement therapy

CBHRT is the practice of personalising HRT based on salivary hormone levels, and perhaps more decisively, the evaluation of individual patient symptoms (Boothby & Doering, 2008:400). The term 'bioidentical hormones' refers to compounds that are natural, plant-derived or chemically identical to the endogenous hormones produced within the human body (Files *et al.*, 2011:674; Sood *et al.*, 2011:202). The 'natural' classification suggests that these are endogenous hormones, when in fact they are synthesised and semi-synthesised (Fugh-Berman & Bythrow, 2007:1030). The use of salivary hormone testing to determine the appropriate therapeutic dosage is considered to be unreliable as the hormone levels in saliva show significant variability within each patient depending on absorption, diet, diurnal variation and the hormone being tested (Files *et al.*, 2011:678; Gavrilova & Lindau, 2009:i99; Pinkerton, 2014:1299). Furthermore, this approach contradicts a worldwide consensus that the lowest effective dose of hormones in line with treatment goals should be prescribed (Bosarge & Freeman, 2009:424; NAMS, 2012:264).

Limited evidence regarding the efficacy and safety of CBHRT is available (Thompson *et al.*, 2017:98). A study conducted by Holtorf (2009:73) stated that bioidentical hormones are associated with a decreased risk of cardiovascular disease and breast cancer, and are more effective than their synthetic counterparts. However, scientific and medical consensus regard the benefits and risks of bioidentical hormones equivalent to conventional hormones (Boothby & Doering, 2008:401; Cirigliano, 2007:600; Thompson *et al.*, 2017:98). Custom-compounded products are not subject to strict regulations by the FDA or other regulatory agencies, which is a prerequisite for the commercial manufacturing and selling of prescription medications (Bhavnani & Stanczyk, 2012:758). In South Africa, no mandatory registration and regulation is required for CBHRT products by the Medicines Control Council. Consequently, these compounds are not tested regularly for purity, safety, potency and efficacy (Guidozzi *et al.*, 2014:540). Postmenopausal women using CBHRT products may be subject to accidental over or under-dosing due to batch-to-batch variation in purity and/or possible human error during compounding (Bhavnani & Stanczyk, 2012:758; Pinkerton, 2014:1298). Moreover, there are no labelling requirements for CBHRT products, whereas conventional HRT products are required to include a package insert that provides information regarding the product's indications for use, pharmacokinetics and potential adverse effects (Bhavnani & Stanczyk, 2012:758).

Numerous studies have found that women are unaware of the safety concerns associated with the use of CBHRT. A survey of 184 participants found that 77% believed that CBHRT is safer compared to conventional HRT (Iftikhar *et al.*, 2011:559). Pinkerton & Santoro (2015:926) found that up to 86% of postmenopausal women had no idea that CBHRT products lack FDA approval. Overall, the lack of randomised, placebo-controlled trials determining the efficacy and safety of CBHRT products, along with the absence of boxed warnings indicating the increased risk of invasive breast cancer, lack of regulatory authorities and reservations about salivary hormone testing have led to the general recommendation against the use of CBHRT products by numerous associations, which include The North American Menopause Society (NAMS), The Endocrine Society and The International Menopause Society (IMS) (Pinkerton, 2014:1298-1299).

2.4 Breast cancer and hormones

The association between oestrogen action and breast cancer was first proposed by Beatson (1896:163) in 1896. Since then, numerous observational studies and clinical trials have demonstrated a positive correlation between the use of conventional HRT and the elevated risk of developing ovarian, endometrial and breast cancers in postmenopausal women (Sood *et al.*, 2014:47). These findings are alarming, since breast cancer is the most frequently diagnosed cancer in women around the world, responsible for nearly 25% of all cancers (Bray *et al.*, 2018:394; Sun *et al.*, 2017:1387). Furthermore, GLOBOCAN 2008 reported that breast cancer is the leading cause of cancer-related deaths among women in both developing and developed countries (Ferlay *et al.*, 2010:2904). Due to universal support of long-term HRT for numerous health benefits, including protection against bone loss and cardiovascular disease, single small observational studies did not provide substantial evidence of the associated risks to change the larger medical view (Goldman, 2010:299; Tao *et al.*, 2011). However, in 2002 the highly publicised results of the WHI trials raised concern regarding the safety of HRT (Rossouw *et al.*, 2002:321). The WHI study included randomised, double-blind, placebo-controlled clinical trials that assessed the health benefits and risks associated with the long-term use of 0.625 mg CEE in hysterectomised postmenopausal women (Anderson *et al.*, 2004:1701) or combination of 0.625 mg CEE and 2.500 mg MPA in postmenopausal women with an intact uterus (Rossouw *et al.*, 2002:321). The strengths of both clinical trials stem from their large-scale participation and long-term follow-up (Roehm, 2015). The results indicated that postmenopausal women using combination therapy had an increased risk of breast cancer, whereas those using oestrogen-only therapy did not (Anderson *et al.*, 2004:1701; Rossouw *et al.*, 2002:321). Contrarily, the Million Women Study demonstrated that the use of both oestrogen-only and combined oestrogen-progestin therapy were associated with an increased risk of breast cancer. Moreover, the results proposed no significant difference in breast cancer risk between specific oestrogens (oestradiol and CEE) or specific progestins (norethisterone, norgestrel and MPA)

(Beral, 2003:426). Although the exact mechanisms whereby hormones stimulate breast cancer development have not been fully elucidated, it has been shown that oestrogen inhibits apoptosis and increases mammary duct epithelium proliferation (Kaaks *et al.*, 2005:1072). Additionally, androgen receptors located in the mammary epithelium are activated by circulating androgens to inhibit breast development (Dimitrakakis *et al.*, 2002:26). Oestrogen inhibits androgen effects on the breast, thus increasing tissue proliferation and the risk of breast cancer (Dimitrakakis *et al.*, 2002:32). Lastly, progesterone has been shown to contribute to the production of cancerous breast cells. However, the role of progesterone in breast cancer is controversial and warrants further investigation (Lange & Yee, 2008:157).

2.5 Human skin and hormones

Human skin is classically considered a target for numerous hormones of which the induced effects have been characterised over years of research (Zouboulis, 2000:230). All physiological functions and development of skin tissue are influenced and regulated by hormones (Deplewski & Rosenfield, 2000:363). The human skin is also categorised as a hormone producer during peripheral androgen metabolism of androstenedione and dehydroepiandrosterone into testosterone and 5 α -dihydrotestosterone (Fritsch *et al.*, 2001:794). The diverse expression of high-affinity receptors in human skin enables interaction with various hormone types (Zouboulis, 2009:250). However, human skin is not a solitary homogenous organ, subsequently the preservation of skin health is determined by interplay of multiple cell types (Brinckat, 2000:114). Exogenous sex hormones have been shown to exert pleiotropic biologic effects on human skin, preserving structural integrity and preventing the clinical manifestations of skin ageing (Thornton, 2013:268; Zouboulis, 2009:250).

2.5.1 Oestrogen

Oestrogens are C-18 steroid hormones comprising of a group of structurally related compounds characterised by an aromatic ring and phenolic hydroxyl group at C-3 (Hall & Phillips, 2005:555); and named for the fundamental role they play in the oestrous cycle of women (Cheskis *et al.*, 2007:610; Enmark & Gustafsson, 1999:133; Morani *et al.*, 2008:128; Ruiz-Cortés, 2012:9). Gonadal hormones are predominantly synthesised from cholesterol (C-26), which is converted to androstenedione (C-19), and lastly to oestradiol (C-18) by means of the successive elimination of carbon side chains (Hall & Phillips, 2005:555; Ruiz-Cortés, 2012:3). Oestradiol is the most potent and abundant oestrogen produced by the biosynthesis process in the ovaries throughout the premenopausal period (Cui *et al.*, 2013:197; Hall & Phillips, 2005:555; Heldring *et al.*, 2007:906). Oestrone is produced from adrenal androstenedione in the adipose tissue and plays a fundamental role in postmenopausal women (Cui *et al.*, 2013:197; Szymezak *et al.*, 1998:320). Oestriol is the weakest oestrogen, produced through

16 α -hydroxylation of oestrone; however, it is the dominant oestrogen during pregnancy when significant quantities are produced by the placenta (Cui *et al.*, 2013:197; Falah *et al.*, 2015:29).

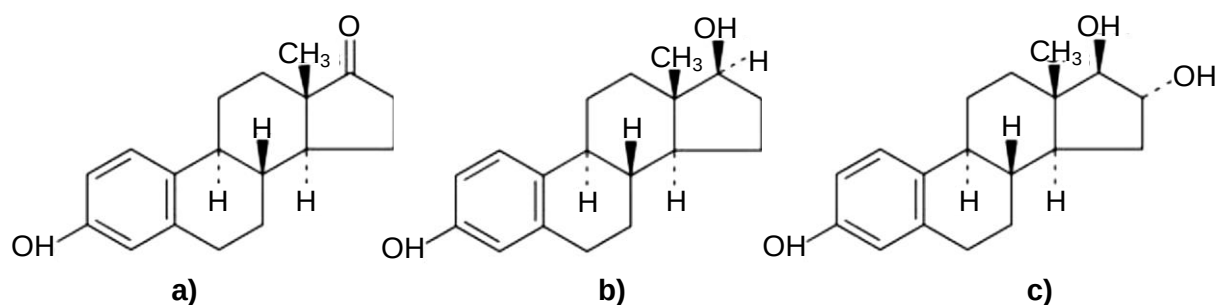


Figure 2.1: Chemical structure of a) oestrone, b) oestradiol and c) oestriol (adapted from Moffat *et al.*, 2004:1350-1352).

Oestrogens are primarily produced by the ovaries, placenta and corpus luteum in premenopausal women and, to a lesser extent, the testes in males (Enmark & Gustafsson, 1999:133; Kendall & Eston, 2002:109). When compared to men, women have a four-fold increase in the amount of oestrogen (Horstman *et al.*, 2012:1142). Oestrogens are fundamentally responsible for the development, maturation and function of the female reproductive system, as well as the development of secondary sexual characteristics (Cui *et al.*, 2013:197; Encyclopaedia Britannica, 2014; Horstman *et al.*, 2012:1142). Furthermore, oestrogens are essential for signalling a wide range of biological effects in various physiological systems in both men and women, including the immune, cardiovascular, central nervous and musculoskeletal systems (Enmark & Gustafsson, 1999:133; Heldring *et al.*, 2007:906; Horstman *et al.*, 2012:1142; Katzenellenbogen *et al.*, 1995:387). The biological effects of endogenous oestrogens are modulated and induced through the activation of oestrogen receptor α (ER α) and oestrogen receptor β (ER β) (Carroll, 2007:159; Couse *et al.*, 1997:4613; Cui *et al.*, 2013:197; Verdier-Sévrain, 2007:291). Common physiological roles are modulated by both oestrogen receptors in the human body, such as ovarian development and cardiovascular protection (Cui *et al.*, 2013:201; Dubey & Jackson, 2001:1880; Paterni *et al.*, 2014:13). However, the ER α has a more profound influence on the uterus and mammary gland, along with preserving skeletal homeostasis and regulating the metabolism (Paterni *et al.*, 2014:13), whereas the ER β has a more prominent role on the immune and central nervous systems, and largely opposes the ER α -induced cell hyperproliferation of uterus and breast tissues (Heldring *et al.*, 2007:911; Paterni *et al.*, 2014:13).

Oestrogens have a profound effect on human skin (Verdier-Sévrain, 2007:289), influencing numerous skin functions, namely pigmentation (Snell & Turner, 1966:155), elasticity (Brincat *et al.*, 2005:113), water-holding capacity (Piérard-Franchimont *et al.*, 1995:151) and vascularity (Haryell *et al.*, 1992:294). The decrease in oestrogen linked to menopause intensifies and

worsens the damaging effects of extrinsic and intrinsic skin ageing (Brincat *et al.*, 2005:110). Multiple studies have shown that oestrogen can combat skin ageing by regulating skin's thickness and moisture (Hall & Phillips, 2005:560). In a study conducted by Affinito *et al.* (1999:241), a decrease in skin collagen content is also linked to the years following the onset of menopause. Postmenopausal women showed a decrease in collagen types I and III, along with a decrease in types III/I ratio when compared to premenopausal women (Affinito *et al.*, 1999:241). In a study conducted by Dunn *et al.* (1997:339), oestrogen use in postmenopausal women is linked to a decrease in the formation of wrinkles and senile dry skin. Topical oestrogen increases skin moisture (Schmidt *et al.*, 1994:25), as well as the thickness and number of elastin fibres in the papillary dermis (Punnonen *et al.*, 1987:39), which play an important role in skin elasticity (Langton *et al.*, 2010:332). Additionally, topical oestrogen decreases the depth of wrinkles (Schmidt *et al.*, 1994:25).

2.5.2 Progesterone

Progesterone is a C-21 steroid hormone synthesised in the gonads, brain and adrenal glands in both males and females (Encyclopaedia Britannica, 2017; Furth *et al.*, 2018:808; Taraborrelli, 2015:9). The first and rate-limiting step in the steroidogenic pathway is the conversion of cholesterol to pregnenolone under the action of cytochrome P-450 cholesterol side-chain cleavage enzyme (Hu *et al.*, 2010:47). Thereafter, pregnenolone is converted to progesterone by 3 β -hydroxysteroid dehydrogenase. A deficiency in either enzyme will have a profound effect on the biosynthesis of all steroid hormones (Henley *et al.*, 2005:52). Progesterone of gonadal origin is secreted into the blood to exert its biological effect, while progesterone synthesised in the adrenal glands is mostly converted into androgens and glucocorticoids (Taraborrelli, 2015:9).

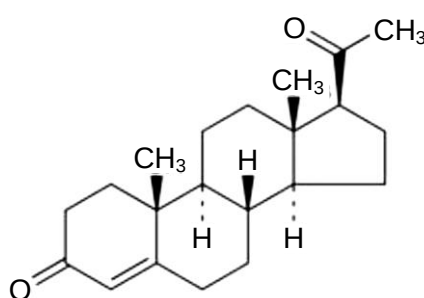


Figure 2.2: Chemical structure of progesterone (adapted from Moffat *et al.*, 2004:1964).

The biological effects of progesterone are primarily mediated through its cognate progesterone receptor (PR), which is expressed as two functional isoforms: PR α and PR β (Scarpin *et al.*, 2009). PRs are widely expressed in both reproductive (i.e. the uterus, ovary, mammary gland and testes) and non-reproductive tissues (i.e. brain, bone, pancreas and the lower urinary tract)

(Bland, 2000:224; Graham & Clarke, 1997:504; Han *et al.*, 2013:127; Tincello *et al.*, 2009:1474). The abundant expression of PRs throughout the human body highlights the fundamental role of progesterone in normal physiology (Scarpin *et al.*, 2009). Both isoforms are generally co-expressed in target cells (Graham & Clarke, 2002:189); however, Mote *et al.* (1999:2963) found that PR α is primarily expressed in the uterine stroma, while PR β is the primary isoform in the glandular epithelium during the mid-secretory phase of the menstrual cycle. Additionally, a study conducted by Mulac-Jericevic *et al.* (2000:1752) demonstrated the distinct functions for the PR isoforms: PR α is required for the development and reproductive function of the uterus and PR β is essential for mammary gland development.

PRs are also present in the human skin (Raine-Fenning *et al.*, 2003:372; Schmidt *et al.*, 1990:97), fibroblasts (Mercier *et al.*, 2002:731) and in keratinocytes (Im *et al.*, 2000:650). With age and the onset of menopause, the expression of receptors decreases in the tissue (Raine-Fenning *et al.*, 2003:372). In a pioneer study by Holzer *et al.* (2005:632) on the effect of progesterone on mature skin, it was demonstrated that there is a significant improvement in skin elasticity and skin firmness. Extracellular MMPs are the main target for progesterone because of the significant role it plays in tissue remodelling (Huber & Gruber, 2001:18). Progesterone hinders the activity and expression of MMPs (Henriet *et al.*, 2002:135), thus suppressing the increased age-related atrophy (Holzer *et al.*, 2005:633). The outcome of the treatment was a decrease in wrinkle depth, as the skin elasticity and skin tissue texture improved over time (Holzer *et al.*, 2005:633).

2.6 Structure and function of human skin

Human skin is a large and complex organ with an average adult surface area of 2 m² (Hadgraft, 2001:1), providing an ideal drug administration site for local and systemic effects (Benson, 2012:3). The skin varies in thickness with age, site and gender; male skin is usually thicker, while females have a thicker subcutaneous fat layer (Mitsui, 1997:13). On average, human skin contains 3 blood vessels, 15 subcutaneous glands, 10 hair follicles, 100 sweat glands and 12 nerves per square centimetre (Asbil & Michniak, 2000:36). The skin functions primarily as a protective layer against free radicals, chemicals, ultraviolet (UV) rays and pathogens (Menon, 2002:4). Furthermore, skin plays an integral role in homeostasis by controlling endocrine synthesis, blood pressure and body temperature by means of regulating TEWL (Benson, 2012:3; Menon, 2002:4). The skin is a sensory organ that identifies environmental changes via the network of nerve cells and multiple receptors for pressure, pain and temperature (Benson, 2012:3), and is composed of four distinctive layers, namely the stratum corneum, viable epidermis, dermis and the hypodermis (Foldvari, 2000:417).

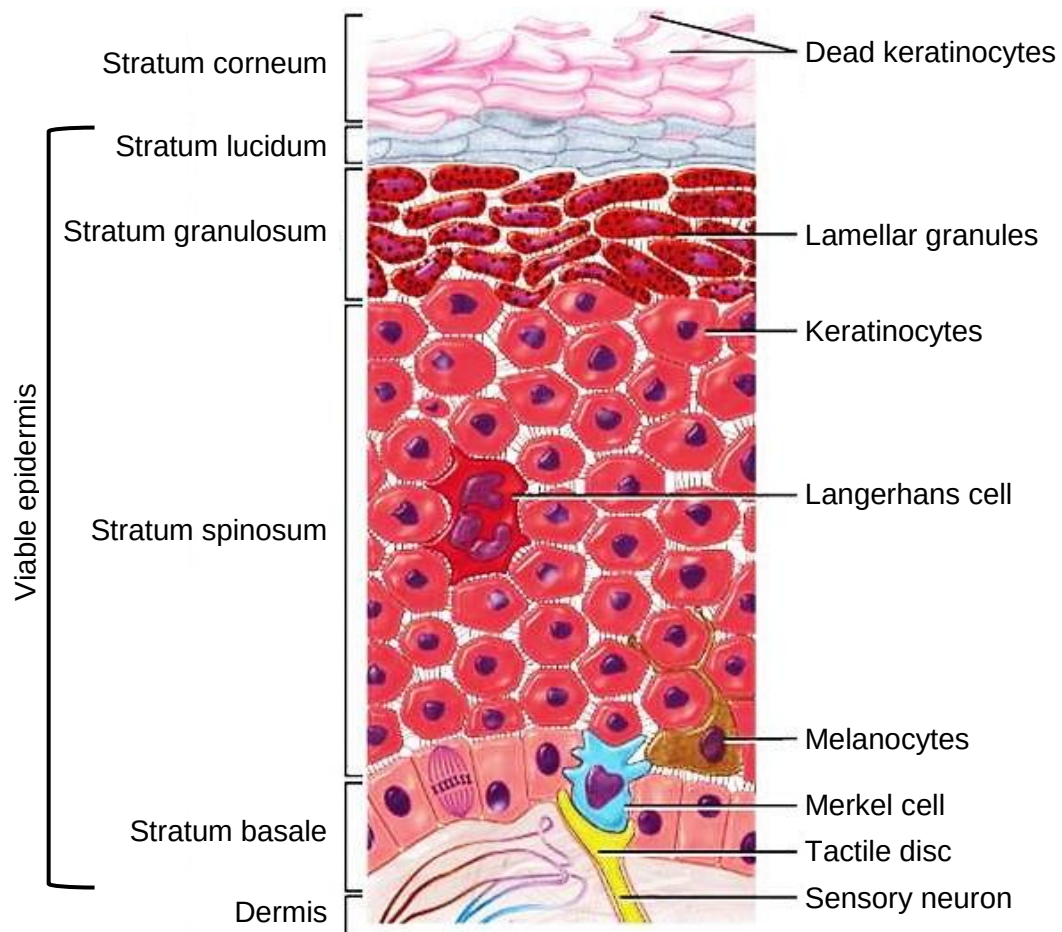


Figure 2.3: Schematic representation of the structure of human skin (adapted from Morrell, 2009).

2.6.1 Stratum corneum

The stratum corneum primarily provides protection and functions as the main barrier, preventing water loss and the penetration of potentially damaging chemicals and pathogens from the surrounding environment into the body (Marks, 2004:2017S; Van Smeden *et al.*, 2004:295). The stratum corneum is composed of 10 – 15 layers of flattened corneocytes with an average thickness ranging between 10 to 20 μm (Ng & Lau, 2015:3). However, depending on hydration levels, the thickness of this layer can vary considerably from 10 – 15 μm when dehydrated to 40 μm when hydrated (Benson, 2005:24). Keratin-filled corneocytes are surrounded by a cornified envelope, stabilised by cross-linked insoluble proteins covalently bound to lipids (Asbil & Michniak, 2000:36). This multifaceted membrane consists of mostly lipids and proteins, characterised by the impermeable interlocking arrangement of the corneocytes (Walters, 1990:82). Due to the high diffusional resistance presented by the stratum corneum, it is considered a rate-limiting barrier in topical drug delivery (Ali *et al.*, 2015:103; Lippold, 1992:294).

The “brick” and “mortar” structure of the stratum corneum poses the most formidable barrier for potential permeates (Benson, 2005:24; El Maghraby *et al.*, 2008:204), with the corneocytes serving as the “bricks” and the intercellular lipid matrix, consisting of cholesterol, ceramides and free fatty acid, as the “mortar” (Bala *et al.*, 2014:1809). Any fluctuations in the lipid composition would result in an abnormal skin barrier function and active permeation (Baroni *et al.*, 2012:259, 261). Permeation enhancement techniques are used to disrupt or circumvent the brick and mortar domain (Barry, 2006:5). Another important component of the stratum corneum is water, which serves as a plasticiser, providing elasticity to the skin, thus preventing this layer from cracking due to mechanical damage (Ali *et al.*, 2015:105). Lipids in the stratum corneum interstices contribute to the barrier function (Pappas, 2009:74). Both SFA and UFA are present, with a characteristic absence of phospholipids (Brooks & Idson, 1991:103; De Jager *et al.*, 2005:2649).

2.6.2 Viable epidermis

Beneath the lipophilic stratum corneum, resides the hydrophobic viable epidermis, which is made up of four distinctive layers, i.e. the stratum lucidum, stratum granulosum, stratum spinosum and the stratum basale, comprising keratinocytes at diverse phases of differentiation (Amsden & Goosen, 1995:1974; Morrell, 2009). Cells are synthesised in the basal layer and travel upwards to form the spinous and granular layer. During the migration process, the cells undergo morphological changes that contribute to the skin barrier function and are primarily associated with intercellular lipid pathways and the partitioning phenomena. The partition of actives from layer to layer after diffusing across the stratum corneum is determined by their solubility (Amsden & Goosen, 1995:1974; Foldvari, 2000:418). The high water content present in the viable epidermis poses a more significant barrier towards lipophilic APIs (N'Da, 2014:20786). Additionally, the epidermis contains Langerhans cells (immune sentinels), melanocytes (skin pigmentation) and Merkel cells (sensory awareness) (Asbil & Michnaik, 2000:36; Mitsui, 1997:13).

2.6.3 Dermis

Located underneath the viable epidermis lies the dermis, with an approximate thickness between 2 000 – 5 000 µm (Jepps *et al.*, 2013:677; Kute & Saudagar, 2013:372). As a result of the abundant presence of elastin fibres and collagen found in the connective tissue, the dermal layer is characteristically elastic (Foldvari, 2000:418; Oikarinen, 1994:47). Fibroblasts, macrophages, leukocytes and mast cells are scattered throughout the dermis (Foldvari, 2000:418). Furthermore, the dermis holds hair follicles and sweat glands, facilitating drug permeation via the appendageal route (El Maghraby *et al.*, 2008:204; Hurley, 2001:47). The hydrophilic nature of the dermis can be ascribed to the mucopolysaccharide gel surrounding the

collagen and elastin (Jepps *et al.*, 2013:677; Williams, 2013:677). Dermal blood supply is essential for the systemic absorption of actives following topical application (Singh & Roberts, 1994:783). This layer is highly vascularised and holds a large network of blood vessels, especially in the papillary layer, resulting in the rapid clearance of actives that cross the stratum corneum (Baroni *et al.*, 2012:260; Barry, 2001:102; Foldvari, 2000:418).

2.6.4 Hypodermis

The fourth and final layer, known as the hypodermis, is composed of subcutaneous fat tissue and functions primarily as an energy source, thermal barrier against extreme temperature fluctuations and protective layer against mechanical trauma (Ali *et al.*, 2015:105; Baroni *et al.*, 2012:260; Williams, 2013:677). While the hypodermis varies in thickness depending on the body site (up to 3 cm in abdominal skin) (Nafisi & Maibach, 2018:50), the average thickness ranges between 4 – 9 mm (Ali *et al.*, 2015:105).

2.7 Drug transport across human skin

Delivery and transport of actives across the skin is an intricate process; aside from the formidable barrier formed by the stratum corneum, several steps are involved (Ruela *et al.*, 2016:529; Zsikó *et al.*, 2019:21):

- effective release of the active from the formulation;
- partitioning of the active into the stratum corneum;
- diffusion of the active across the stratum corneum;
- partitioning of the active from the stratum corneum into the viable epidermis;
- diffusion of the active across the viable epidermis into the dermis, and
- the absorption of the active into the systemic circulation.

Traditional routes of drug penetration across the stratum corneum include the follicular, transcellular and intercellular pathway (illustrated in Figure 2.4) (Haque & Talukder, 2018:171).

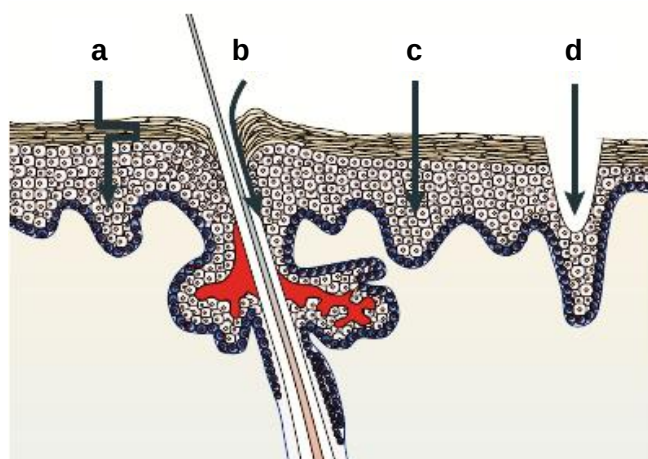


Figure 2.4: Schematic representation of the penetration pathways through the skin: a) transcellular; b) follicular; c) intercellular penetration and d) direct diffusion into hydrophilic epidermis and dermis layers (adapted from Prausnitz *et al.* (2004:119).

2.7.1 Follicular route

The follicular route, also commonly referred to as the transappendageal route, comprises transport through the hair follicles and sweat glands, bypassing the stratum corneum (Hueber *et al.*, 1992:100). The transappendageal pathway has long been considered to be relatively insignificant, as hair follicles cover only 0.1% of the entire skin surface area (Liu *et al.*, 2011:769; Mohd *et al.*, 2016:33). However, hair follicles are associated with sebaceous glands, which produce and secrete sebum, creating a lipid-rich environment, which in turn, facilitates the diffusion of lipophilic APIs (Gay *et al.*, 2014:1588; Knorr *et al.*, 2009:175).

2.7.2 Transcellular route

The transcellular route involves transport through the layered corneocytes within the lipid-rich extracellular matrix (Ng and Lau, 2015:9). Due to the hydrophilic nature of corneocytes, it is expected that hydrophilic actives would cross the stratum corneum easily. These cells are, however, surrounded by a lipophilic matrix, preventing hydrophilic actives from crossing (N'Da, 2014:20783).

2.7.3 Intercellular route

The intercellular route involves transport through the lipid matrix within the stratum corneum, which presents a tortuous pathway for potential permeates due to the interlocking arrangement of the corneocytes (Morrow *et al.*, 2007:38). Moreover, the intercellular domain consists of alternating structures of bilayers (Haque & Talukder, 2018:171). As a result, an active must sequentially undergo partition and diffusion through the lipid and aqueous domains (Morrow *et*

al., 2007:38). This penetration route is therefore more suitable for small uncharged molecules (Jhawar *et al.*, 2013:48).

2.8 Topical drug delivery

Skin permeation influences the capability of actives to reach the target area (Mathur *et al.*, 2010:173). Topical drug delivery is the transport of actives to the viable epidermis located below the stratum corneum, the dermal blood vessels or the lymphatic network. Transport is driven by diffusion; thus no active transport is involved during permeation. The lipophilic stratum corneum determines the permeation rate of hydrophilic formulations, whereas the hydrophilic epidermis and dermis determine the permeation rate for lipophilic formulations (WHO, 2006:23). Topical drug delivery proves to be advantageous over the systemic administration of female hormones for the treatment of skin ageing in postmenopausal women. These advantages are:

- Avoidance of hepatic first-pass metabolism and pH variations associated with the gastrointestinal tract (Singh *et al.*, 2016:213).
- Controlled drug delivery over a prolonged period of time, which ensures steady API levels, reducing systemic adverse effects (Brown *et al.*, 2006:177).
- Therapeutically effective concentrations are delivered directly to the target site, e.g. treatment of cutaneous ageing in postmenopausal women (Nair *et al.*, 2013:423).
- Increased suitability for self-medication (Kaur *et al.*, 2016:6355).
- Reduced administration frequency and improved patient compliance (Leite-Silva *et al.*, 2012:384).
- Painless, non-invasive and easily administered (Gelfuso *et al.*, 2016:735).
- Minimal inter- and intra-patient variations (Kaur *et al.*, 2016:6355).
- Ability to easily discontinue API input should an adverse reaction occur (systemic or local) (Brown *et al.*, 2006:177).

In general, APIs that are considered to be efficient following topical administration are those of adequate lipophilicity, low MW and high potency (i.e. have the ability to initiate a desired therapeutic effect in the micrograms per millilitre concentration range) (Gelfuso *et al.*, 2016:735; Polonini *et al.*, 2014:332). The primary challenge of topical drug delivery is to overcome the stratum corneum, as the intracellular lipid region limits the diffusion of most API molecules through the skin (Gelfuso *et al.*, 2016:735; Ng, 2018:51; Roohnikan *et al.*, 2019:257; Zsikó *et al.*, 2019:20). Additionally, tight junctions that seal the intercellular space between neighbouring keratinocytes in the stratum granulosum could present difficulties for API diffusion; however, this

barrier is considered to be insignificant when the stratum corneum is undamaged (Andrews *et al.*, 2012:1108; Brandner *et al.*, 2015; Gelfuso *et al.*, 2016:765). Other challenges associated with the topical delivery of actives are:

- Actives with large MW (> 500 g/mol) are less likely to permeate through the stratum corneum (Shin *et al.*, 2019:332).
- Actives must attain adequate aqueous and lipid solubility to effectively permeate through the stratum corneum and underlying skin layers (Brown *et al.*, 2006:177). Naik *et al.* (2000:319) stated that an API with a log P value between 1 and 3 is ideal for topical delivery; however, according to Williams (2013:680) a log P value up to 4 is also considered favourable.
- Enzymes in the skin might metabolise APIs into therapeutically inactive forms, reducing their efficacy (Brown *et al.*, 2008:121).
- Skin irritation or sensitisation may occur (Paudel *et al.*, 2010:116).
- Skin hydration and age influence the percutaneous absorption of APIs.
- Inter- and intra-patient variability in the percutaneous absorption of APIs due to factors such as skin hydration, ethnicity and age, which could lead to inconsistent/inadequate dosing (Singh & Morris, 2011:4; Thomas & Finnin, 2004:700).

2.9 Physicochemical properties

Scheuplein and his colleagues first demonstrated that the primary diffusional pathway for drug molecules across the skin is lipophilic in nature. Since then, various studies have continued to characterise the relationship between the physicochemical properties of an API and flux through the skin (Peck & Higuchi, 267). Over the years, it has been established that an API's ability to permeate the skin (flux), is directly related to its physicochemical properties; consequently, not all actives are suitable candidates for topical delivery (Williams, 2013:680).

Table 2.5: Ideal physicochemical properties for topical delivery (Naik *et al.*, 2000:319).

Properties	Ideal range ^a
MW (g/mol)	< 500
Melting point (°C)	< 200
Aqueous solubility (mg/ml)	> 1
Log P	1 – 3

Table 2.6: Physicochemical properties of selected female hormones

	Oestriol	Oestradiol	Progesterone
MW (g/mol)	288.4 ^a	272.4 ^a	314.5 ^b
Melting point (°C)	280 ^a	173 – 179 ^a	121 ^b
Aqueous solubility	Practically insoluble ^a ; 13.25 µg/ml ^c	Practically insoluble ^a ; 1.51 µg/ml ^d	Practically insoluble ^b ; 8.10 µg/ml ^e
Log P	2.4 ^a	4.0 ^a	3.9 ^b

a) Moffat *et al.*, 2004:1350-1351; b) Moffat *et al.*, 2004:1964; c) Pubchem, 2005; d) Shareef *et al.*, 2006:879; e) NTP, 2011:363.

The ideal physicochemical properties for topical delivery is summarised in Table 2.5. The human skin forms a formidable barrier that hinders the penetration of numerous molecules; however, smaller molecules can surpass percutaneously (Bos & Meinardi, 2000:165). Various studies have demonstrated drug flux as a function of MW, where the percutaneous absorption of an active increased with a decrease in MW (Alkilani *et al.*, 2015:450). From Table 2.6, it can be observed that the female hormones presented with ideal MW for topical delivery.

It has been established that a direct correlation exists between the aqueous solubility of an active and its melting point (Batisai *et al.*, 2014:9992). In general, APIs with a lower melting point display good aqueous solubility and percutaneous absorption and *vice versa* (Chu & Yalkowsky, 2009:24). Hence, oestradiol and progesterone are favourable for topical delivery as both presented with ideal melting points (< 200 °C).

Solubility describes the ability of a given compound to dissolve within a medium, and consequently, be available for absorption through the skin (Savjani *et al.*, 2012:1; Khadka *et al.*, 2014:304; Steele & Austin, 2009:24). Encyclopaedia Britannica (2016) stated that the permeation of an active is closely related to its solubility; consequently, good aqueous solubility leads to enhanced skin permeation (Kulkarni & Nagarsenker, 2008:466; Orme, 1984:60; Savjani *et al.*, 2012:1). The solubility of an active is influenced by its lipophilic nature (Stocks, 2013:96). In general, high lipophilicity (log P > 5) contributes to low solubility (Gao *et al.*, 2017:461). The poor aqueous solubility of the hormones could hinder topical delivery.

The tendency of an API to dissolve in a biphasic system made up of two immiscible solvents (i.e. water and lipid) can be referred to as the log P value (Bannan *et al.*, 2016:4015). Skin is a complex multi-layered structure with both lipophilic (stratum corneum) and hydrophilic (viable epidermis and dermis) regions (Abd, 2016:10). Lipophilic APIs will partition into the stratum corneum with ease; however, the viable epidermis will present a significant barrier, resulting in decreased permeation rates (Holmgaard & Nielsen, 2009:20). Therefore, potential drug candidates for topical delivery should have an affinity for both lipophilic and hydrophilic environments (Yavuz *et al.*, 2018:462). According to Naik *et al.* (2000:319), only oestriol possesses an ideal log P value; however, Williams (2013:680) stated that a log P value

between 1 and 4 is acceptable. Therefore, it can be stated that all three hormones possessed log P values that are considered ideal for topical delivery.

2.10 Formulation of simple oil-in-water emulsions

Emulsions can be described as heterogeneous systems consisting of two main components of water and oil, which is stabilised by hydrophilic, as well as lipophilic surfactants due to their biphasic nature (Buchmann, 2001:151; Magdassi & Garti, 1999:156). An emulsion can be formulated with either oil or water as the core component, depending on the solubility of the incorporated API (Saxena *et al.*, 2017:142). For the purpose of this study, an o/w emulsion was formulated using the selected natural oils as the core component, together with the lipophilic female hormones. Of the wide assortment of cosmetic and skin care products, o/w emulsions are undoubtedly the most used due to their many advantages, which include (Buchmann, 2001:151; Hewitt, 2013:1; Jones, 2016:61; Whitby, 2019:127):

- ability to solubilise and deliver both lipid and water-insoluble compounds;
- improved skin absorption and permeation;
- good skin spreadability;
- less greasy after topical application (compared to w/o emulsions);
- pleasant skin feel (immediate feeling of hydration due to large continuous aqueous phase);
- reduced skin irritation (irritant is incorporated into the internal phase of an o/w emulsion);
- produced in large volumes;
- cosmetically acceptable and consumer appeal (easily administered with favourable texture and visual appearance);
- formulation process tends to be short and uses low energy, and
- cost-effective (water accounts for 60% of cosmetic o/w emulsion).

Regardless of the numerous advantages presented by o/w emulsions, they are thermodynamically unstable due to the natural tendency of liquid-liquid colloidal dispersions to minimise interfacial interactions, and therefore phase separation can occur after a period of time through coalescence, creaming or flocculation (Goodarzi & Zendeboudi, 2018:284; Ngan *et al.*, 2014:1). Decreasing the droplet size has been shown to significantly increase the stability of emulsions (Saari *et al.*, 2019). Smaller droplets can be attained with the use of high-energy emulsification methods (Hu *et al.*, 2017:17). For this study, high-pressure homogenisation was used to reduce the droplet size and improve the homogeneity in the o/w emulsions by forcing

the dispersion to flow at high velocity through a narrow opening. Consequently, more elegant and kinetically stable o/w emulsions were formulated (Berk, 2009:189; Engman *et al.*, 1998; Hebishy *et al.*, 2013:166). Figure 2.5 illustrates the multi-step formulation process of the o/w emulsions.

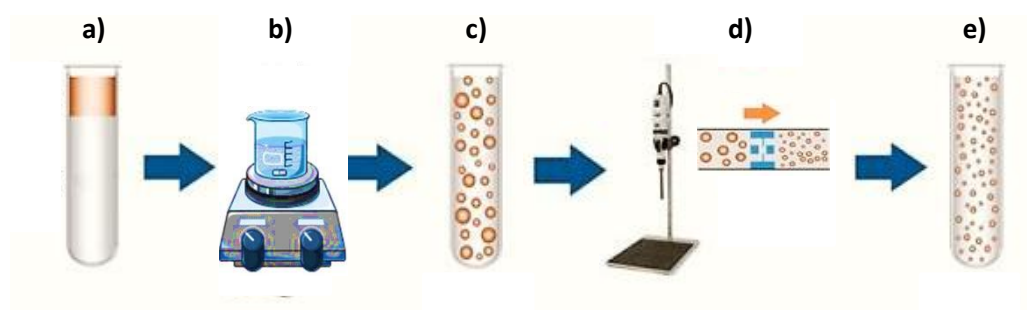


Figure 2.5: Schematic representation of formulation process of o/w emulsions: a) oil (top) and water (bottom) phase; b) high-speed stirring; c) coarse o/w emulsion; d) high-pressure homogenisation, and e) fine o/w emulsion (adapted from Juttulapa *et al.*, 2017:22).

Table 2.7: Advantages and disadvantages of high-pressure homogenisation

Advantages	Disadvantages
Good reproducibility ^a	High temperature process ^a
Organic solvent-free method ^a	High energy input ^a
Effective droplet size diminution ^b	Possible degradation of excipients ^a
Low contamination ^b	Expensive equipment ^c
Large scale production ^b	Potential breakdown of equipment ^d
Quick process ^a	Requires high surfactant concentrations ^d

a) Michalak *et al.*, 2017:302; b) Kumar, 2019:250; c) Michalski & Janeul, 2006:426; d) Ocampo-Salinas *et al.*, 2016:651.

Natural oils are multipart compounds consisting of SFA and UFA (Montoya *et al.*, 2019:403) and commonly utilised as penetration enhancers in cosmetic products due to their safety profile and natural origin (Dreger & Wielgus, 2013:150; Viljoen *et al.*, 2015:2045). These fatty acids reversibly reduce the barrier function of the stratum corneum by disrupting its lipid domains, thereby effectively enhancing topical drug delivery (Dayan, 2005:104). For this study, coconut, castor and emu oil was selected to serve as the oil phase and penetration enhancer in the o/w emulsions, respectively.

2.11 Conclusion

Oestrogen deficiency brought about by menopause alters skin function and structural stability, accelerating the clinical manifestations of skin wrinkling, dryness and sagging (Farage *et al.*,

2013:5; Irrera *et al.*, 2017:622). HRT has long been regarded as one of the cornerstones of anti-ageing medicine, preventing and improving signs of skin ageing. However, the increased risk of breast cancer associated with elevated hormone levels in postmenopausal women has raised concerns regarding the safety of HRT. To avoid systemic exposure, and therefore, the risk of hormone-induced breast cancer, female hormones should be delivered topically (hence, not transdermally). The stratum corneum presents as a selectively permeable barrier, and therefore as a rate-limiting step during topical drug delivery (Ali *et al.*, 2015:103; Lippold, 1992:294). The choice of API, excipients and delivery system (vehicle) is essential to ensure effective drug delivery. Chemical penetration enhancers are utilised to enable the formulation of topical delivery systems for APIs that are otherwise inefficiently skin-permeable (Ghafourian *et al.*, 2004:113).

For the purpose of this study, it was aimed to formulate simple o/w emulsions containing female hormones (oestriol, oestradiol and progesterone) and selected natural oils (coconut, castor and emu) in an effort to overcome the stratum corneum barrier and deliver the hormones within the skin (epidermis-dermis). Consequently, the lipophilic hormones are incorporated within the oil phase. Natural oils are rich in fatty acids, which have been shown to possess penetration-enhancing effects. It is expected that the o/w emulsions will overcome the stratum corneum and deliver the female hormones topically.

Moreover, complex formulations containing female hormones will be provided by Idexis, which include a **(FS)**, **(EC)** and **(MC)**. Idexis aimed to formulate topical products that deliver female hormones to the epidermis-dermis to initiate an anti-ageing effect, while preventing systemic exposure, therefore reducing the risk of breast cancer in postmenopausal women. Consequently, the simple o/w emulsions will be compared to the complex Idexis products.

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CHAPTER 3:

ARTICLE FOR THE PUBLICATION IN THE EUROPEAN JOURNAL OF PHARMACEUTICAL SCIENCES

Chapter 3 is written in article format for publication in the *European Journal of Pharmaceutical Sciences*. The complete author's guide is listed in Appendix F. According to the author's guide, the use of either US or UK English is acceptable; UK English was used. The text has been justified for ease of reading.

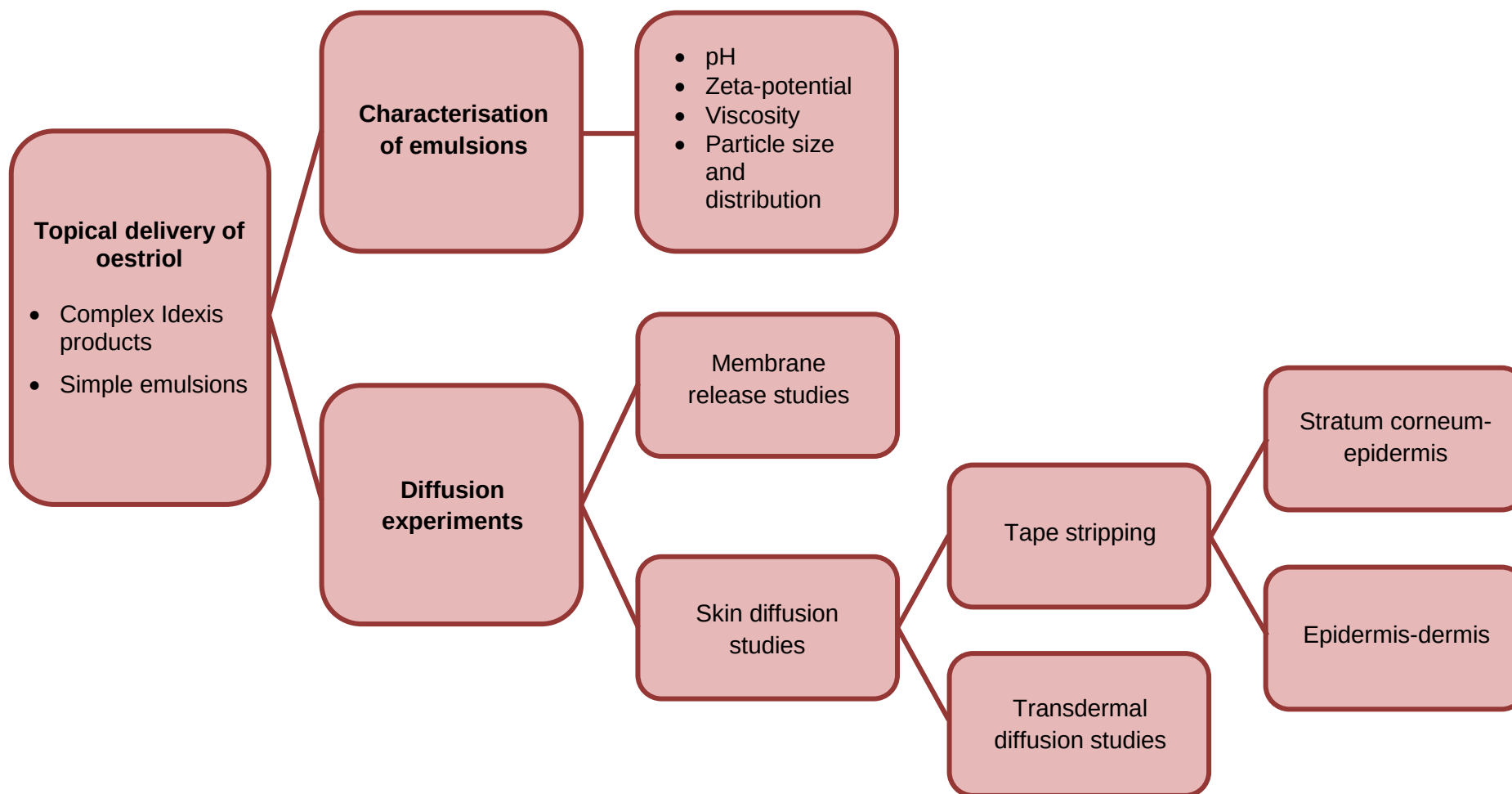
Formulation and topical delivery of oestriol against skin ageing

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Graphical abstract



Abstract

The use of conventional hormone replacement therapy (HRT) poses a great risk of hormone-induced breast cancer in postmenopausal women. Idexis (compounding pharmacy) aimed to formulate topical systems that deliver oestriol to the epidermis-dermis (ED) to exert an anti-ageing effect, and thereby, limit systemic exposure and breast cancer risk. For this study, three complex formulations were provided by Idexis. However, due to the classified nature of this study, limited information was made available. The aim of this study was therefore to formulate simple oil-in-water (o/w) emulsions containing 2% oestriol and selected natural oils (coconut, castor or emu) as the oil phase and as penetration enhancer, and to determine which formulation (Idexis products *versus* o/w emulsions) proved more advantageous for topical delivery, and in addition, to determine the efficacy of the polymers used in the Idexis products to inhibit systemic absorption. During this study, it was found that the o/w emulsion containing castor oil displayed the best-targeted delivery in the ED, with the lowest concentrations delivered transdermally. For all three Idexis products higher concentrations were delivered transdermally (not topically). Consequently, it can be concluded that the polymers were ineffective. Idexis might need to consider a simple formulation.

Keywords: Anti-ageing; Postmenopausal; Breast cancer; Oestriol; Topically

1 Introduction

Skin ageing is an inevitable deteriorating process resulting from two types of ageing: intrinsic ageing, which occurs over time at a genetically determined rate (Farage *et al.*, 2008), and extrinsic ageing, which is induced by environmental factors, such as ultraviolet radiation (UVR), tobacco smoking and air pollution (Jenkins, 2002). The cumulative effect of intrinsic and extrinsic factors leads to the gradual loss of structural integrity and physiological function (Naidoo & Birch-Machin, 2017). The clinical manifestations, however, are different. Chronologically aged skin is thin, finely wrinkled and atrophic, while extrinsically aged skin appears coarse with irregular hyperpigmentation and large pores (Mukherjee *et al.*, 2006). Oestrogen deficiency is the main consequence of menopause and is inextricably linked to the acceleration of skin ageing (Dalal & Agarwal, 2015; Thornton, 2013). Oestrogen has been shown to modulate skin physiology significantly; therefore, the drastic decline in circulating levels following menopause causes atrophic skin changes; skin becomes thin and wrinkled with a decrease in elasticity and vascularity (Stevenson & Thornton, 2007; Thornton, 2013).

The use of hormone replacement therapy (HRT) and its imperative role in the prevention of skin ageing has been confirmed by numerous studies, demonstrating amongst other things an increase in elasticity and a decrease in the probability of senile dry skin in postmenopausal women (Calleja-Agius *et al.*, 2007; Stevenson & Thornton, 2007). Breast cancer is the most common malignancy among women worldwide (Bray *et al.*, 2018; Sun *et al.*, 2017). This finding is alarming, as studies have shown the increased risk of breast cancer in postmenopausal women is associated with elevated circulating oestrogen levels (Folkerd *et al.*, 2014). Therefore, women diagnosed with breast cancer or those at risk due to genetic proneness cannot use standard HRT. Considering an alternative route of administration, such as topical delivery, could prove to be advantageous in reducing the risk of hormone-induced breast cancer as topical delivery aims to retain the active within the skin, and thereby, reduces systemic absorption (Garg *et al.*, 2015). In addition, topical delivery systems offer numerous advantages including avoidance of hepatic first-pass

metabolism, sustained drug delivery and reduced systemic adverse effects (Leite-Silva *et al.*, 2012).

Human skin, covering an average surface area of 2 m², is considered to be the largest and most accessible organ for drug delivery (Hadgraft, 2001; Schoellhammer *et al.*, 2014). Nevertheless, the skin constitutes a formidable permeation barrier, limiting the bioavailability of topically applied active pharmaceutical ingredients (APIs) (Shiozuka *et al.*, 2013). While the skin is a multi-layered structure, the barrier function is predominantly mediated by the stratum corneum, providing the rate-limiting step in topical drug delivery (Ali *et al.*, 2015; Lippold, 1992). To overcome the stratum corneum and reach the target-site, the intended drug candidate must possess certain physicochemical properties to ensure successful topical delivery (Alkilani *et al.*, 2015). These ideal physicochemical properties include a molecular weight (MW) of <500 g/mol, a melting point of <200°C, an aqueous solubility of >1 mg/ml, and an octanol-water partition coefficient (log P) between 1 and 3 (Naik *et al.*, 2000). However, oestriol does not meet these requirements, and so the selection of an appropriate vehicle is essential to enhance topical delivery (Dodou, 2012; Moffat *et al.*, 2004; Pubchem, 2005).

Table 1

Emulsions are colloidal dispersions composed of two or more immiscible liquids (water and oil), in which one liquid is homogenously dispersed (internal/dispersed phase) throughout the other (external/continuous phase) (Smith, 2003). Emulsions having an aqueous internal phase are designated water-in-oil (w/o) emulsions, whereas oil-in-water (o/w) emulsions have an oleaginous internal phase (Alexander, 2009). Emulsions exhibit several advantages, including the ability to carry and deliver both hydrophilic and lipophilic APIs, and consequently, improve the percutaneous absorption and bioavailability of oil- and water-insoluble compounds (Buchmann, 2001; Shaker *et al.*, 2019). However, emulsions are thermodynamically unstable, exhibiting phase separation over time through coalescence, flocculation and creaming (Goodarzi & Zendehboudi, 2018; Ngan *et al.*, 2014). Emulsion

stability is negatively associated with the droplet size of the dispersed phase (Goodarzi & Zendehboudi, 2018). Smaller droplets can be obtained using a high-energy input (Hu *et al.*, 2017), and so high-pressure homogenisation was utilised during this study to yield more elegant and kinetically stable colloidal systems by reducing the droplet size (Ding & Kan, 2017; Engman *et al.*, 1998; Hebishy *et al.*, 2013).

Chemical penetration enhancers allow the formulation of topical delivery systems for actives that are otherwise inadequately skin-permeable, by disrupting the barrier function of the stratum corneum, and in turn, enhancing the percutaneous absorption of actives (Ghafourian *et al.*, 2004; Patel *et al.*, 2019). Natural oils are multipart mixtures composed of saturated fatty acids (SFA) and unsaturated fatty acids (UFA) (Montoya *et al.*, 2019) and widely used in cosmetic preparations due to their natural origin and safety profile (Dreger & Wielgus, 2013:150). These fatty acids have the ability to reversibly reduce the permeability barrier of the stratum corneum by means of perturbation and fluidisation, thereby enhancing topical drug delivery (Correa *et al.*, 2014; Dayan, 2005; Haque & Talukder, 2018; Kezutyte *et al.*, 2013). For the purpose of this study, simple o/w emulsions were formulated using selected natural oils (coconut, castor or emu oil) as the oil phase and as a penetration enhancer in an effort to overcome the barrier resistance of the stratum corneum and deliver oestriol topically. The selected natural oils formed the core component, together with the lipophilic oestriol. In addition to the simple emulsions, complex formulations were also investigated. These formulations were supplied by Idexis (compounding pharmacy) and included a face serum (**FS**), eye cream (**EC**) and male cream (**MC**). Idexis aimed to formulate a topical oestriol delivery system that would not diffuse through the skin into the systemic circulation and still initiate an anti-ageing effect. Consequently, the simple o/w emulsions were compared to the complex Idexis products to evaluate the efficacy of the polymers (used in the Idexis products) to reduce/inhibit systemic absorption, and so reduce the risk of hormone-induced breast cancer in postmenopausal women.

2 Materials and Methods

2.1 Materials

Oestriol was supplied by Thonson Technology Ltd (Shanghai, China). Coconut, castor and emu oils were purchased from ENCO Fuels CC (Potchefstroom, South Africa), Allied Drug Company Ltd (Durban, South Africa) and Emuphoria (Potchefstroom, South Africa), respectively. Span[®] 60, Tween[®] 80 and glycerine were all obtained from Sigma Aldrich (Johannesburg, South Africa), whilst stearic acid and polyethylene glycol (PEG) 400 were obtained from Saarchem (Krugersdorp, South Africa). Crodafos[™] MCK was obtained from Croda (Boksburg, South Africa) and Veegum[®] HV from R.T Vanderbilt Co, Inc (Norwalk, United States). High performance liquid chromatography (HPLC) grade methanol, HPLC-grade acetonitrile, orthophosphoric acid (H₃PO₄) and cetyl alcohol, in addition to sodium hydroxide (NaOH) pearls and potassium dihydrogen orthophosphate (KH₂PO₄) (used to prepare phosphate buffer solution (PBS)), were attained from Merck Laboratory Supplies (Midrand, South Africa) and Merck Chemicals (Germiston, South Africa). Deionised HPLC-grade water was obtained by using a Milli-Q[®] water purification system (Millipore, Milford, United States). Parafilm[®], Dow Corning[®] high vacuum grease and Whatman[®] filter paper were obtained from Separations (Randburg, South Africa). All other solvents and reagents used were of analytical grade. The **(FS)**, **(EC)** and **(MC)** were provided by Idexis (compounding pharmacy) (Pretoria, South Africa).

2.2 Methods

2.2.1 Analysis of oestriol

An HPLC analytical method for oestriol was developed and validated by making use of an Agilent[®] 1200 Series HPLC system (Agilent Technologies, United States of America), fitted with an Agilent[®] 1200 gradient pump, ultraviolet (UV) detector (set at 210 nm) and autosampler. Chromatograms were analysed using ChemStation Rev. A.10.03 data acquisition and analysis software. A Venusil[®] XBP C₁₈ (2) (150 x 4.6 mm, 5 µm) column was utilised (Agela Technologies, United States of America) and the default injection volume was set at 20 µl and the flow rate at 1.0 ml/min. The two mobile phases comprised of Milli-Q[®]

water (in-lab) with 0.1% H₃PO₄, and HPLC-grade acetonitrile; HPLC-grade methanol was used as the solvent. Gradient elution was carried out starting at 25% acetonitrile, while increasing linearly to 75% after 4 min, followed by a 4-min hold and re-equilibration at starting conditions (runtime of 12 min). The retention time of oestriol was ± 3.837 min. The LOD (lowest limit of detection) and LOQ (lowest limit of quantification) obtained for oestriol were 0.0022 $\mu\text{g/ml}$ and 0.0067 $\mu\text{g/ml}$, respectively. HPLC analysis of all samples for this study was performed at a controlled temperature of 25°C.

2.2.1.1 Standard preparation

A standard solution was prepared prior to each analysis by weighing approximately 10 mg of oestriol in a clean 200 ml volumetric flask, whereafter it was dissolved and filled to volume with methanol. The standard solution was injected at different volumes (1.0, 5.0, 10.0, 15.0, 20.0 μl) to create a standard curve.

2.2.2 Formulation of oil-in-water emulsions

For this study, three o/w emulsions were formulated containing 2.0% (w/v) oestriol and 13.0% (w/v) of the selected natural oil (coconut, castor or emu oil). Excipients of the respective phases are listed in Table 2. The o/w emulsions were prepared by dissolving the excipients within the respective phases (A, B and C) on a hot plate (70 to 75°C) with a magnetic stirrer to ensure continuous stirring. Oestriol was added to the preheated phase B. After the excipients were entirely dissolved, phase B was added to phase C in a drop wise manner to form a coarse o/w emulsion. The emulsion was slowly added to phase A to form a cream base. Thereafter, the cream was homogenised at 9500 rpm (for 5 min), resulting in a finer o/w emulsion that was left to stir until it reached room temperature.

Table 2

The o/w emulsions were referred to as **(CoOE)** (o/w emulsion containing coconut oil), **(CaOE)** (o/w emulsion containing castor oil) and **(EOE)** (o/w emulsion containing emu oil). Collectively, these emulsions were referred to as **(NOE)** (natural oil emulsions).

2.2.3 Characterisation of oil-in water-emulsions

The three o/w emulsions were characterised in terms of pH, zeta-potential, particle size and distribution, and viscosity.

2.2.3.1 pH

A Mettler Toledo[®] pH meter (Mettler Toledo, United States of America), fitted with a Mettler Toledo[®] InLab[®] 410 electrode (Mettler Toledo, United States of America), was used to determine the pH values of the respective emulsions. Prior to measurements, the electrode was calibrated at pH 4, 7 and 10. Triplicate measurements were taken and averaged.

2.2.3.2 Zeta-potential

A Malvern Zetasizer Nano ZS (Malvern Instruments, United Kingdom) was utilised to determine the zeta-potential of each emulsion. A dilution of each emulsion was prepared (20 mg with 30 ml Milli-Q[®] water) and placed in an ultrasonic bath (for 15 min) to ensure proper dissolution. Thereafter, each dilution was analysed by injecting 2 ml into a clear disposable zeta-cell (DTS1070 folded capillary cell). Measurements were performed in triplicate and averaged.

2.2.3.3 Particle size and distribution

A Malvern Mastersizer 2000 Particle Size Analyser (Malvern Instruments, United Kingdom), fitted with a Hydro 2000SM Dispersion Unit (Malvern Instruments, United Kingdom), was utilised to determine the average particle size of the three emulsions. A dilution of each emulsion was prepared by adding 1 ml of the formulation into a polytop containing 10 ml Milli-Q[®] water and mixed thoroughly. Small quantities of the dilution were added to the Hydro 2000SM Dispersion Unit until the obscuration level was between 10-20%. The speed of the pump was set to 1200 rpm. Measurements were performed in triplicate to calculate the average particle size for each formulation.

2.2.3.4 Viscosity

A Brookfield Viscometer DV2T LV Ultra (Middleboro, United States of America), linked to a thermostatic water bath ($\pm 25^{\circ}\text{C}$), was used to determine the viscosity of the different emulsions. A sample of each formulation was placed in the bath to acclimatise 60 min

preceding the measurements. Thereafter, a T-E spindle was immersed into the pre-heated formulation and connected to the viscometer. The viscosity was measured at a spindle rotation spindle of 30 rpm. Rheocalc T1.2.19 software was pre-set to measure and gather multipoint data at 10 s intervals for 2 min; consequently, 12 readings were obtained and averaged to determine the viscosity of each emulsion.

2.2.4 Diffusion experiments

2.2.4.1 Membrane release studies

Prior to conducting the membrane release studies, the six formulations (3 simple **(NOE)** and 3 complex Idexis products) were pre-heated to 32°C in a regulated water bath (Grant Instruments, United Kingdom), while the receptor phase (PBS (pH 7.4)) was pre-heated to 37°C (in a second water bath). Twelve vertical Franz cells, consisting of a donor and receptor chamber, were used during this study: 10 cells contained the respective formulation, while the other two cells contained water (served as a control). Dow Corning® high vacuum grease was applied to both chambers on the connecting side. A magnetic stirrer was placed in the receptor chamber before placing the polyvinylidene fluoride (PVDF) synthetic membrane (Pall® Life Sciences, United States of America) upwards onto its greased side. The donor chamber was positioned on top of the receptor chamber, followed by the generous application of vacuum grease to seal the sides of the two connecting chambers (to prevent leakage). A metal horseshoe clamp was used to securely fasten the two chambers together. The receptor chamber had a filling capacity of ± 2 ml, and therefore, 2 ml of the pre-heated PBS (pH 7.4) was accurately measured to fill the receptor chamber. Before filling the donor chamber with the selected formulation (± 1 ml), each receptor chamber was visually inspected to ensure no trapped air bubbles were present. Thereafter, a piece of Parafilm® and a plastic cap was used to seal each donor chamber to prevent evaporation of constituents. The Franz cells were placed in a cell stand within the pre-heated water bath (37°C) on a magnetic stirring plate (Variomag, United States of America). Hourly for 6 h, the entire receptor phase was extracted and refilled with pre-heated PBS.

The extracted samples were placed in vials and analysed using HPLC to determine the concentration and release rate of oestriol from the different formulations.

2.2.4.2 Skin preparation

Female Caucasian skin obtained from cosmetic abdominal surgery, after receiving informed consent, was utilised for this study (Ethics no: NWU-00111-17-A1-06). The sole use of female skin minimises the variation in the results obtained and is easier to come by as abdominoplasty is a procedure regularly performed on females. Prior to the preparation of the skin, the donated samples were closely inspected to ensure there were no abnormalities or damage that could alter the results of the study. A Zimmer™ electric dermatome (Zimmer TDS, United Kingdom) was used to obtain dermatomed samples with a continuous thickness of 400 µm, by pressing the apparatus onto the skin at a 30-45° angle. The samples were then placed on Whatman® filter paper and enclosed with aluminium foil to be stored at -20°C for future use. Prior to each diffusion studies, the samples were thawed and cut into circular pieces (15 mm diameter), so that each circle could fit between the receptor and donor chamber of a vertical Franz cell.

2.2.4.3 Skin diffusion

The same technique used during membrane release studies (explained in Section 2.2.4.1) was employed for *in vitro* skin diffusion studies; however, the PVDF synthetic membranes were replaced with circular dermatomed skin samples (with the stratum corneum facing upwards) between the donor and receptor chamber. A single extraction took place 12 h after the study had commenced. The extracted samples were placed in vials and analysed using HPLC to determine the amount of oestriol that diffused through the abdominal skin after 12 h.

2.2.5 Tape stripping

The aim of tape stripping was to determine whether topical delivery of oestriol occurred within either the stratum corneum-epidermis (SCE) and/or the epidermis-dermis (ED). Following the completion of the skin diffusion study, the Franz cell chambers were disconnected, and the skin was visually examined. Undamaged skin samples were patted

dry with a paper towel to remove any remaining formulation and stapled to a solid surface on a piece of Parafilm®. 3M Scotch® Magic™ Tape was cut into 16 strips in such a way that it could sufficiently cover the diffusion area. The first strip was discarded after use to prevent any possible contamination. The remaining 15 strips, containing oestriol, stratum corneum and segments of the epidermis, were added to a polytop containing 5 ml of the extraction solution (methanol) and cut into smaller pieces. An additional polytop containing 5 ml of the same extraction solution was used for the remaining skin, which was cut into smaller pieces. All of the polytops were stored overnight (± 8 h) in the fridge (at 4°C), after which a small volume was extracted from each polytop and filtered through a 0.45 μ m polytetrafluoroethylene (PTFE) membrane filter into an amber vial for HPLC analysis (Pellett *et al.*, 1997).

2.2.6 Data analysis

The samples obtained from each study (membrane release, skin diffusion and tape stripping) were analysed by means of HPLC to obtain a linear line, which was then used to determine the oestriol concentration in the respective Franz cells (Ng *et al.*, 2010). For membrane release studies, the average flux ($\mu\text{g}/\text{cm}^2\cdot\text{h}$) of oestriol, that diffused through the membrane, was determined according to the slope of the linear line plotted against time (h) (at hourly intervals for 6 h) (Ng *et al.*, 2010; Shakeel *et al.*, 2007). For skin diffusion studies, the amount per area diffused ($\mu\text{g}/\text{cm}^2$) of oestriol that permeated through the skin after 12 h was determined, together with the average concentration ($\mu\text{g}/\text{ml}$) that permeated into the skin after 12 h. For this study, the median values will be used to describe the data obtained during membrane release studies, diffusion studies and tape stripping, as it is unaffected by extreme outliers (Manikandan, 2011).

2.2.7 Statistical analysis

Descriptive analysis comprises the calculation of mean (including standard deviation) and median values relating to the flux ($\mu\text{g}/\text{cm}^2\cdot\text{h}$) obtained during membrane release studies, amount per area diffused ($\mu\text{g}/\text{cm}^2$) during skin diffusion, and concentration ($\mu\text{g}/\text{ml}$) in the ED and SCE where tape stripping is concerned (Sheskin, 2000). Inferential analysis entails

analysis of variance (ANOVA) and the non-parametric Kruskal-Wallis test. A statistically significant effect is indicated by a p-value of ≤ 0.05 (Concato & Hartigan, 2016). Box-plots were used to illustrate summarised data. Logarithmic transformation of data was performed during this study to determine whether statistically significant effects occurred during diffusion studies and tape stripping. For the purpose of this study, the following three comparisons were investigated regarding the respective formulations: 1) **(FS)**, **(EC)** and **(MC)**; 2) **(CoOE)**, **(CaOE)** and **(EOE)**, and 3) **(FS)**, **(CoOE)**, **(CaOE)** and **(EOE)**. One-way ANOVA was performed to determine whether the flux values varied significantly between the different formulations, followed by a normal probability plot to visually test for the assumption of normality. Levene's test was used to test for the assumption of homogeneity of variances in order to apply ANOVA. If the assumption of homogeneity was met, Tukey's Unequal (N) honest significance difference (HSD) post-hoc test was used. The same statistical analyses were performed during skin diffusion studies. For tape stripping, a two-way ANOVA was applied by means of TIBCO Statistica™ (data analysis software system), version 13.3 (StatSoft, 2018) to determine the effects of different formulations and the skin layers (i.e. SCE and ED) on the accumulation of oestriol in the tape strips. The interaction showed no statistical significance, therefore one-way ANOVAs were performed for the respective skin layers. The assumption of normality did not hold; therefore, the Kruskal-Wallis test was applied with multiple comparisons for the ED. In order to compare the two Idexis products in terms of the SCE, a t-test was performed, followed by the Mann-Whitney U test for confirmation.

3 Results and Discussion

3.1 Formulation of oil-in water emulsions

The three formulations contained 2% (w/v) oestriol and 13% (w/v) of the respective natural oil (coconut, castor or emu oil). The **(NOE)** presented as smooth homogenous creams that were white in colour. By visual examination, both the **(CoOE)** and **(EOE)** presented as more viscous formulations compared to the **(CaOE)**. The **(NOE)** were formulated in an attempt to overcome the stratum corneum and deliver oestriol topically.

3.2 Characterisation of oil-in-water emulsions

The results obtained from the pH, zeta-potential, particle size and distribution, and viscosity analysis are tabulated in Table 3.

Table 3

It is essential to determine the pH of formulations intended for topical use, as values outside the acceptable range (3-9) could compromise the integrity of skin (Barry, 2002; Barry, 2007). All three **(NOE)** exhibited acceptable pH values, consequently, it can be stated that the emulsions were safe to use and non-irritating in nature (Nair *et al.*, 2013). According to Yang *et al.* (2007), higher pH provides more stability to an emulsion, and therefore the **(CoOE)** is considered to be more stable. The highly negative zeta-potential values displayed by all three **(NOE)** were indicative of excellent stability and increased skin permeability (Duangjit *et al.*, 2011; Silva *et al.*, 2012). While the **(CoOE)** exhibited more favourable skin permeability due to its highly negative value, the lower zeta-potential of the **(EOE)** would be more advantageous if postmenopausal women were to apply the emulsions, as it would not penetrate the skin as effectively as the **(CoOE)**. Particle size influences several physicochemical characteristics of an emulsion, such as stability, viscosity, appearance and effective drug delivery (Malvern Instruments Limited, 2015). The smaller average particle size of the **(CaOE)** was associated with higher stability and enhanced skin permeability (Adib *et al.*, 2016). Contrarily, the **(CoOE)** would be more favourable for limited skin permeation due to its larger average particle size. The lower span values of **(CoOE)** and **(EOE)** were indicative of more monodispersed emulsions, providing higher stability and uniform drug distribution when applied to the skin (Buchmann, 2001; Liu *et al.*, 2010; Ramin *et al.*, 2009). Yalcin *et al.* (2012) suggested a correlation between viscosity and the fatty acid composition of oils. Viscosity plays an essential role in controlling skin permeation; as viscosity increases, permeability decreases (Welin-Berger *et al.*, 2001). Therefore, the higher viscosities of the **(CoOE)** and **(EOE)** would be more advantageous if the aim was to

formulate emulsions that did not increase skin permeability of oestriol due to the increased risk of breast cancer.

3.3 Membrane release experiments

Figure 1

During the membrane release experiments, oestriol concentrations were quantified in the receptor phase of the Franz cells for all six formulations. It can therefore be proposed that detectable amounts of oestriol were released from the respective vehicles, followed by diffusion through the PVDF synthetic membranes. For the complex Idexis products, the **(MC)** obtained the highest median flux, followed by the **(FS)**, and lastly, the **(EC)**. The viscosity of the Idexis products increased in order of **(FS) < (EC) < (MC)**, whereas the median flux decreased in order of **(MC) > (FS) > (EC)**. Consequently, the lack of correlation indicated that the viscosity of the respective Idexis products was not a primary factor influencing the diffusion of oestriol across the membrane, but mainly drug-vehicle interactions (El Gendy *et al.*, 2002). For the simple **(NOE)**, the **(EOE)** presented with the highest median flux, followed by **(CoOE)** (2.847 µg/cm².h) and then **(CaOE)**. These results correlated well with those of Viljoen *et al.* (2015) and Larsen *et al.* (2001). Viljoen *et al.* (2015) reported that preparations containing emu oil exhibited higher median flux values than those containing coconut oil. Larsen *et al.* (2001) demonstrated that castor oil caused a marked decrease in drug release from oil vehicles; a likely cause for the low median flux exhibited by the **(CaOE)**. The viscosity of the **(NOE)** increased in order of **(CaOE) < (EOE) < (CoOE)**, whereas the median flux decreased in order of **(EOE) > (CoOE) > (CaOE)**. Consequently, the lack of correlation suggests that the viscosity of the **(NOE)** did not attribute to the diffusion of oestriol across the synthetic membrane. When the complex **(FS)** and the simple **(NOE)** were compared, the **(FS)** presented with a significantly higher median flux, which could be explained by its lower viscosity, facilitating the diffusion of drug molecules from the drug carrier towards the skin surface (El Gendy *et al.*, 2002).

3.4 Diffusion experiments

3.4.1 Diffusion study

Figure 2

For the complex Idexis products, the **(MC)** displayed the highest median amount per area diffused, followed by the **(EC)** and lastly, **(FS)**. The high median amount per area diffused displayed by the **(MC)** could be attributed to the increased availability of oestriol from the formulation, resulting in an increased concentration gradient, and in turn, enhanced diffusion across the skin (Fu & Kao, 2010). The viscosities of the Idexis products increased in order of **(FS) < (EC) < (MC)**, whereas the median amount per area diffused decreased in order of **(MC) > (EC) > (FS)**. Batheja *et al.* (2011) reported that the viscosity of a formulation is positively related to skin permeation. This observation made by Batheja *et al.* (2011) correlates with the results obtained from this study, as the more viscous **(MC)** exhibited the highest median amount per area diffused. However, according to Welin-Berger *et al.* (2001), skin permeability increases with decreasing viscosity. Consequently, the exact mechanisms responsible for the varied results are not known.

For the **(NOE)**, the **(CoOE)** displayed the highest median amount per area diffused, followed by the **(CaOE)**. For **(EOE)**, no oestriol concentration was quantified within the receptor phase. Consequently, it can be stated that the **(EOE)** did not deliver oestriol transdermally. Coconut oil is rich in lauric acid (C12:0) and myristic acid (C14:0), which could have attributed to the high median amount per area diffused ($\mu\text{g}/\text{cm}^2$) displayed by the **(CoOE)** (Viljoen *et al.*, 2015). Kravchenko *et al.* (2003) found that formulations containing lauric acid displayed increased percutaneous absorption, which the authors attributed to an increased bioavailability of the marker from the applied matrix. The low median amount per area diffused exhibited by **(CaOE)** could be attributed to the linoleic acid (C18:2) present in castor oil, which has two double bonds in its hydrocarbon chain (Akinshina *et al.*, 2016). These double bonds introduce conformational kinks in the structure of the fatty acid (Croguennec, 2016:43), which makes it more difficult for the fatty acid to insert itself along the stratum

corneum lipid chains, resulting in an increased lag time. However, once the fatty acids are packed within the skin, they disrupt the ordered structure of the lipid domain due to their bent structure, increasing the fluidity of the stratum corneum, and in turn, the API flux across the skin (Haque & Talukder, 2018). The **(EOE)** displayed no apparent diffusion across the skin. Emu oil contains high levels of palmitic acid (C16:0), which has a linear appearance and low solubility. Consequently, this lowers its ability to disrupt the barrier function of the stratum corneum by inserting itself into the lipid bilayers (Cho & Shin, 2012), resulting in little to no effect (Viljoen *et al.*, 2015).

When the complex **(FS)** and simple **(NOE)** were compared, the **(FS)** exhibited the highest median amount per area diffused, followed by the **(CoOE)** and lastly, **(CaOE)**. The difference in the results obtained could be due to the different viscosities of the formulations, which increased in order of **(FS) < (CaOE) < (CoOE)**. Baroli *et al.* (2010) stated that viscous formulations reduce the movement of drug molecules in the vehicle, impeding its skin partitioning and subsequent absorption. The lack of correlation between the median amount per area diffused and the viscosity of the formulation indicates that another mechanism contributed to the diffusion of oestriol across the skin.

3.5 Tape stripping

3.5.1 Stratum corneum-epidermis

Figure 3

For the complex Ilexis products, the **(MC)** displayed the highest median concentration within the SCE, followed by the **(EC)** and lastly, the **(FS)**, for which no concentration was detected within the SCE. Due to the ideal physicochemical properties of oestriol (see Table 1) (Moffat *et al.*, 2004; Naik *et al.*, 2000), it was expected that the lipophilic hormone would partition into the stratum corneum with ease, and then slowly partition into the viable epidermis (Benson *et al.*, 2005). The low oestriol concentrations in the SCE from the **(MC)** and **(EC)** could be because of slow diffusion into the ED (Benson *et al.*, 2005).

For the **(NOE)**, the **(CaOE)** displayed the highest median concentration within the SCE, followed by the **(CoOE)** and finally, the **(EOE)**. Fatty acids are commonly used as penetration enhancers, as they have the ability to disrupt the barrier function of the stratum corneum through perturbation and fluidisation, which in turn, increases the skin permeability of actives (Correa *et al.*, 2014; Haque & Talukder, 2018; Kezutyte *et al.*, 2013). Castor oil contains high concentrations of ricinoleic acid (Naik *et al.*, 2018), which could have attributed to increased concentrations of the **(CaOE)** within the SCE. However, not all fatty acids present with equal penetration enhancing effects. A study led by Viljoen *et al.* (2015) reported that lower drug concentrations were obtained in the stratum corneum with preparations containing emu and coconut oil, respectively. These findings correlate with the results obtained from this study (although the **(CoOE)** and **(CaOE)** concentrations in the SCE did not differ much).

When comparing the simple **(NOE)** and complex **(FS)**, the **(CaOE)** displayed the highest median concentration within the SCE, followed by the **(CoOE)**, **(EOE)**, and lastly, the **(FS)**, for which no concentration was quantified within the SCE. The difference in the concentrations could be due to the amount of oestriol released from the respective vehicles. The use of polymers in the Idexis products to limit skin penetration and systemic absorption could have attributed to lack of oestriol in the SCE from the **(FS)**. Moreover, the high oestriol concentrations from the **(NOE)** could be attributed to the penetration enhancing effects of fatty acids (in natural oils).

3.5.2 Epidermis-dermis

Figure 4

When comparing the Idexis products, the **(MC)** displayed the highest median concentration in the ED, followed by the **(FS)** and then, the **(EC)**. Binder *et al.* (2019) stated that the penetration depths of an incorporated API decrease with increasing formulation viscosity. The viscosity of the Idexis products increased in order of the **(FS)** < **(EC)** < **(MC)**.

Consequently, the lack of correlation indicated that the viscosity of the respective IDEXIS products was not a primary factor influencing the penetration depth of oestriol.

When comparing the **(NOE)**, the **(CaOE)** displayed the highest median concentration in the ED, followed by the **(CoOE)** and lastly, the **(EOE)**. Castor oil is rich in ricinoleic acid (Naik *et al.*, 2018), which has been shown to penetrate easily into the skin and enhance the percutaneous absorption of other constituents (Barbosa *et al.*, 2018; Baynes & Riviere, 2004). It can be proposed that the use of castor oil attributed to the high median concentration obtained in the ED for the **(CaOE)**. Coconut oil contains high levels of palmitic (C16:0), myristic (C14:0) and lauric acid (C12:0). These fatty acids have a linear shape, which consequently reduce their ability to disrupt the barrier lipids of the stratum corneum, and in turn, decrease the skin permeability of oestriol (Chi, 1995). Consequently, the lipid composition of coconut oil could have attributed to the low median concentration exhibited in the ED for the **(CoOE)**. Lastly, emu oil contains considerably high concentrations of SFAs (i.e. stearic and palmitic acid). Longer chain SFAs (>C₁₄) have higher melting points, reducing their solubility in oil, and consequently, their ability to disrupt the skin barrier function (Viljoen *et al.*, 2015). Hence, the lower median concentration obtained in the ED for the **(EOE)**, could be due to the decreased penetration ability of the SFAs present in the emu oil.

When comparing the simple **(NOE)** and complex **(FS)**, the **(CaOE)** displayed the highest median concentration, followed by the **(CoOE)**, **(EOE)** and finally, the **(FS)**. The higher median concentrations exhibited by the **(NOE)** could be due to the penetration enhancing effects of the fatty acids (in natural oils) (Correa *et al.*, 2014; Haque & Talukder, 2018; Kezutyte *et al.*, 2013). The lower median concentration displayed by the **(FS)** could be due to the low percentage recovery of oestriol from the formulation, leading to a lower concentration gradient, and in turn, lower oestriol accumulation in the ED (Fu & Kao, 2010).

3.6 Statistical analysis

3.6.1 Membrane release studies

Statistical comparison of the mean flux values using one-way ANOVA revealed statistically significant interactions ($p < 0.001$) between the means of the Idexis products. The homogeneity of variances assumption was satisfied as determined by Levene's test, $F(2, 23) = 6.871$, $p < 0.005$. Tukey's N HSD post-hoc test followed to determine the significant differences between all possible pair of means of the Idexis products. Consequently, the means of the Idexis products ((**FS**), (**EC**) and (**MC**)) differed significantly from each other.

Statistical comparison of the flux values by means of one-way ANOVA revealed no significant interactions ($p > 0.05$) between the means of the (**NOE**). The normal probability plot illustrated that the data was reasonably normally distributed. The homogeneity of variances assumption was met as confirmed by Levene's test, $F(2, 27) = 1.713$, $p = 0.199$. Consequently, Tukey's N HSD post-hoc test was not performed; therefore, no significant difference exists between the means of the (**CoOE**), (**CaOE**) and (**EOE**).

Statistical comparison of the flux values by use of one-way ANOVA revealed statistically significant interactions ($p < 0.001$) between the means of the (**NOE**) and the (**FS**). The assumption of normality was not met; therefore, the Kruskal-Wallis test was utilised to determine the differences between the (**NOE**) and (**FS**) medians. The test revealed statistically significant interactions ($p < 0.05$) between the (**FS**) and the respective (**NOE**) medians.

3.6.2 Skin diffusion studies

Logarithmic transformation was applied to transform skewed data to conform to normality (Feng *et al.*, 2014). Statistical comparison of the amount per area diffused values by means of one-way ANOVA revealed significant interactions ($p < 0.001$) between the means of the Idexis products. The assumption of normality was held. Thereafter, the homogeneity of variance assumption was met, as determined by Levene's test, $F(2,24) = 1.039$, $p = 0.369$. Consequently, Tukey's N HSD post-hoc test was performed. The means of the (**FS**) and

(EC) were not significantly different; however, a significant difference could be observed between both products and the **(MC)**.

Logarithmic transformation was used to reduce skewness, hence, the distribution of the **(CoOE)** and **(CaOE)** approximately conformed to normality. A t-test was used to compare the mean amount per area diffused values of the **(CoOE)** and **(CaOE)**, which revealed a significant effect $t(7.86) = 3.058$, $p = 0.016$.

Statistical comparison of the amount per area diffused values by means of one-way ANOVA showed significant interactions ($p < 0.001$) between the means of the **(NOE)** and the **(FS)**. The data was approximately normally distributed. The homogeneity of variances assumption was satisfied based on Levene's test, $F(2, 20) = 1.743$, $p = 0.201$. Consequently, Tukey's N HSD post-hoc test was applied to determine the significant differences between all possible pairs of the mean amount per area diffused values of the **(CoOE)**, **(CaOE)** and **(FS)**. The means of all three formulations were significantly different.

3.6.3 Tape stripping

A two-way ANOVA applied on the skin layers (SCE and ED) and the Idexis products revealed no statistical significant interaction ($F(1,36) = 3.18$, $p = 0.083$ (> 0.05). In order to compare the mean concentrations of the **(MC)** and **(EC)** in terms of the SCE, a t-test was performed, which revealed a significant effect, $t(11.439) = -3.768$, $p = 0.003$, which was followed by the Mann-Whitney U test ($p < 0.001$) for confirmation. The distribution of the **(EC)** approximately conformed to normality, whereas the distribution of the **(MC)** skewed left.

One-way ANOVA was utilised to determine the effect of the ED and the Idexis products during tape stripping studies, which revealed a significant effect ($p < 0.05$). Thereafter, the Kruskal-Wallis test was utilised to determine the differences between the Idexis products medians in the ED, which revealed a significant effect ($p < 0.05$). Consequently, the **(MC)** median differed significantly from that of the **(EC)**; the **(FS)** did not differ significantly from any of the products.

Statistical comparison of the concentrations in the ED by means of one-way ANOVA indicated significant differences ($p < 0.001$) between the **(NOE)** and **(FS)** means. The

Kruskal-Wallis test was used to determine the differences between the **(FS)** and **(NOE)** medians in the ED, which showed a significant effect ($p < 0.05$). The **(CaOE)** median differed significantly from that of the **(EOE)** ($p = 0.005$) and the **(FS)** ($p = 0.002$). The Mann-Whitney U test was used to compare the median concentrations of the SCE and ED for the **(NOE)** and Ilexis products, respectively. Significant differences were observed for the **(MC)** ($p = 0.009$) and the **(CaOE)** ($p = 0.021$).

4 Conclusion

Ilexis aimed to formulate topical systems that deliver oestriol to the ED to exert an anti-ageing effect, while inhibiting systemic exposure, and therefore, reducing the breast cancer risk in postmenopausal women. The aim of this study was to determine whether oestriol in the complex **(FS)**, **(EC)** and **(MC)** could only be delivered topically. Due to the classified nature of this study, limited information was made available regarding the excipients and vehicle used for the respective Ilexis products. Consequently, simple o/w emulsions were formulated containing 2% (w/v) oestriol and 13% (w/v) selected natural oils, which served as the oil phase and as a penetration enhancer in an attempt to overcome the skin barrier function. The simple **(NOE)** were compared to the complex Ilexis products to evaluate the efficacy of the polymers used to deliver oestriol topically to the ED and limit systemic exposure.

During membrane release studies, it was established that oestriol release occurred from all six formulations. The **(FS)** released significantly higher amounts of oestriol compared to the simple **(NOE)**, which could be ascribed to the low viscosity of the **(FS)**, facilitating the movement of drug molecules from the formulation to the surface of the skin (El Gendy *et al.*, 2002). As a result, a larger amount of oestriol was available for percutaneous absorption from the **(FS)** compared to the **(NOE)**, resulting in an increased API flux across the skin and into the systemic circulation.

It can be concluded from the skin diffusion studies that oestriol concentrations ($\mu\text{g/ml}$) were detected in the receptor phase for all the Ilexis products, therefore it can be stated that the polymers incorporated to limit the systemic absorption of oestriol were unsuccessful.

Oestriol concentrations were detected in the receptor phase for the **(CaOE)** and **(CoOE)**. The **(EOE)** was the only formulation not transdermally delivering oestriol. Considerably higher quantities of oestriol diffused across the skin from the **(FS)** compared to the **(NOE)**. Hence, higher oestriol concentrations were delivered transdermally from the **(FS)** compared to the **(NOE)**. The difference could be attributed to the higher occlusion effect of the oily vehicle of the **(FS)** compared to the continuous aqueous phase of the **(NOE)** (Jiménez *et al.*, 2004). The low viscosity of the **(FS)** could also have contributed to the higher oestriol concentrations, as skin permeation increases with decreasing viscosity (Gallagher & Heard, 2005).

During tape stripping studies, oestriol concentrations were detected in the SCE and ED for the **(NOE)**. Higher concentrations were obtained in the ED than in the SCE for the **(EOE)** and **(CaOE)**, confirming the penetration enhancing effects of the fatty acids (in natural oils). This could be attributed to the high levels of ricinoleic acid (C18:3) in castor oil, which effortlessly penetrated deep into the skin (Barbosa *et al.*, 2018; Baynes & Riviere, 2004). Emu oil contains extremely high concentrations of oleic acid and is the only selected natural oil that contains palmitoleic acid. Katsuta *et al.* (2005:1009) found that the topical application of palmitoleic and oleic acid caused apparent scaling, which signified abnormal epidermal differentiation. This modification could have contributed to the increased accumulation of oestriol in the ED for the **(EOE)**. A lower oestriol concentration was obtained in the ED compared to the SCE for the **(CoOE)** (although the concentrations did not differ much). This could be attributed to the higher levels of SFAs in coconut oil (Petrauskaitė *et al.*, 2000). Contrary to what was predicted, the **(FS)** did not display a higher affinity for the stratum corneum, as oestriol permeated rapidly (within 12 h) into the viable epidermis. From visual examination, it was apparent the **(FS)** was primarily composed of an oily base, which could have resulted in greater skin occlusion, and in turn, enhanced percutaneous absorption of oestriol (Hafeez & Maibach, 2013).

For the **(NOE)**, higher oestriol concentrations were retained within the skin layers compared to the amount that had diffused across the skin into the receptor phase. For the Idexis

products, higher oestriol concentrations were delivered transdermally compared to the amount retained within the skin layers. The excipients used during the formulation of the respective Idexis products may have disrupted the stratum corneum barrier function, and in turn, enhanced the percutaneous absorption of oestriol (Bigucci *et al.*, 2014; Yanase & Hatta, 2018). Due to the classified nature of this study, the excipients were not known, and therefore the degree of their penetration enhancing effects remains unclear.

Upon comparison of the **(NOE)**, the **(EOE)** exhibited the best drug release; however, drug delivery to the SCE and ED was the most limited. It can be proposed that an inadequate amount of oestriol was available for transdermal delivery. Both **(CoOE)** and **(CaOE)** released relatively equal concentrations of oestriol. However, the **(CaOE)** delivered higher oestriol concentrations to the SCE, and especially to the ED (target-site). Compared to the **(CoOE)**, the **(CaOE)** delivered lower concentrations transdermally. To conclude, the **(CaOE)** exhibited the best-targeted delivery, as high oestriol concentrations were retained within the ED and a limited amount diffused through the skin to be delivered transdermally.

Upon comparison of the Idexis product, the **(MC)** exhibited the best drug release, in addition to the best transdermal (4.4 times higher than the **(FS)** and 2.1 times higher than the **(EC)**) and topical delivery (SCE and ED). Both the **(FS)** and **(EC)** exhibited good drug release, and despite the low delivery in the SCE (**(FS)** had no delivery) and ED, the transdermal delivery was significantly higher than topical delivery for all three products tested. It can be concluded that the polymers (systemic absorption inhibitors) were unsuccessful. It is recommended that Idexis should consider a simple formulation that better targets the ED and delivers less transdermally.

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577 **Conflict of interest**

578 The authors declare no conflict of interest.

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812 **Tables**813 **Table 1:**

814 Physicochemical properties of oestriol

Oestriol	
MW (g/mol)	288.4 ^a
Melting point (°C)	280 ^a
Aqueous solubility	Practically insoluble ^a ; 13.25 µg/ml ^b
Log P	2.4 ^a

815 a) Moffat *et al.*, 2004; b) Pubchem, 2005;

816 **Table 2:**

817 Excipients used during the formulations of the **(NOE)**

	Ingredient	%w/v	Function
A	Stearic acid	4.0%	Thickening agent
	Cetyl alcohol	0.5%	Thickening agent
	Crodafos™ MCK	1.7%	Emulsifier
	Span® 60	0.4%	Emulsifier
B	Natural oil*	13.0%	Penetration enhancer and oil phase
	PEG** 400	5.0%	Humectant
	Oestriol	2.0%	Active
C	Glycerine	10.0%	Humectant
	Tween® 80	3.2%	Hydrophilic surfactant
	Veegum®	0.5%	Thickening agent
	dH ₂ O	60.0%	Water phase and solvent

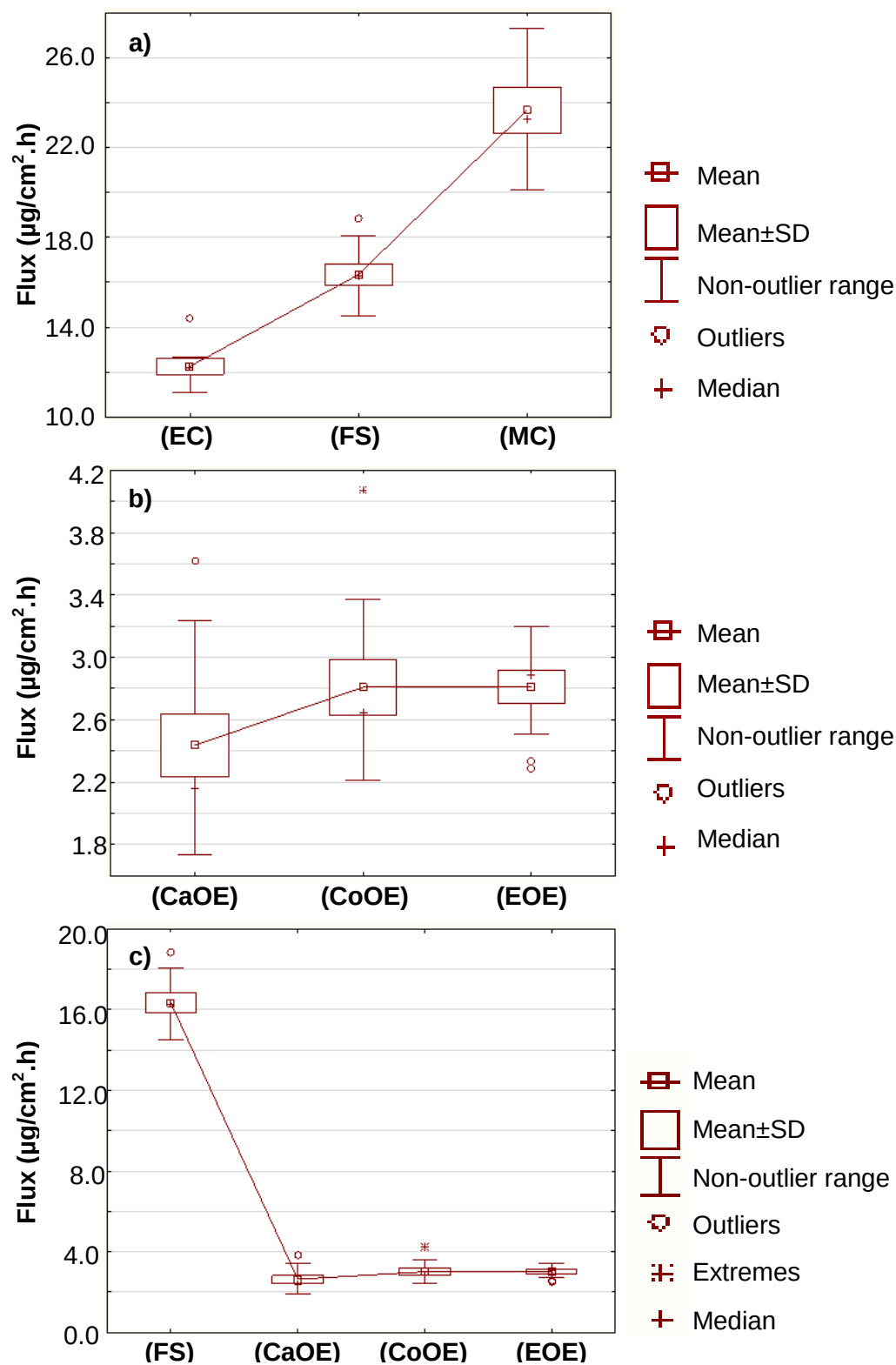
818 * Coconut-/castor-/emu oil; ** Polyethylene glycol

819 **Table 3:**

820 Characterisation results of the **(NOE)**

Formula	pH	Zeta- potential (mV)	Particle size (µm)	Span	Viscosity (cP)
(CoOE)	6.75	-98.2	14.23	2.286	3734.42
	(±0.358)	(±1.71)	(±0.137)	(±0.010)	(±11.975)
(CaOE)	6.58	-90.9	10.36	4.643	2438.83
	(±0.307)	(±0.85)	(±1.603)	(±0.541)	(±61.227)
(EOE)	6.30	-77.5	13.79	2.744	3334.58
	(±0.008)	(±1.85)	(±0.202)	(±0.043)	(±84.150)

821



823

824 **Figure 1:** Box-plot indicating the flux ($\mu\text{g}/\text{cm}^2\cdot\text{h}$) of oestriol from a) the Idexis products, b) the

825 **(NOE)** and c) the **(FS)** together with the **(NOE)** during membrane release studies over 6 h

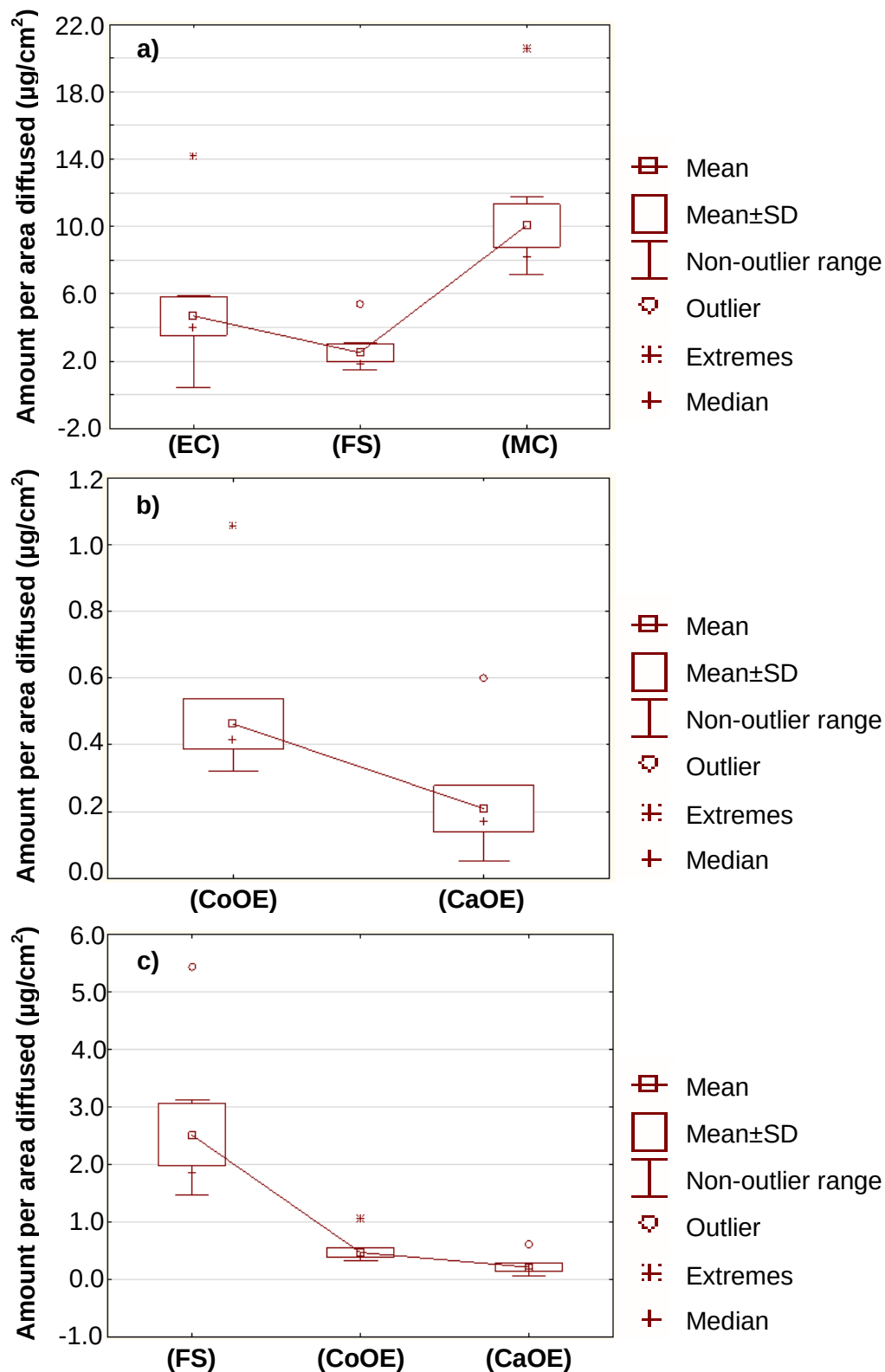


Figure 2: Box-plot indicating the mean and median amount per area diffused ($\mu\text{g}/\text{cm}^2$) of oestriol from a) the Idexis products, b) the **(NOE)** and c) the **(FS)** together with the **(NOE)** after 12 h

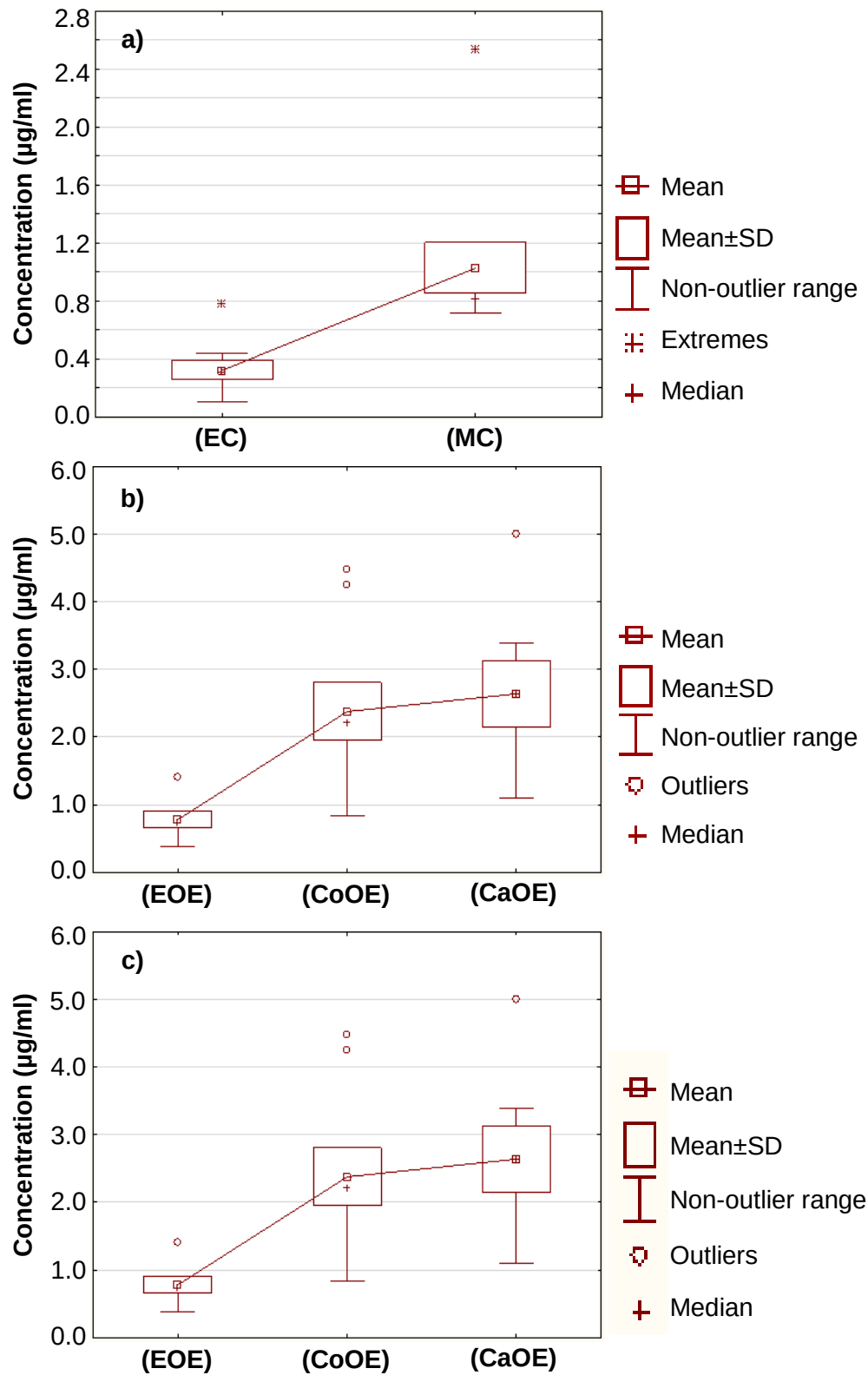


Figure 3: Box-plot indicating the mean and median concentration (µg/ml) of oestriol present in the SCE from a) the Idexis products, b) the **(NOE)** and c) the **(FS)** together with the **(NOE)**

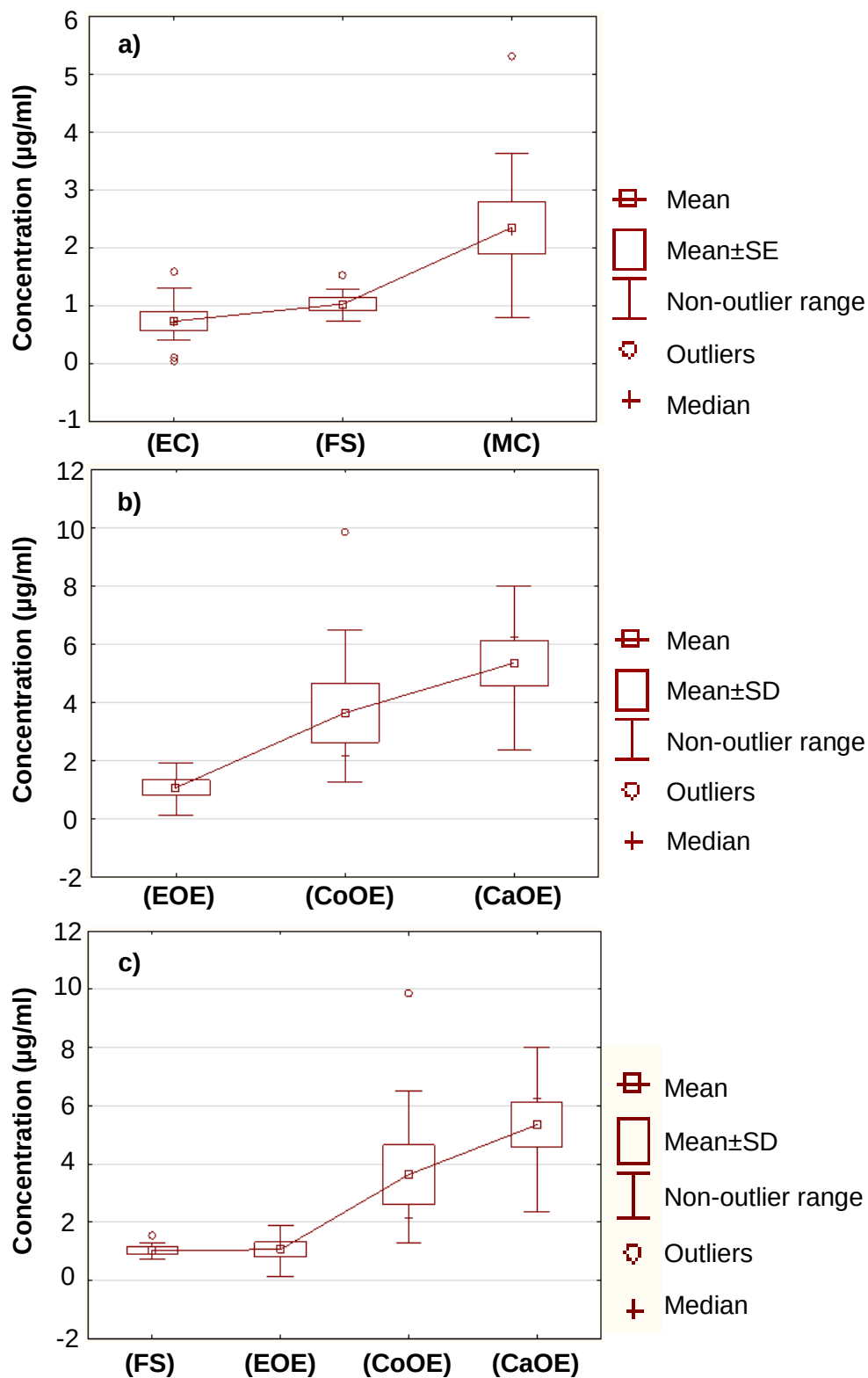


Figure 4: Box-plot indicating the mean and median concentration (µg/ml) of oestriol present in the ED from a) the Idexis products, b) the **(NOE)** and c) the **(FS)** together with the **(NOE)**

CHAPTER 4:

CONCLUSION AND FUTURE PROSPECTS

Skin ageing is a deteriorating process associated with the loss of both structural stability and function in the skin caused by the synergistic action of intrinsic and extrinsic factors (Bocheva *et al.*, 2019:2798; Zhang & Duan, 2018:729). Intrinsic ageing is the result of unavoidable physiological changes that occur over time at a genetically determined rate (Farage *et al.*, 2008:88; Sjerobabski-Masnec & Šitum, 2010:515). Extrinsic ageing is the result of continued exposure to damaging environmental factors, such as UVR, tobacco smoke and air pollution (Krutmann *et al.*, 2017:158; Sjerobabski-Masnec & Šitum, 2010:516). Oestrogen deficiency due to menopause causes a series of atrophic changes in the skin, such as skin wrinkling, thinning and dryness (Farage *et al.*, 2013:5; Irrera *et al.*, 2017:622). The anti-ageing effects of HRT on the skin of postmenopausal women have been well documented (Brincat *et al.*, 2005:110; Holzer *et al.*, 2005:633; Kanda & Watanabe, 2005:6). However, concerns exist regarding the safety of HRT, as numerous studies have reported a positive relationship between elevated hormone levels and breast cancer risk in postmenopausal women (Kaaks *et al.*, 2005:1071; Missmer *et al.*, 2004:1862). Consequently, HRT cannot be used by women previously diagnosed with breast cancer or those who are genetically predisposed. Idexis aimed to formulate topical preparations that deliver female hormones within the skin, avoiding systemic exposure; thus, decreasing the risk of hormone-induced breast cancer.

The aim of this study was to investigate whether each female hormone (oestriol, oestradiol and progesterone) in a complex **(FS)**, **(EC)** and **(MC)** formulation (provided by Idexis) could only be delivered topically (hence, not transdermally). The classified nature of this study, however, resulted in limited information disclosed to the student. In order to evaluate the efficacy of the polymers used in the complex Idexis products to inhibit systemic absorption, and thereby, reduce the risk of hormone-induced breast cancer in postmenopausal women, three simple o/w emulsions of different formulas were formulated and characterised, after which an optimised o/w emulsion was selected. This formula was then applied to formulate three o/w emulsions containing female hormones and selected natural oils (coconut, castor and emu oil), serving as the oil phase and as a penetration enhancer in an effort to overcome the stratum corneum. Accordingly, the complex Idexis products and simple o/w emulsions were investigated and their diffusion into and through the skin compared to determine the efficacy of the vehicle and polymers to inhibit systemic absorption. In addition, accelerated stability studies were performed on the Idexis products to determine the effect of elevated temperature and humidity variations on product stability.

The following objectives were set to achieve the aforementioned aims:

- To develop a validated HPLC method to determine the concentrations of the selected female hormones.
- To formulate three simple o/w emulsions containing female hormones and selected natural oils (separately) as the oil phase and as penetration enhancer.
- To characterise the three simple o/w emulsions (pre-formulated and optimised) with regard to particle size and distribution, pH, zeta-potential and viscosity.
- To characterise and conduct stability studies on the respective Idexis products at predetermined time intervals (months 0, 1, 2 and 3), evaluating the concentration assay, visual appearance, mass loss, pH, particle size and distribution, zeta-potential and viscosity.
- To determine the release of the selected female hormones from both the Idexis products and simple o/w emulsions by means of membrane release studies.
- To determine the transdermal and topical delivery of the selected female hormones by means of Franz cell skin diffusion studies and tape stripping, respectively.

An HPLC method for oestriol, oestradiol and progesterone was successfully validated and found to be sensitive, precise and reliable for the quantification of female hormones in different topical preparations.

Upon investigation of the physicochemical properties, it became apparent that the female hormones possessed ideal properties for topical delivery, such as low MW and adequate lipophilicity (Naik *et al.*, 2000:319). However, inadequacies with regard to poor aqueous solubility and high melting points were observed. For the purpose of this study, an emulsion was chosen as a possible delivery system, as it has the ability of solubilising both hydrophilic and lipophilic compounds (Buchmann, 2001:149; Shaker *et al.*, 2019:20). Three o/w emulsions of different formulas were formulated and characterised, after which the optimised o/w emulsion formula was selected as it presented with the most favourable characteristics for effective topical delivery, which included a highly negative zeta-potential (-55.90 ± 1.8980), lower viscosity (9471.2 ± 251.6) and a pH (5.543 ± 0.0058) within the acceptable range for safe topical use (3 – 9) (Barry, 2002:512).

The optimised formula was then applied to formulate three simple o/w emulsions using selected natural oils as the oil phase and as a penetration enhancer. Each natural oil emulsion contained 2% (w/v) oestriol, 0.2% (w/v) oestradiol, 2.5% (w/v) progesterone and 13% (w/v) of the respective natural oil. Characterisation of the optimised o/w emulsions containing coconut, castor and emu oil respectively, was indicative of advantageous characteristics, such as highly

negative zeta-potential values below - 30 mV and pH values that were considered safe for topical application (3 – 9) (Barry, 2002:512; Mahbubul, 2019:77). If postmenopausal women (with an increased risk of breast cancer) were to apply the optimised o/w emulsions, the higher viscosity and lower zeta-potential of the o/w emulsion containing emu oil would be more favourable as it would not penetrate the skin as efficiently and rapidly compared to the other emulsions.

Upon receiving the Idexis products, characterisation tests were performed and used as reference values. Thereafter, accelerated stability studies commenced at three different storage conditions (25 °C/60% RH, 30 °C/65% RH and 40 °C/75% RH) over a period of 3 months. The same characterisation tests were repeated at predetermined time intervals (month 1, 2 and 3). The concentration assay results for oestriol, oestradiol and progesterone did not remain within the acceptable 5% variation limit during the 3-month period (ICH, 2003:9). Increased concentrations were displayed by all three hormones, at all three climatic conditions for all three products. All three products stored at all three conditions displayed a significant change in mass and did not remain within the acceptable 5% variation limit. The visual appearance of the **(MC)** and **(EC)** changed significantly over the 3-month period, whereas the **(FS)** displayed no significant change in colour; however, phase separation was observed in the sample stored at 40 °C/75% RH. The initial slight, yet not unpleasant odour emitted by the products intensified to an unpleasant, sulphurous odour. Furthermore, the viscosity of all three products increased, whereas the mass decreased at all three storage conditions over the 3-month period. Both the **(EC)** and **(MC)** samples stored at 40 °C/75% RH displayed an increase in particle size, while the samples stored at 25 °C/60% RH and 30 °C/65% RH displayed a decrease over the 3-month period. The **(FS)** stored at all three storage conditions showed an increase in particle size. All three Idexis products presented with highly negative zeta-potential values. Although the values increased over the 3-month period, which is indicative of reduced product stability, the values were within the acceptable range. No significant changes were observed in the pH of all three products stored at different conditions over the 3-month period. It can be proposed that the physicochemical changes observed in the **(FS)**, **(EC)** and **(MC)** during stability testing compromised the quality, efficacy and safety of the products. None of the products completely met the criteria for stability as set by the ICH.

Oestriol was the only quantifiable female hormone during *in vitro* diffusion studies. Membrane release studies were performed to determine the release of oestriol from the different formulations. It was confirmed that oestriol was released from all three Idexis products and all three natural oil emulsions. The simple natural oil emulsions released considerably lower amounts of oestriol in comparison to the complex **(FS)**.

The transdermal and topical delivery of oestriol was determined by means of *in vitro* skin diffusion studies and tape stripping, respectively. Oestriol was delivered transdermally from all three Idexis products, and to a lesser degree from both the castor oil and coconut oil emulsions. Oestriol was not delivered transdermally from the emu oil emulsion. During tape stripping, oestriol was quantified in the stratum corneum-epidermis (SCE) and epidermis-dermis (ED) for the **(MC)**, **(EC)** and all three natural oil emulsions. No amount of oestriol was detected in the SCE for the **(FS)**. For the natural oil emulsions, lower oestriol concentrations were delivered transdermally compared to the amount retained within the skin layers (hence, topically). For the Idexis products, lower oestriol concentrations were retained within the skin layers compared to the amount delivered transdermally.

To conclude, the emu oil emulsion was the only formulation that successfully achieved the aim of topical delivery. The Idexis products were unsuccessful in reaching the set aim. It can therefore be stated that the polymers used in the Idexis products to inhibit systemic absorption (hence, transdermal delivery), were ineffective.

Future prospects might include:

- Performing stability testing at different climatic conditions (i.e. cool and dry) to determine the ideal temperature and humidity for optimal preservation.
- Investigating the use of sealable amber glass containers as a better vessel for maintaining product stability during storage.
- Considering a basic formulation that better targets the ED and delivers a reduced amount of the female hormones transdermally.
- Increasing the concentrations of the polymers used in the respective Idexis products.
- Using polymers with a higher degree of inhibitory effects.
- Conducting a long-term clinical efficacy study.

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APPENDIX A:

VALIDATION OF A HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR FEMALE HORMONE QUANTIFICATION IN TOPICAL FORMULATIONS

A.1 Objective

Validation is the process of verifying that a method is satisfactory for its proposed intent by acquiring experimentally justified evidence of the capability of the method to provide accurate and reproducible results (Betz *et al.*, 2011:44; Épshtein, 2004:212; Green, 1996:305A, 309A). The objective was to validate an HPLC analytical method to determine the sensitivity and suitability of the method with regard to the quantification of female hormones in topical formulations (APVMA, 2004:3; ICH, 2005:1). The required validation criteria that were investigated included linearity, accuracy, precision, ruggedness, specificity, limit of quantification (LOQ) and limit of detection (LOD) (ICH, 2005:2). Furthermore, the analytical method was used to establish and quantify the concentration of each female hormone present in the topical formulations during the course of stability testing (month 0, 1, 2 and 3), and in the receptor phase collected after the completion of *in vitro* diffusion studies.

A.2 Chromatographic conditions

This HPLC analytical method for female hormones was developed and validated under the supervision and with the support of Prof. J.L. du Preez at the Analytical Technology Laboratory (ATL), North-West University, Potchefstroom Campus. Method validation was completed in conjunction with Ms A.D. Bosch, as both research studies included the use of female hormones as actives. Despite the similarity, it should be mentioned that the documentation and analysis of data were done independently by each student. Various chromatography conditions were controlled during the validation of this analytical method, which included the analytical instrument used, mobile phase, the column, flow rate, detection wavelength and the injection volume. Method validation was performed under the following chromatographic conditions:

Analytical instrument: The HPLC analysis of the female hormones was completed by making use of an Agilent® 1200 Series HPLC system (Agilent Technologies, United States of America) equipped with an Agilent® 1200 gradient pump, autosampler injection mechanism and UV detector. ChemStation Rev. A.10.03 data acquisition and analysis software were used to analyse the chromatograms. A constant temperature of 25 °C was maintained in the laboratory.

Column:	A reverse phase Venusil® XBP C ₁₈ (2) column containing pure silica (150 x 4.6 mm) with a particle size of 5 µm was used (Agela Technologies, United States of America).
Gradient:	The gradient elution started at 25% acetonitrile, followed by a linear increase to 75% after 4 min and kept until 8 min elapsed and re-equilibrated at starting conditions.
Mobile phase A:	Milli-Q® water (in-lab) with 0.1% orthophosphoric acid (H ₃ PO ₄)
Mobile phase B:	HPLC-grade acetonitrile
Solvent:	HPLC-grade methanol
Injection volume:	20 µl
Flow rate:	1.0 ml/min
Detection:	The UV detector was set at 210 nm
Retention time:	± 3.837, 5.149 and 7.518 min for oestriol, oestradiol and progesterone, respectively
Run time:	12 min

A.3 Standard and sample preparation

A.3.1 Standard preparation

The standard solution containing oestriol, oestradiol and progesterone was prepared prior to each analysis as follows:

1. Weigh approximately 10 mg of oestradiol in a clean 50 ml volumetric flask, dissolve and fill to volume with methanol (Solution 1).
2. Weigh approximately 10 mg of oestriol and 20 mg of progesterone in a 200 ml volumetric flask and dissolve in methanol (Solution 2).
3. Add 10 ml of Solution 1 to Solution 2 using a volumetric glass pipette and fill to volume with methanol (Standard solution).
4. Transfer standard solution into an autosampler vial and analyse.

A.3.2 Sample preparation for analysis

The formulations used throughout this study were provided by Idexis (compounding pharmacy), which included a **(FS)**, **(EC)** and **(MC)**. The known ingredients and quantities present in each formulation are shown in Tables A.1 – A.2. To prepare a sample, the appropriate amount of formulation was weighed off into a 200 ml volumetric flask and diluted with methanol. The

sample was placed in an ultrasonic bath to ensure proper dissolution and made up to volume with methanol. Each sample was filtered through a 0.45 µm polytetrafluoroethylene (PTFE) filter into an autosampler vial and analysed.

Table A.1: Total percentage (%) of hormones present in Idexis formulations

Formulations	Hormones (%)		
	Oestriol	Oestradiol	Progesterone
FS	2.0	0.2	2.5
EC	0.5	0.1	2.0
MC	1.0	0.2	2.0

Table A.2: Total percentage (%) of antioxidants present in Idexis formulations

Formulations	Antioxidant (%)				
	VEA	ALA	COQ10	CL	GTE
FS	1.5	1.0	0.5	3.0	-
EC	1.5	1.5	1.0	2.0	0.2
MC	0.5	0.5	0.5	-	-

Vitamin E acetate (VEA), alpha lipoic acid (ALA), co-enzyme Q10 (CoQ10), caffeine liposomes (CL) and green tea extract (GTE)

A.3.3 Placebo preparation

A placebo formulation for both the cream and serum were supplied by Idexis. Samples were prepared as mentioned in Section A.3.2.

A.3.4 Sample preparation for stability testing

The assay analysis of all the formulations at all the storage conditions was performed at three time intervals (month 0, 1, 2 and 3). The samples were prepared as described in Section A.3.2.

A.3.5 Sample preparation for diffusion studies

Samples extracted from the receptor phase of every Franz diffusion cell were transferred directly into autosampler vials for analysis without being filtered or diluted.

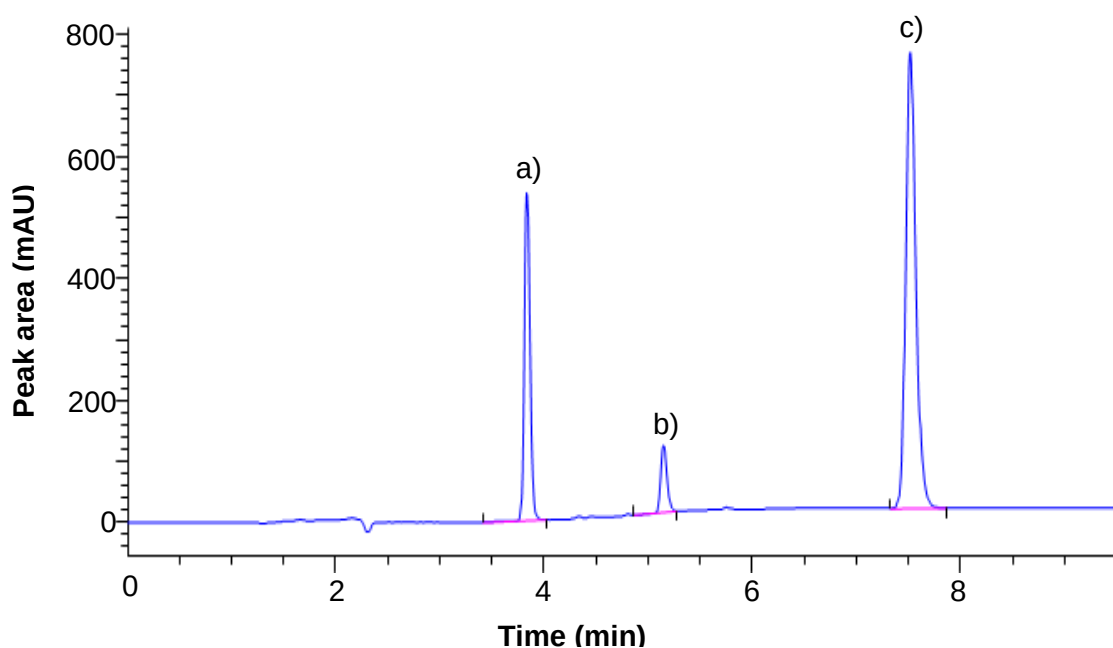


Figure A.1: Representative chromatogram of a standard solution showing the peak area and retention time of a) oestriol (3.837 min), b) oestradiol (5.149 min) and c) progesterone (7.518 min)

A.4 Validation criteria

A.4.1 Linearity

Linearity can be defined as the ability of an analytical method to procure test results that are directly equivalent to the analyte concentration ($\mu\text{g/ml}$) in the sample over a given range (APVMA, 2004:4; ICH, 2005:5; Paithankar, 2013:234). Linearity was determined by analysing the data plots formed by the peak areas (y-axis) as a function of the corresponding analyte concentration (x-axis) on the linear regression curve (González & Herrador, 2007:230), which should form a straight line with a coefficient of determination (R^2) ≥ 0.99 (APVMA, 2004:4; González & Herrador, 2007:230; Taverniers *et al.*, 2004:546). Method stability and a high degree of linearity are indicated if the R^2 is within the acceptable criteria, confirming a direct linear proportionality between the response (peak area) and the concentration of the analyte (APVMA, 2004:4).

The regression curve was interpreted by utilising the peak area (y-axis) *versus* the analyte concentration (x-axis). The linear regression equation (Equation A.1) can best describe the plotted regression curve (González & Herrador, 2007:229).

$$y = mx + c$$

Equation A.1

Where:

y = peak area ratios of the different female hormones

m = slope

x = concentration of the different female hormones

c = y-intercept

To determine the linearity of the analytical method, a stock solution was prepared containing oestriol (9.75 mg), oestradiol (9.35 mg) and progesterone (9.46 mg). The female hormones were weighed, dissolved in a 50 ml volumetric flask and made up to volume with methanol. Further dilutions were made as follows:

- From the stock solution, 5 ml was transferred to a 50 ml volumetric flask and made up to volume with methanol (Dilution 1).
- From Dilution 1, 5 ml was transferred to a 50 ml volumetric flask and made up to volume with methanol (Dilution 2).

Table A.3: Concentration ($\mu\text{g/ml}$) of each female hormone obtained after dilution

Hormone	Stock solution ($\mu\text{g/ml}$)	Dilution 1 ($\mu\text{g/ml}$)	Dilution 2 ($\mu\text{g/ml}$)
Oestriol	195.0	19.50	1.950
Oestradiol	187.0	18.70	1.870
Progesterone	189.2	18.92	1.892

The stock solution, together with the two dilutions, was injected into the HPLC eight times. The stock solution and Dilution 1 samples were injected at the following injection volumes in duplicate:

- 5.0 μl
- 10.0 μl
- 15.0 μl
- 20.0 μl

Dilution 2 was injected at the following injection volumes in duplicate:

- 1.0 μl
- 2.5 μl
- 5.0 μl
- 10.0 μl

- 15.0 µl
- 20.0 µl

The following concentration ranges were obtained for each of the female hormones:

- Oestriol: 0.098 – 195.000 µg/ml
- Oestradiol: 0.094 – 187.000 µg/ml
- Progesterone: 0.095 – 189.200 µg/ml

Figures A.2 – A.4 and Tables A.4 – A.6 demonstrate the linearity of each female hormone over the concentration ranges mentioned above.

Table A.4: Linearity results of oestriol

Sample	Concentration (µg/ml)	Peak area 1 (mAU)	Peak area 2 (mAU)	Average peak area (mAU)
13	0.098	5.60	6.20	5.9
12	0.244	14.70	14.20	14.5
11	0.488	28.20	27.90	28.1
10	0.975	55.30	55.40	55.4
9	1.463	82.60	82.80	82.7
8	1.950	109.70	109.90	109.8
7	4.875	262.00	269.30	265.7
6	9.750	527.10	517.60	522.4
5	14.625	770.20	769.70	770.0
4	19.500	1018.80	1017.90	1018.4
3	48.750	2457.90	2482.60	2470.3
2	97.500	4610.90	4594.10	4602.5
1	146.250	6128.80	6206.40	6167.6
Stock solution	195.000	7770.00	7595.80	7682.9
R²				0.9927
y-intercept				119.4
Slope				40.86

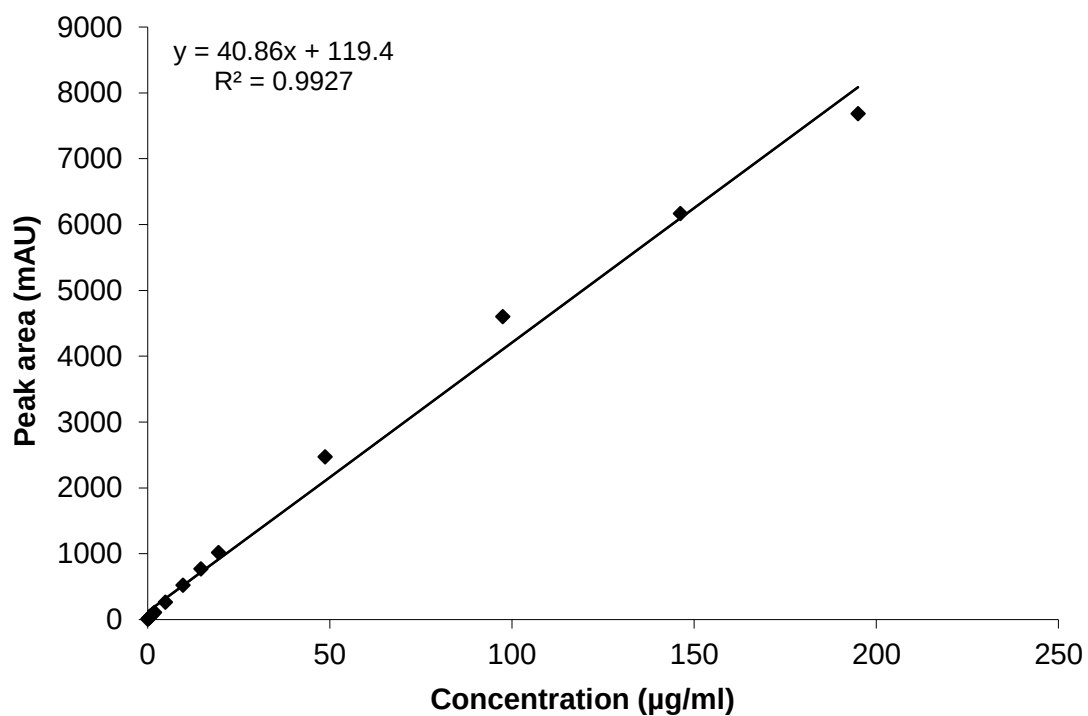


Figure A.2: Linear regression curve of oestriol

Table A.5: Linearity results of oestradiol

Sample	Concentration (µg/ml)	Peak area 1 (mAU)	Peak area 2 (mAU)	Average peak area (mAU)
13	0.094	5.10	5.30	5.2
12	0.234	13.40	14.10	13.8
11	0.468	27.70	27.80	27.8
10	0.935	56.50	54.70	55.6
9	1.403	84.30	83.30	83.8
8	1.870	111.70	111.70	111.7
7	4.675	269.20	278.10	273.7
6	9.350	541.60	532.60	537.1
5	14.025	794.00	792.90	793.5
4	18.700	1049.90	1051.70	1050.8
3	46.750	2602.00	2635.70	2618.9
2	93.500	4918.80	4921.60	4920.2
1	140.250	6800.50	6824.10	6812.3
Stock solution	187.000	8462.70	8338.50	8400.6
R²				0.9943
y-intercept				104.4584
Slope				46.687

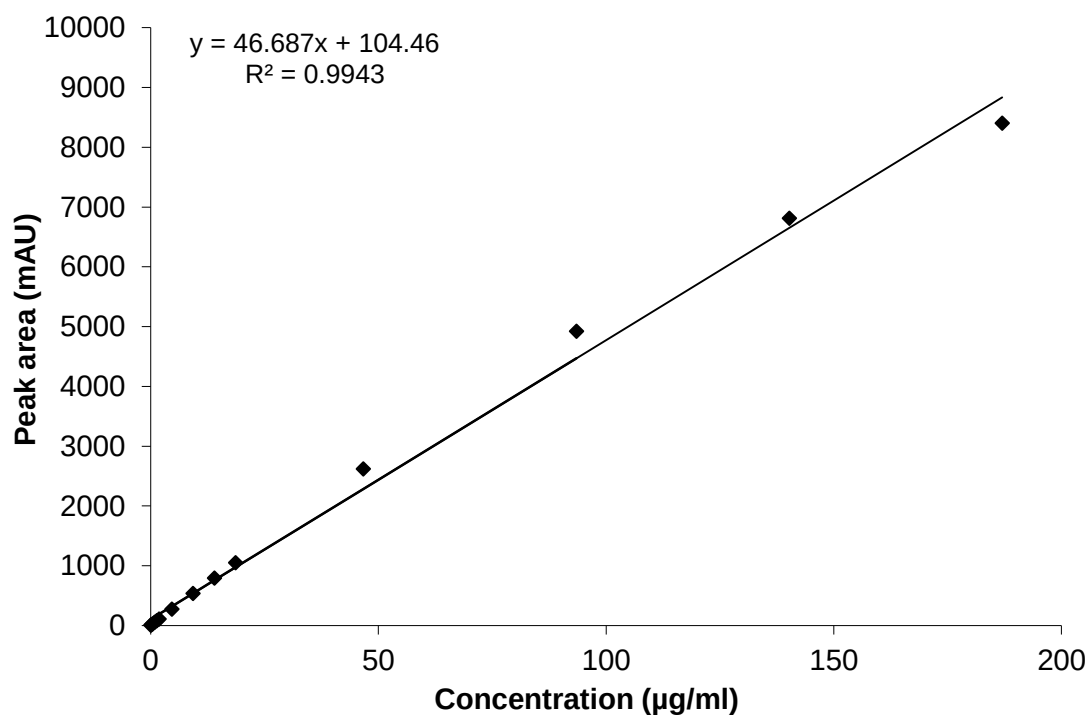


Figure A.3: Linear regression curve of oestradiol

Table A.6: Linearity results of progesterone

Sample	Concentration (µg/ml)	Peak area 1 (mAU)	Peak area 2 (mAU)	Average peak area (mAU)
13	0.095	6.00	6.60	6.3
12	0.237	15.60	15.60	15.6
11	0.473	30.40	30.50	30.5
10	0.946	60.00	60.00	60.0
9	1.419	89.40	89.70	89.6
8	1.892	119.20	119.10	119.2
7	4.730	282.10	289.80	286.0
6	9.460	573.60	563.00	568.3
5	14.190	846.40	845.40	845.9
4	18.920	1127.30	1128.30	1127.8
3	47.300	2831.50	2853.30	2842.4
2	94.600	5703.30	5713.90	5708.6
1	141.900	8483.30	8470.50	8476.9
Stock solution	189.200	11022.00	11022.90	11022.5
R²				0.9997
y-intercept				17.87942
Slope				58.91

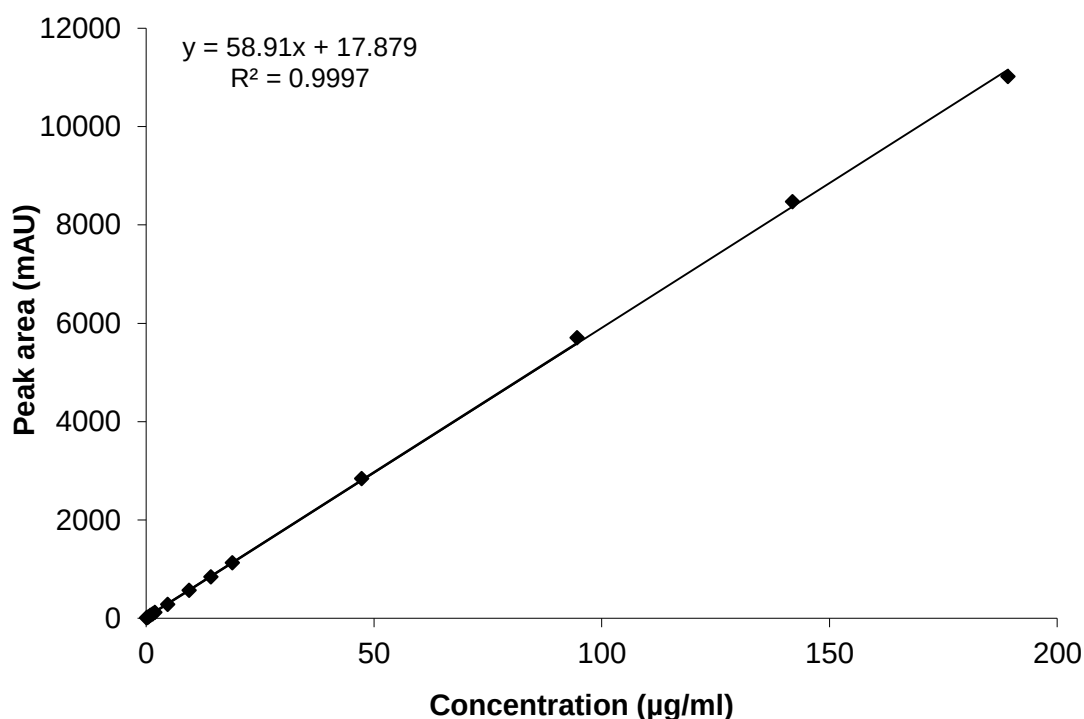


Figure A.4: Linear regression curve of progesterone

Linear regression aims to obtain the best-fitting straight line, also known as the trendline, through a scatter plot of data that best indicates the relationship between those points (Casson & Farmer, 2014:593). The trendline for each female hormone is shown in Figures A.2 – A.4. The R^2 describes the proportion of variability in the dependent variable (peak area), which is explained by the independent variable (analyte concentration) in the linear relationship (Young, 2011:59). The accepted criteria for linearity include an $R^2 \geq 0.99$ for the range of 80 – 120% of the target concentration (APVMA, 2004:4; Shabir, 2005:317). All the female hormones obtained an R^2 value above 0.99 (as listed in Table A.7), which is indicative of a positive linear relationship.

Table A.7: R^2 values of each female hormone

Hormone	R^2
Oestriol	0.9927
Oestradiol	0.9943
Progesterone	0.9997

Although the analytical method meets the criteria for linearity based on the R^2 , the trendlines for both oestriol and oestradiol (as seen in Figures A.2 – A.3) did not comply with Beer's law; progesterone was the only female hormone to comply with Beer's law (as seen in Figure A.4).

Beer's law states that the absorbance value for each hormone is directly proportional to the concentration of the hormone in the solution (Myers *et al.*, 2013:5). Deviations of Beer's law can occur at high concentrations due to change in the absorptivity of an analyte and the refractive index of the solution (Ball, 2006:65; Garcia-Rubio, 1992:2608; Mehta, 2012). Note that the refraction index of a solution can be defined as the ratio of the speed of light in a vacuum to that in a defined solution (Hasanuzzaman *et al.*, 2016:8).

The trendline for both oestriol and oestradiol was plotted against lower concentration values (as seen in Figures A.5 – A.6), which obtained better R^2 values (as seen in Table A.8).

Table A.8: Improved R^2 values

Hormone	R^2	Improved R^2
Oestriol	0.9927	0.9997
Oestradiol	0.9943	1.0000

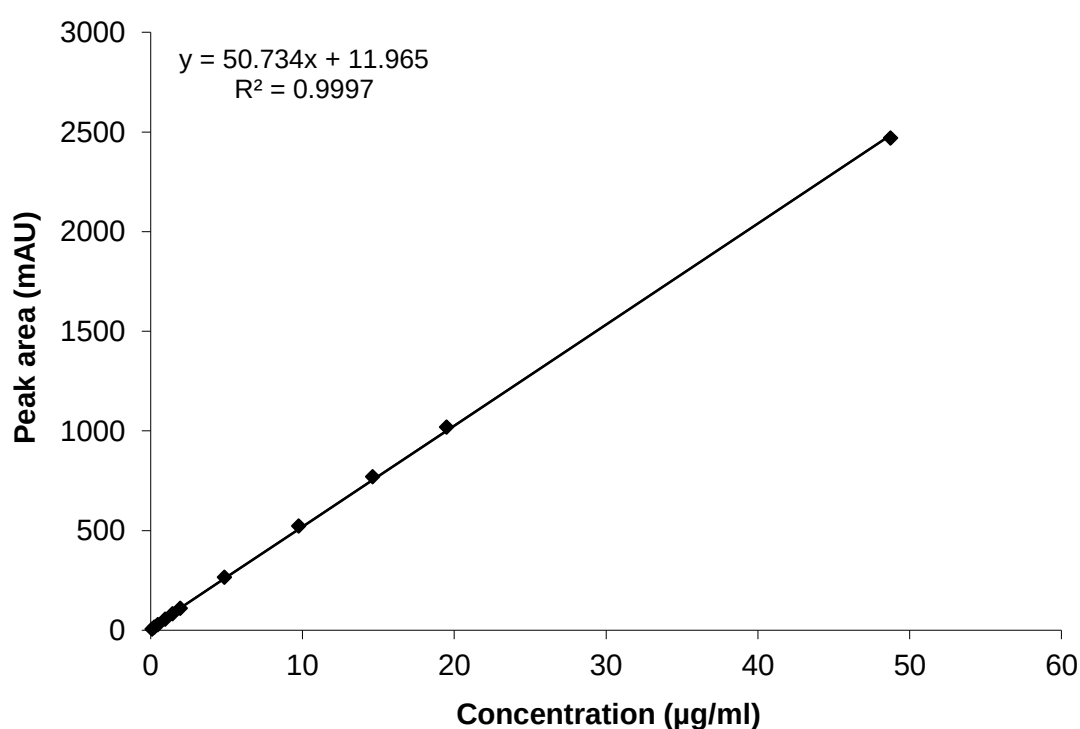


Figure A.5: Linear regression curve of oestriol plotted against lower concentrations

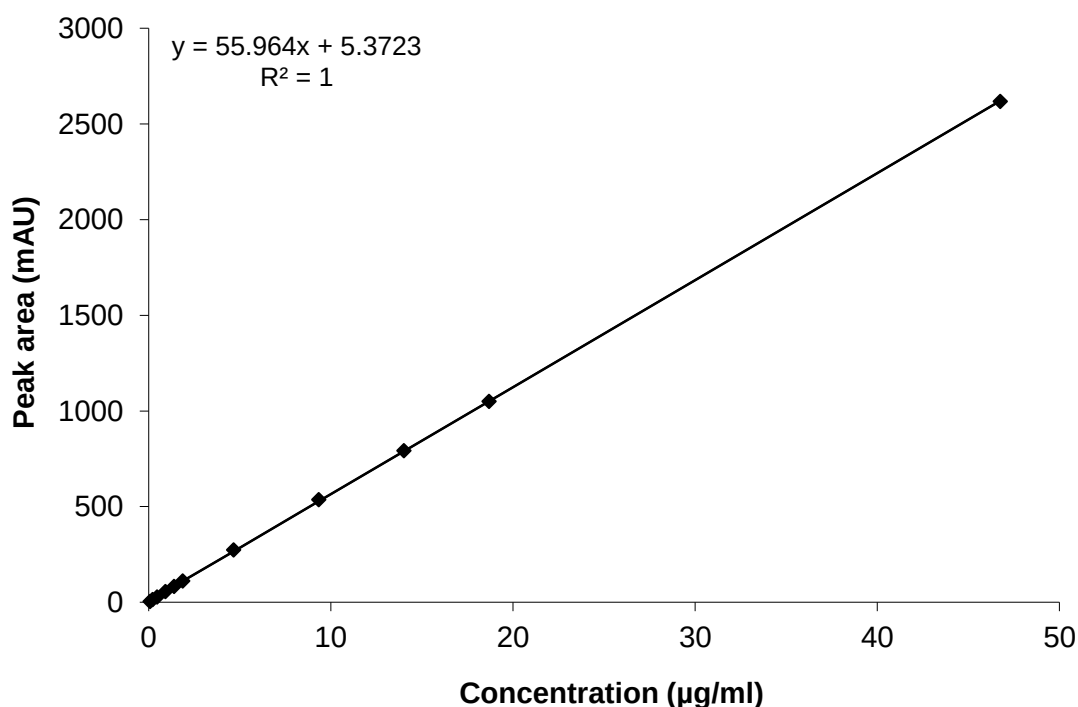


Figure A.6: Linear regression curve of oestradiol plotted against lower concentrations

A.4.2 Accuracy

The accuracy of an analytical method can be described as the closeness of agreement between the test result obtained and the true value of the property being analysed (APVMA, 2004:4; ICH, 2005:4). The ICH (2005:10) recommends that a minimum of nine samples spiked at three different concentrations, covering the full calibration curve, should be analysed to determine accuracy (Bansal & DeStefano, 2007:E111; Snyder *et al.*, 1997:691). The mean recovery percentage of the samples is required to be within a certain acceptance criteria (APVMA, 2004:5), as shown in Table A.9, which is indicative of the method's accuracy.

Table A.9: Acceptance criteria for accuracy validation (AVPMA, 2004:5)

%Active/impurity content	Acceptable mean recovery
≥ 10.0	98 – 102%
≥ 1.0	90 – 110%
0.1 – 1.0	80 – 120%
< 0.1	75 – 125%

The placebo formulations used to determine accuracy were provided by Idexis with limited information about the excipients and formulation methods used due to the classified nature of this study. The accuracy of the analytical method for the estimation of female hormones in the

presence of numerous excipients was investigated for both the placebo cream and serum due to the distinct difference in the formulations (as seen in Figure A.7).

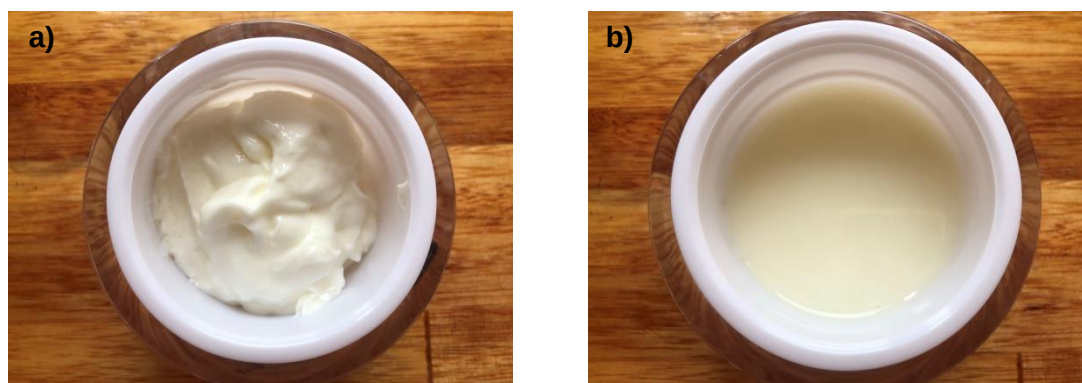


Figure A.7: a) Placebo cream and b) placebo serum formulations supplied by Idexis

A.4.2.1 Accuracy analysis of the placebo cream

A spike solution containing each female hormone was prepared by dissolving 103.72 mg oestradiol in 50 ml methanol; 10 ml was transferred to a 200 ml flask containing 105.90 mg oestriol and 200.80 mg progesterone, and made up to volume with methanol.

The following concentrations of each female hormone were obtained in the spike solution:

- Oestradiol: 103.72 µg/ml
- Oestriol: 529.50 µg/ml
- Progesterone: 1004.00 µg/ml

The placebo cream samples were prepared as follows:

1. Weigh approximately 0.8, 1.0 and 1.2 g of the placebo cream separately into 200 ml volumetric flasks in triplicate.
2. Using a volumetric glass pipette, add 15 ml of the spike solution to every 200 ml volumetric flask containing 0.8 g placebo cream and make up to volume with methanol.
3. Using a volumetric glass pipette, add 20 ml of the spike solution to every 200 ml volumetric flask containing 1.0 g placebo cream and make up to volume with methanol.
4. Using a volumetric glass pipette, add 25 ml of the spike solution to every 200 ml volumetric flask containing 1.2 g placebo cream and make up to volume with methanol.
5. Repeat this method for each female hormone to procure nine samples per hormone.
6. The samples were filtered through a 0.45 µm PTFE filter into autosampler vials.

The standard solution was prepared by dissolving 9.77 mg oestradiol in 50 ml methanol; thereafter 10 ml was transferred with a glass pipette into a 200 ml volumetric flask containing 9.80 mg oestriol and 21.60 mg progesterone, which was filled to volume with methanol and placed in an autosampler vial.

The placebo cream sample yielded three different spiked concentrations of each female hormone (see Table A.10). Each sample was injected in duplicate at a default injection volume of 20 μ l. The samples were analysed against the abovementioned standard solution to determine accuracy.

Table A.10: Spiked concentrations (μ g/ml) obtained of each female hormone during the sample preparation of the placebo cream

Volume	Concentration spiked (μ g/ml)		
	Oestriol	Oestradiol	Progesterone
15 ml	39.7	7.8	75.3
20 ml	53.0	1.4	100.4
25 ml	66.2	13.0	125.5

Conferring to the spiked concentrations of each female hormone (as seen in Table A.10) and the acceptance criteria for accuracy (as seen in Table A.9), the mean recovery percentage is required to be within 75 – 125% as it expresses the ratio between the obtained and estimated results (APVMA, 2004:5). The measure of agreement among replicate analysis of a test sample is expressed as the standard deviation (SD) or percentage relative standard deviation (%RSD) (Clontz, 2008:195). Tables A.11 – A.16 demonstrate the accuracy of each female hormone over the concentration ranges for the placebo cream

Table A.11: Accuracy results of oestriol in the spiked placebo cream

Concentration spiked (μ g/ml)	Peak area (mAU)			Recovery	
	Area 1	Area 2	Average	μ g/ml	%
39.7	2210.8	2217.9	2214.3	44.6	112.2
39.7	2203.4	2197.3	2200.4	44.3	111.5
39.7	2231.7	2224.0	2227.8	44.8	112.9
53.0	2912.5	2916.6	2914.6	59.2	111.7
53.0	2939.6	2942.8	2941.2	59.7	112.7
53.0	2859.1	2851.6	2855.4	58.0	109.5
66.2	3613.1	3607.6	3610.4	73.7	111.3
66.2	3620.8	3628.4	3624.6	74.0	111.8
66.2	3585.0	3579.8	3582.4	73.1	110.5

Table A.12: Statistical analysis results of oestriol in the spiked placebo cream

Statistical analysis	
Mean	111.6
SD	1.0
%RSD	0.9
95% confidence intervals	
Lowest limit	109.5
Highest limit	112.9
Median	111.7
Confidence level (95%)	0.83

The recovery of oestriol ranged between 109.5 – 112.9%, with a mean recovery percentage of 111.6% (as seen in Table A.12). Although the mean recovery percentage of oestriol was higher compared to the other hormones, it complied with the acceptance criteria of 75 – 125% (APVMA, 2004:5).

Table A.13: Accuracy results of oestradiol in the spiked placebo cream

Concentration spiked (µg/ml)	Peak area (mAU)			Recovery	
	Area 1	Area 2	Average	µg/ml	%
7.8	462.4	462.1	462.2	8.3	106.7
7.8	459.4	453.1	456.3	8.2	105.3
7.8	466.8	463.3	465.1	8.4	107.3
10.4	609.2	609.3	609.2	11.1	106.7
10.4	619.7	619.6	619.6	11.3	108.5
10.4	597.1	596.9	597.0	10.9	104.5
13.0	764.6	760.0	762.3	13.8	105.8
13.0	764.5	763.1	763.8	13.8	106.0
13.0	757.6	758.1	757.8	13.7	105.2

Table A.14: Statistical analysis results of oestradiol in the spiked placebo cream

Statistical analysis	
Mean	106.2
SD	1.1
%RSD	1.1
95% confidence intervals	
Lowest limit	104.5
Highest limit	108.5
Median	106.0
Confidence level (95%)	0.94

The recovery of oestradiol ranged between 104.5 – 108.5%, with a mean recovery percentage of 106.2% (as seen in Table A.14), which falls within the acceptance criteria of 75 – 125% and serves as an indicator of the method's accuracy (APVMA, 2004:5).

Table A.15: Accuracy results of progesterone in the spiked placebo cream

Concentration spiked (µg/ml)	Peak area (mAU)			Recovery	
	Area 1	Area 2	Average	µg/ml	%
75.3	4945.3	4949.0	4947.2	77.1	102.4
75.3	4936.6	4926.6	4931.6	76.8	102.0
75.3	4983.7	4983.5	4983.6	77.6	103.1
100.4	6583.8	6575.5	6579.6	102.6	102.2
100.4	6652.1	6635.6	6643.8	103.6	103.2
100.4	6440.1	6438.6	6439.4	100.4	100.0
125.5	8209.1	8203.3	8206.2	128.2	102.1
125.5	8237.4	8210.1	8223.7	128.5	102.4
125.5	8047.4	8050.2	8048.8	125.7	100.2

Table A.16: Statistical analysis results of progesterone in the spiked placebo cream

Statistical analysis	
Mean	102.0
SD	1.1
%RSD	1.0
95% confidence intervals	
Lowest limit	100.0
Highest limit	103.2
Median	102.2
Confidence level (95%)	0.87

The recovery of progesterone ranged between 100.0 – 103.2%, with a mean recovery percentage of 102.0% (as seen in Table A.16), which complied with the acceptance criteria of 75 – 125% (APVMA, 2004:5).

A.4.2.2 Accuracy analysis of the placebo serum

A spike solution containing each female hormone was prepared by dissolving 99.2 mg oestradiol in 50 ml methanol; 10 ml was transferred to a 200 ml flask containing 102.6 mg oestriol and 199.8 mg progesterone, which was made up to volume with methanol.

The following concentrations of each female hormone were obtained in the spike solution:

- Oestradiol: 99.2 µg/ml
- Oestriol: 513.0 µg/ml
- Progesterone: 999.0 µg/ml

The same method as described in Section A.4.2.1 was used to prepare and analyse the placebo serum samples. A standard solution was prepared by dissolving 10.44 mg oestradiol in 50 ml methanol; thereafter 10 ml of the solution was transferred with a glass pipette into a clean 200 ml volumetric flask, where 10.20 mg oestriol and 19.40 mg progesterone were added and made up to volume with methanol.

Table A.17: Spiked concentrations (µg/ml) obtained of each female hormone during the sample preparation of the placebo serum

Volume	Concentration spiked (µg/ml)		
	Oestriol	Oestradiol	Progesterone
15 ml	38.5	7.4	74.9
20 ml	51.3	9.9	99.9
25 ml	64.1	12.4	124.9

In accordance with the criteria for accuracy (seen in Table A.9) and the concentrations of each hormone spiked (seen in Table A.17), the mean recovery percentage is required to range between 75 – 125% (APVMA, 2004:5). Tables A.18 – A.23 establish the accuracy of each female hormone over the concentration ranges for the placebo serum.

Table A.18: Accuracy results of oestriol in the spiked placebo serum

Concentration spiked (µg/ml)	Peak area (mAU)			Recovery	
	Area 1	Area 2	Average	µg/ml	%
38.5	2044.6	2039.9	2042.3	40.8	106.1
38.5	2041.3	2036.2	2038.7	40.7	105.9
38.5	2024.9	2022.5	2023.7	40.4	105.1
51.3	2653.3	2666.2	2659.7	53.6	104.4
51.3	2636.6	2660.7	2648.6	53.3	104.0
51.3	2746.3	2729.8	2738.1	55.1	107.5
64.1	3291.9	3319.2	3305.6	67.4	105.1
64.1	3259.4	3259.6	3259.5	66.5	103.7
64.1	3276.7	3264.3	3270.5	66.7	104.0

Table A.19: Statistical analysis results of oestriol in the spiked placebo serum

Statistical analysis	
Mean	105.1
SD	1.2
%RSD	1.1
95% confidence intervals	
Lowest limit	103.7
Highest limit	107.5
Median	105.1
Confidence level (95%)	0.96

The recovery of oestriol ranged from 103.7 – 107.5%, with an acceptable mean recovery percentage of 105.1% (as seen in Table A.19), which was in accordance with the acceptance criteria of 75 – 125% (APVMA, 2004:5).

Table A.20: Accuracy results of oestradiol in the spiked placebo serum

Concentration spiked ($\mu\text{g/ml}$)	Peak area (mAU)			Recovery	
	Area 1	Area 2	Average	$\mu\text{g/ml}$	%
7.4	426.1	424.6	425.4	7.1	96.3
7.4	421.7	424.9	423.3	7.1	95.8
7.4	415.5	419.6	417.6	7.0	94.5
9.9	545.4	561.2	553.3	9.3	94.1
9.9	543.8	559.1	551.4	9.3	93.8
9.9	568.2	570.5	569.3	9.6	96.8
12.4	691.6	695.9	693.8	11.7	94.7
12.4	692.5	689.2	690.9	11.7	94.3
12.4	698.3	682.1	690.2	11.7	94.2

Table A.21: Statistical analysis results of oestradiol in the spiked placebo serum

Statistical analysis	
Mean	94.9
SD	1.0
%RSD	1.1
95% confidence intervals	
Lowest limit	93.8
Highest limit	96.8
Median	94.5
Confidence level (95%)	0.83

As indicated in Table A.21, the recovery of oestradiol ranged from 93.8 – 96.8%. The mean recovery percentage of 94.9% was within the acceptable limits of 75 – 125% (APVMA, 2004:5).

Table A.22: Accuracy results of progesterone in the spiked placebo serum

Concentration spiked (µg/ml)	Peak area (mAU)			Recovery	
	Area 1	Area 2	Average	µg/ml	%
74.9	4674.9	4667.2	4671.1	80.1	106.8
74.9	4641.7	4639.6	4640.7	79.5	106.2
74.9	4615.2	4612.4	4613.8	79.1	105.5
99.9	6110.0	6138.5	6124.3	105.1	105.2
99.9	6062.1	6120.3	6091.2	104.6	104.7
99.9	6265.3	6260.1	6262.7	107.5	107.6
124.9	7605.3	7713.7	7659.5	131.8	105.5
124.9	7584.6	7596.8	7590.7	130.6	104.6
124.9	7592.4	7516.3	7554.3	129.9	104.1

Table A.23: Statistical analysis results of progesterone in the spiked placebo serum

Statistical analysis	
Mean	105.6
SD	1.1
%RSD	1.0
95% confidence intervals	
Lowest limit	104.1
Highest limit	107.6
Median	105.5
Confidence level (95%)	0.88

The recovery of progesterone ranged between 104.1 – 107.6% with a mean recovery percentage of 105.6% (as seen in Table A.23). The accuracy of progesterone (105.6%) was within the acceptance criteria (APVMA, 2004:5).

A.4.3 Precision

The precision of an analytical method can be described as the closeness of agreement achieved between a series of measurements from numerous sampling of the same homogeneous sample, under the specified conditions (APVMA, 2004:5; Naveen *et al.*, 2018:90; Snyder *et al.*, 1997:690). The precision of this analytical method was considered in terms of intra-day precision (repeatability) and inter-day precision (reproducibility).

Table A.24: Acceptance criteria for precision validation (APVMA, 2004:5)

%Analyte in sample	Precision (%RSD)
≥ 10.0%	≤ 2%
1.0 – 10.0%	≤ 5%
0.1 – 1.0%	≤ 10%
< 0.1%	≤ 20%

A.4.3.1 Intra-day precision (repeatability)

The repeatability of an analytical method defines precision under comparable operating conditions over a short period (ICH, 2005:4). To determine repeatability, a minimum of nine samples, comprising of three different concentrations, are prepared and analysed in duplicate on the HPLC on the same day. Acceptable repeatability of an analytical method is indicated by a %RSD value ≤ 2% (APVMA, 2004:5; Belouafa *et al.*, 2017:6; Snyder *et al.*, 1997:691).

A.4.3.1.1 Repeatability analysis of cream

A standard solution was prepared by dissolving 10.6 mg oestradiol in 50 ml methanol; 10 ml was transferred to a 200 ml flask containing 9.8 mg oestriol and 19.5 mg progesterone and made up to volume with methanol.

The cream samples were prepared as follows:

1. Weigh approximately 0.8, 1.0 and 1.2 g of the cream into 200 ml volumetric flasks in triplicate and fill up to volume with methanol.
2. Place the flasks in an ultrasonic bath for a few minutes to ensure proper dissolution.
3. Filter each sample through a 0.45 µm PTFE filter into an autosampler vial for HPLC analysis.

Table A.25: Amount of cream used (g) to prepare samples of three different concentrations in triplicate for repeatability analysis

Amount of cream used (g)		
Lowest concentration (± 0.8 g)	Intermediate concentration (± 1.0 g)	Highest concentration (± 1.2 g)
0.863	0.913	1.275
0.801	1.066	1.307
0.826	1.022	1.277

Table A.26: Repeatability results of oestriol in cream

Amount of cream used (mg)	Peak area (mAU)			Recovery	
	Area 1	Area 2	Mean	µg/ml	%
863.1	2438.3	2442.6	2440.5	55.8	139.5
800.8	2219.9	2226.7	2223.3	54.8	137.1
826.1	2328.8	2329.9	2329.4	55.7	139.1
913.2	2556.7	2550.7	2553.7	68.9	137.9
1066.2	3067.3	3068.0	3067.7	70.8	141.6
1022.1	2830.2	2831.5	2830.9	68.2	136.4
1274.6	3511.3	3498.9	3505.1	81.1	135.2
1306.5	3576.6	3564.7	3570.6	80.6	134.3
1277.4	3479.1	3798.4	3638.8	84.0	140.0
				Mean	137.6
				SD	2.2
				%RSD	1.6

Table A.27: Repeatability results of oestradiol in cream

Amount of cream used (mg)	Peak area (mAU)			Recovery	
	Area 1	Area 2	Mean	µg/ml	%
863.1	586.0	588.1	587.1	11.0	137.8
800.8	532.6	533.5	533.0	10.8	135.0
826.1	560.4	560.1	560.3	11.0	137.5
913.2	613.9	611.0	612.5	13.6	135.7
1066.2	738.5	738.1	738.3	14.0	139.8
1022.1	680.0	678.6	679.3	13.4	134.3
1274.6	846.1	845.8	845.9	16.0	133.7
1306.5	862.0	857.8	859.9	15.9	132.6
1277.4	842.7	842.2	842.4	15.9	132.9
				Mean	135.8
				SD	2.2
				%RSD	1.6

Table A.28: Repeatability results of progesterone in cream

Amount of cream used (mg)	Peak area (mAU)			Recovery	
	Area 1	Area 2	Mean	µg/ml	%
863.1	5530.3	5536.5	5533.4	82.1	102.6
800.8	5099.6	5099.5	5099.5	81.8	102.2
826.1	5268.5	5276.1	5272.3	81.9	102.3
913.2	5784.1	5799.7	5791.9	101.4	101.4
1066.2	6988.4	6992.3	6990.3	104.2	104.2
1022.1	6464.3	6463.8	6464.1	100.7	100.7
1274.6	7989.3	7974.5	7981.9	119.0	99.2
1306.5	8202.9	8206.3	8204.6	119.3	99.4
1277.4	7973.1	7975.1	7974.1	118.6	98.9
				Mean	101.5
				SD	1.6
				%RSD	1.6

As demonstrated in Tables A.26 – A.28, all three female hormones obtained a %RSD value of 1.6%, which complies with the acceptance criteria of repeatability (%RSD ≤ 2%) (APVMA, 2004:5).

A.4.3.1.2 Repeatability analysis of serum

A standard solution was prepared by dissolving 10.4 mg oestradiol in 50 ml methanol; 10 ml was transferred to a 200 ml flask containing 10.2 mg oestriol and 19.4 mg progesterone and made up to volume with methanol. The serum samples were prepared as described in Section A.4.3.1.1 and analysed by means of HPLC on the same day.

Table A.29: Amount of serum used (g) to prepare samples of three different concentrations in triplicate for repeatability analysis

Amount of serum used (g)		
Lowest concentration (± 0.8 g)	Intermediate concentration (± 1.0 g)	Highest concentration (± 1.2 g)
0.869	1.000	1.219
0.805	1.014	1.241
0.820	1.057	1.261

Table A.30: Repeatability results of oestriol in serum

Amount of serum used (mg)	Peak areas (mAU)			Recovery	
	Area 1	Area 2	Mean	µg/ml	%
868.5	3420.4	3429.8	3425.1	63.8	79.8
804.9	3200.4	3201.9	3201.2	64.3	80.3
820.2	3260.0	3283.5	3271.8	64.5	80.6
1000.7	3940.1	3916.4	3928.2	79.6	79.6
1014.4	3974.7	3977.0	3975.9	79.5	79.5
1056.8	4286.7	4270.2	4278.5	82.2	82.2
1218.5	4674.6	4719.4	4697.0	94.0	78.4
1240.5	4882.2	4907.6	4894.9	96.3	80.3
1261.3	4974.3	4963.4	4968.9	96.2	80.1
				Mean	80.1
				SD	1.0
				%RSD	1.3

Table A.31: Repeatability results of oestradiol in serum

Amount of serum used (mg)	Peak areas (mAU)			Recovery	
	Area 1	Area 2	Mean	µg/ml	%
868.5	1248.6	1240.4	1244.5	20.8	26.0
804.9	1160.2	1161.0	1160.6	20.9	26.1
820.2	1173.9	1194.0	1184.0	20.9	26.2
1000.7	1438.4	1411.6	1425.0	25.9	25.9
1014.4	1452.5	1448.0	1450.2	26.0	26.0
1056.8	1557.6	1559.9	1558.8	26.9	26.9
1218.5	1701.3	1732.2	1716.8	30.9	25.7
1240.5	1795.2	1792.5	1793.9	31.7	26.4
1261.3	1829.5	1820.0	1824.8	31.7	26.4
				Mean	26.2
				SD	0.3
				%RSD	1.3

Table A.32: Repeatability results of progesterone in serum

Amount of serum used (mg)	Peak areas (mAU)			Recovery	
	Area 1	Area 2	Mean	µg/ml	%
868.5	7208.1	7219.2	7213.7	111.0	138.7
804.9	6723.0	6727.5	6725.3	111.6	139.5
820.2	6823.4	6878.7	6851.1	111.6	139.4
1000.7	8327.9	8228.7	8278.3	138.3	138.3
1014.4	8356.8	8386.2	8371.5	137.9	137.9
1056.8	9060.7	9039.5	9050.1	143.2	143.2
1218.5	9899.7	9993.8	9946.8	163.9	136.6
1240.5	10390.7	10410.5	10400.6	168.9	140.3
1261.3	10541.2	10526.8	10534.0	167.7	139.7
				Mean	139.3
				SD	1.7
				%RSD	1.2

The repeatability results obtained of each female hormone (as seen in Tables A.30 – A.32) were within the acceptance criteria (%RSD ≤ 2%) (APVMA, 2004:5):

- Oestriol: 1.3%
- Oestradiol: 1.3%
- Progesterone: 1.2%

A.4.3.2 Inter-day precision (reproducibility)

Reproducibility of the analytical method was determined by preparing three standardised samples with approximately similar known concentrations for HPLC analysis on three consecutive days. The results express the method precision between different laboratories (ICH, 2005:5). For the purpose of this study, three samples of intermediate concentrations (as described in Section A.4.3.1.1) were prepared.

According to the FDA (2013:6), the acceptance criteria for reproducibility of a bio-analytical method should not exceed 15% of the %RSD (ICH, 2019:12). Due to the use of biological actives in this research study (oestriol, oestradiol and progesterone), the acceptance criteria for bio-analytical method validation applies (ICH, 2019:12).

A.4.3.2.1 Reproducibility analysis of cream

The intermediate concentration samples were prepared as mentioned in Section A.4.3.1.1 for three consecutive days. Table A.33 illustrates the amount of cream used to prepare the samples.

Table A.33: Amount of cream used (g) to prepare samples in triplicate for reproducibility analysis

Days	Amount of cream used (g)		
	Sample 1	Sample 2	Sample 3
Day 1	0.913	1.066	1.022
Day 2	1.079	1.150	1.036
Day 3	1.040	1.018	1.102

Table A.34: Reproducibility results of oestriol in cream

Days	%Recovery			Mean	SD	%RSD
Day 1	137.9	141.6	136.4	138.63	2.18	1.58
Day 2	118.7	118.1	119.3	118.70	0.49	0.41
Day 3	117.4	117.6	119.3	118.10	0.85	0.72
Between days:				125.14	9.64	7.70

Table A.35: Reproducibility results of oestradiol in cream

Days	%Recovery			Mean	SD	%RSD
Day 1	135.7	139.8	134.3	136.60	2.31	1.69
Day 2	148.5	150.6	150.2	149.78	0.90	0.60
Day 3	149.1	147.5	150.7	149.11	1.32	0.88
Between days:				145.16	6.28	4.32

Table A.36: Reproducibility results of progesterone in cream

Days	%Recovery			Mean	SD	%RSD
Day 1	101.4	104.2	100.7	102.10	1.50	1.47
Day 2	97.2	95.5	97.7	96.80	0.94	0.96
Day 3	96.1	96.7	98.1	96.97	0.84	0.86
Between days:				98.62	2.71	2.75

The %RSD value obtained for each female hormone (as seen in Tables A.34 – A.36) was within the acceptance criteria for reproducibility (%RSD ≤ 15%) (ICH, 2019:12; FDA, 2013:6):

- Oestriol: 7.70%
- Oestradiol: 4.32%
- Progesterone: 2.75%

A.4.3.2.2 Reproducibility analysis of serum

The intermediate concentration samples were prepared as described in Section A.4.3.1.1 for three consecutive days. Table A.37 illustrates the amount of serum used to prepare the samples.

Table A.37: Amount of serum used (g) to prepare samples in triplicate for reproducibility analysis

Days	Amount of serum used (g)		
	Sample 1	Sample 2	Sample 3
Day 1	1.001	1.014	1.057
Day 2	0.994	1.051	1.058
Day 3	1.077	1.088	1.071

Table A.38: Reproducibility results of oestriol in serum

Days	%Recovery			Mean	SD	%RSD
Day 1	79.6	79.5	82.2	80.43	1.25	1.55
Day 2	82.7	83.4	81.8	82.63	0.65	0.79
Day 3	83.4	82.2	82.6	82.73	0.50	0.60
Between days:				81.93	1.37	1.67

Table A.39: Reproducibility results of oestradiol in serum

Days	%Recovery			Mean	SD	%RSD
Day 1	25.9	26.0	26.9	26.27	0.45	1.71
Day 2	24.2	24.5	24.0	24.23	0.21	0.85
Day 3	24.8	24.1	24.2	24.37	0.31	1.27
Between days:				24.96	0.99	3.96

Table A.40: Reproducibility results of progesterone in serum

Days	%Recovery			Mean	SD	%RSD
Day 1	138.3	137.9	143.2	139.80	2.41	1.72
Day 2	131.8	133.2	130.5	131.83	1.10	0.84
Day 3	133.4	131.0	131.8	132.07	1.00	0.76
Between days:				134.57	4.05	3.01

As demonstrated in Tables A.38 – A.40, all three female hormones obtained a %RSD value below 15%, which complies with the acceptance criteria of reproducibility ($\%RSD \leq 15\%$) (ICH, 2019:12; FDA, 2013:6):

- Oestriol: 1.67%
- Oestradiol: 3.96%
- Progesterone: 3.01%

A.4.4 Ruggedness

Ruggedness describes the ability of an analytical method to reproduce results when performed under actual chromatographic conditions; therefore, it determines the variability of the method (Snyder *et al.*, 1997:701). Ruggedness is validated in terms of sample stability and system repeatability.

A.4.4.1 Sample stability

The ability of a sample to retain its initial properties by resisting physical disintegration, chemical changes or decomposition is known as sample stability (UNODC, 2009:65). Stability is further described as the absence of an influence of time on the analyte concentration in the prepared sample (Hartmann *et al.*, 1998:195). Sample stability is essential throughout the entire analytical procedure to ensure reliable quantification (Peters *et al.*, 2007:220). The acceptance criteria for sample stability is a %RSD value $\leq 2\%$, which is representative of the amount of degradation that occurred (Paithankar, 2013:238). Samples are analysed to determine the time it takes to degrade with 2% and should no longer be used.

The sample was prepared by dissolving ± 10 mg oestradiol in 50 ml methanol; thereafter 10 ml was transferred into a 200 ml volumetric flask containing ± 10 mg oestriol and ± 20 mg progesterone, made up to volume with methanol and placed in an autosampler vial to be analysed. The analysis was performed under pre-established conditions and in hourly intervals for a period of 24 h.

Tables A.41 – A.43 demonstrate the results obtained after injecting each female hormone hourly for 24 h. The %RSD values for all the female hormones were within the acceptance criteria (%RSD $\leq$ 2%):

- Oestriol: 1.33%
- Oestradiol: 1.35%
- Progesterone: 1.72%

Table A.41: Sample stability results of oestriol

Time (h)	Peak area (mAU)	%Remaining
0	1254	100.0
1	1255.5	100.1
2	1249.7	99.7
3	1285.9	102.5
4	1294.7	103.2
5	1280.5	102.1
6	1292.8	103.1
7	13005	103.7
8	1287.9	102.7
9	1297.2	103.4
10	1295.7	103.3
11	1307.0	104.2
12	1291.5	103.0
13	1306.3	104.2
14	1304.7	104.0
15	1301.0	103.7
16	1305.4	104.1
17	1306.9	104.2
18	1291.7	103.0
19	1302.8	103.9
20	1318.7	105.2
21	1300.2	103.7
22	1307.5	104.3
23	1310.4	104.5
24	1302.2	103.8
Mean	1294.0	103.2
SD	17.23	1.37
%RSD	1.33	1.33

Table A.42: Sample stability results for oestradiol

Time (h)	Peak area (mAU)	%Remaining
0	282.6	100.0
1	280.5	99.3
2	293.6	103.9
3	290.3	102.7
4	291.0	103.0
5	288.3	102.0
6	292.4	103.5
7	293.9	104.0
8	291.0	103.0
9	293.4	103.8
10	295.6	104.6
11	293.9	104.0
12	292.1	103.4
13	298.8	105.7
14	295.4	104.5
15	294.7	104.3
16	296.3	104.8
17	295.4	104.5
18	291.4	103.1
19	291.3	103.1
20	297.1	105.1
21	291.7	103.2
22	292.4	103.5
23	292.8	103.6
24	294.2	104.1
Mean	292.4	103.5
SD	3.94	1.39
%RSD	1.35	1.35

Table A.43: Sample stability results of progesterone

Time (h)	Peak area (mAU)	%Remaining
0	2875.9	100.0
1	2866.6	99.7
2	2857.3	99.4
3	2811.5	97.8
4	2812.5	97.8
5	2960.6	102.9
6	2960.9	103.0
7	2915.2	101.4
8	2932.9	102.0
9	2974.8	103.4
10	2936.5	102.1
11	2947.5	102.5
12	2959.3	102.9
13	2983.1	103.7
14	2938.9	102.2
15	3000.5	104.3
16	2993.9	104.1
17	2951.2	102.6
18	2961.4	103.0
19	2960.9	103.0
20	2954.1	102.7
21	2954.3	102.7
22	2964.1	103.1
23	2966.4	103.1
24	2963.2	103.0
Mean	2936.1	102.1
SD	50.43	1.75
%RSD	1.72	1.72

A.4.4.2 System repeatability

To determine the repeatability of the system, a sample was prepared, as described in Section A.3.1, and injected into the HPLC six consecutive times. This analysis was performed to evaluate the repeatability of the peak area and the retention time of each female hormone present in the sample on the same day and under uniform conditions. Shabir (2003:62) stated a %RSD value $\leq 2\%$ is considered acceptable. Tables A.44 – A.46 reflect the repeatability of the system was acceptable for each female hormone as the %RSD values for both the retention times and peak areas were lower than 1%.

Table A.44: System repeatability results of oestriol

Injection	Peak area (mAU)	Retention time (min)
1	1261.0	3.816
2	1262.0	3.881
3	1259.9	3.810
4	1259.2	3.809
5	1253.2	3.805
6	1255.7	3.808
Mean	1258.5	3.822
SD	3.08	0.027
%RSD	0.24	0.702

Table A.45: System repeatability results of oestradiol

Injection	Peak area (mAU)	Retention time (min)
1	281.5	5.159
2	281.9	5.165
3	282.1	5.139
4	284.9	5.138
5	281.8	5.147
6	283.8	5.137
Mean	282.7	5.148
SD	1.24	0.011
%RSD	0.44	0.212

Table A.46: System repeatability results of progesterone

Injection	Peak area (mAU)	Retention time (min)
1	2892.4	7.617
2	2888.2	7.626
3	2895.6	7.579
4	2892.0	7.610
5	2858.1	7.597
6	2887.2	7.613
Mean	2885.6	7.607
SD	12.60	0.015
%RSD	0.44	0.200

Table A.47: Summarised system repeatability results for each female hormone

Hormone	%RSD for peak area	%RSD for retention time
Oestriol	0.24	0.702
Oestradiol	0.44	0.212
Progesterone	0.44	0.200

A.4.5 Specificity

Specificity can be defined as the ability of an analytical method to detect an analyte concentration accurately in the presence of the matrix, impurities or degradants (ICH, 2005:4). Acceptable specificity is considered the true identification and differentiation of analytes with closely related structures in a complex mixture (ICH, 2005:4; Ravisankar *et al.*, 2015:13). An analytical method is viewed as specific when there is no interference of peaks with the retention times of the analytes in the chromatogram (Jahan *et al.*, 2014:77; Snyder *et al.*, 1997:700). Specificity is required to consider a method as acceptable (Snyder *et al.*, 1997:696).

To determine specificity, the samples were prepared as follows:

1. Standard solution:
 - Solution 1 was prepared by dissolving 9.72 mg oestradiol in 50 ml methanol.
 - Solution 2 was prepared by transferring 10 ml of Solution 1 to a 100 ml volumetric flask, thereafter 9.83 mg oestriol and 19.6 mg progesterone were added and made up to volume with methanol.
 - The standard solution was prepared by transferring 10 ml of Solution 2 to a 20 ml volumetric flask and made up to volume with methanol.
2. Of the standard solution, 1 ml was added to three individual test tubes and diluted as follows:
 - 1.0 ml standard solution (Sample 1)
 - 1.0 ml standard solution + 0.5 ml 2 M hydrochloric acid (HCl) (Sample 2)
 - 1.0 ml standard solution + 0.5 ml 2 M sodium hydroxide (NaOH) (Sample 3)
 - 1.0 ml standard solution + 0.5 ml 2 M hydrogen peroxide (H₂O₂) (Sample 4)
3. The samples were mixed and left to stand for a few minutes and neutralised as follows:
 - Nothing was added to Sample 1
 - 0.5 ml NaOH to Sample 2
 - 0.5 ml HCl to Sample 3

- 0.5 ml H₂O₂ to Sample 4
4. Thereafter all the samples were injected into the HPLC and analysed in duplicate with a run time of 12 min.

Table A.48: Specificity data for oestriol

Sample	Retention time (min)
Standard solution	3.812
HCl	3.820
NaOH	3.810
H ₂ O ₂	3.812

Table A.49: Specificity data for oestradiol

Sample	Retention time (min)
Standard solution	5.142
HCl	5.145
NaOH	5.141
H ₂ O ₂	5.143

Table A.50: Specificity data for progesterone

Sample	Retention time (min)
Standard solution	7.615
HCl	7.623
NaOH	7.599
H ₂ O ₂	7.615

As demonstrated in Figure A.8, the degradation of the samples caused an additional peak to form (± 1.80 min), however, it did not affect the retention times of each female hormone:

- Oestriol: ± 3.81 min
- Oestradiol: ± 5.14 min
- Progesterone: ± 7.61 min

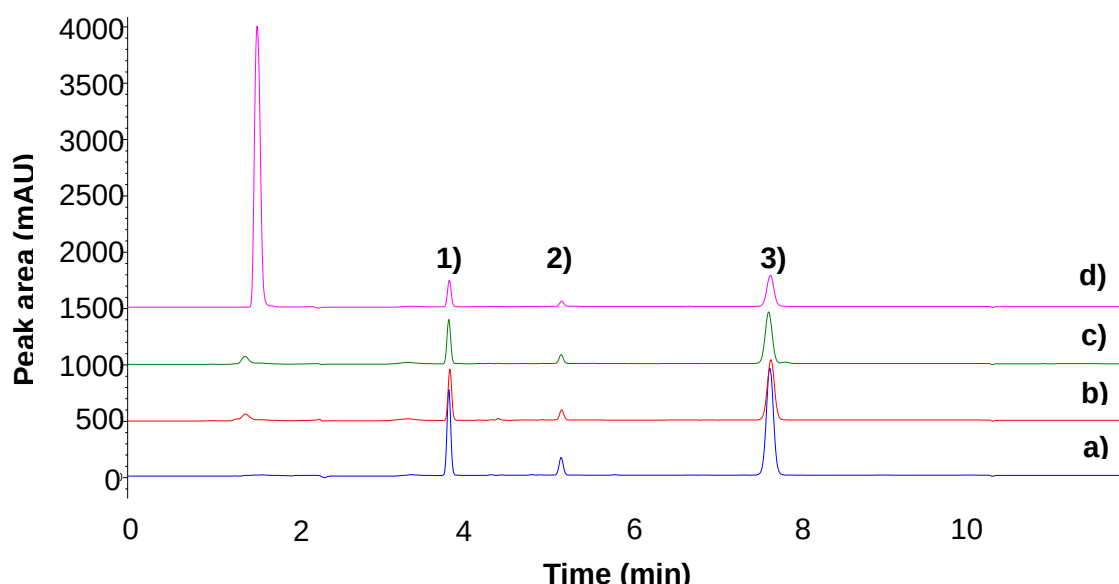


Figure A.8: HPLC chromatogram displaying specificity data obtained for: a) the standard sample, following the samples stressed with b) HCl c) NaOH and d) H₂O₂ for 1) oestriol, 2) oestradiol and 3) progesterone

The female hormones were subjected to stress conditions of acid hydrolysis (HCl), base hydrolysis (NaOH) and oxidation (H₂O₂) (Gautam & Purvis, 2017:7). From Figure A.8 it is evident that the sample stressed with HCl experienced some degree of degradation and formed an additional peak (± 1.80 min). This could be expected as acid hydrolysis can destroy certain oestrogenic steroids (Ismail & Watson, 1974:1128). According to a study by Gautam and Purvis (2017:10), progesterone does not degrade in an acidic medium. Zargar-Shoshtari (2011:62) also established the absence of degradation of progesterone in HCl.

Furthermore, degradation occurred in the sample containing NaOH (± 1.80 min). Progesterone is highly unstable in an alkaline medium and degrades rapidly (Zargar-Shoshtari, 2011:62). There is evidence that oestrogenic steroids are also prone to degradation when exposed to a strong base, such as NaOH (Gautam & Purvis, 2017:10).

The sample stressed with H₂O₂ to induce oxidation presented with the highest degree of degradation, as observed from the prominent additional peak at ± 1.80 min (Figure A.8). Progesterone did not reveal any degradation in H₂O₂ at room temperature (Zargar-Shoshtari, 2011:62); however, Shappell *et al.* (2008:1296) and Ma *et al.* (2015:12019) both found evidence of accelerated degradation of oestrogens in the presence of H₂O₂.

Specificity is the most important parameter of an analytical method utilised for assay and stability determination. The specificity of this method was determined by confirming the absence of interference by any peaks in the stressed samples and resolving any potential degradation peaks from the individual female hormone peaks (Gautam & Purvis, 2017:7). This

analytical method can be viewed as specific, since the retention times for each respective female hormone was not affected by any of the added degradation compounds and additional peak areas (as seen in Figure A.8).

A.4.6 Robustness

Robustness can be described as the measurement of an analytical method's capacity to withstand small, though deliberate variations in the chromatographic conditions specific to the method, which is indicative of the method's suitability and reliability during normal usage (César & Pianetti, 2009:235; ICH, 2005:5). Therefore, robustness is an indicator of the effect of small parameter variations on peak retention times on a chromatogram (Snyder *et al.*, 1997:702). For this validation test, a standard solution was prepared as described in Section A.3.1, and placed in an ultrasonic bath for a few minutes to ensure proper dissolution. A volume of the standard solution was placed in an autosampler vial and analysed under normal conditions by means of HPLC (as described in Section A.2) to obtain a reference chromatogram.

The following variations were made to the chromatographic conditions of the HPLC method:

- Wavelength: 205 – 215 nm
- Injection volume: 15 – 25 μ l
- Flow rate: 0.8 – 1.2 ml/min

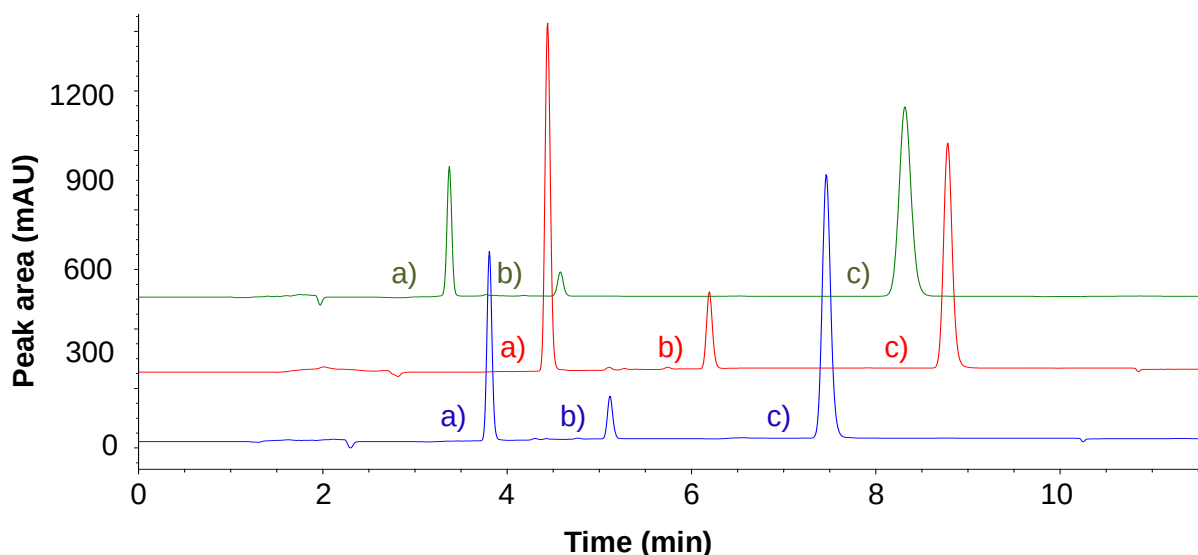


Figure A.9: HPLC chromatogram representing the robustness data of a standard solution containing a) oestriol, b) oestradiol and c) progesterone analysed at different chromatographic conditions: normal conditions of 1.0 ml/min flow rate, 20 μ l injection volume and 210 nm wavelength (blue); 1.2 ml/min flow rate, 25 μ l injection volume and 215 nm wavelength (red) and 0.8 ml/min flow rate, 15 μ l injection volume and 205 nm wavelength (green)

The shifts in retention times for each female hormone were as follows:

- Oestriol: 3.4 – 4.5 min
- Oestradiol: 4.6 – 6.3 min
- Progesterone: 7.5 – 8.8 min

Figure A.9 represents the data obtained after injecting the standard solution under altered conditions. To determine the robustness of a method, the sample chromatograms obtained under the altered chromatographic conditions must be compared to the sample chromatogram obtained under normal chromatographic conditions. The method is considered as reliable due to the minimal changes that occurred in the peaks areas and retention times of each female hormone when compared to the reference chromatogram (as seen in Figure A.9).

A.4.7 Lower limit of detection and quantification

The lower limit of quantification (LLOQ) is the lowest concentration of an analyte in a sample that can be determined quantitatively with a stated accuracy and precision, and is indicative of the method's sensitivity (APVMA, 2004:6; ICH, 2005:5; Lozano & Cantero, 1997:619). The lower limit of detection (LLOD) is the lowest concentration of an analyte in a sample that can be reliably detected, but not necessarily quantified (APVMA, 2004:4; Thompson & Ramsey, 1999:494). To determine the LLOQ and LLOD, a solution was prepared containing 10.1 mg oestriol, 11.3 mg oestradiol and 10.7 mg progesterone dissolved in 50 ml methanol. Thereafter, 5 ml was transferred with a glass volumetric pipette to a clean volumetric flask and made up to volume with methanol; this process was repeated. A volume of the last dilution was placed in an autosampler vial for HPLC analysis and injected six times at different injection volumes:

- 1.0 μ l
- 2.5 μ l
- 5.0 μ l
- 10.0 μ l
- 15.0 μ l
- 20.0 μ l

The acceptance criteria for LLOQ is a %RSD value $\leq 20\%$ (Saadati *et al.*, 2013:65). Both limits were calculated based on the standard deviation of the response (σ) and the calibration curve slope (S) approach, as described in the ICH guidelines (2005:11). The following formulas were applied to determine the LLOD (Equation A.2) and LLOQ (Equation A.3), respectively (Batravi *et al.*, 2017:2; Swartz & Krull, 2012:70-71).

$$DL \text{ (detection limit)} = 3.3 \times \sigma/S$$

Equation A.2

$$QL \text{ (quantification limit)} = 10 \times \sigma/S$$

Equation A.3

Where:

σ = standard deviation of y-intercepts

S = slope

Table A.51: LLOD and LLOQ results obtained for oestriol

Injection volume (µl)	1.0	2.5	5.0	10.0	15.0	20.0
Concentration (µg/ml)	0.101	0.253	0.505	1.010	1.515	2.020
Peak area (mAU)	4.939	11.586	22.814	44.998	67.351	89.823
	5.170	12.016	22.817	45.177	67.297	89.764
	5.265	12.024	22.907	45.118	67.200	89.284
	5.037	11.849	22.865	45.219	67.502	89.879
	5.193	11.711	22.811	45.291	67.517	89.924
	5.100	11.781	22.854	45.199	67.700	89.516
Mean	5.12	11.83	22.84	5.17	67.42	89.70
SD	0.11	0.16	0.03	0.09	0.17	0.23
RSD%	2.09	1.33	0.15	0.20	0.25	0.25

Table A.52: Statistical analysis of oestriol

Summary output		
Regression statistics		
Multiple R	0.999999	
R square	0.999999	
Adjusted R square	0.999998	
Standard error	0.044233	
Observations	6	
ANOVA	df	SS
Regression	1	5552.7088
Residual	4	0.0078263
Total	5	5552.7167
	Coefficients	Standard error
Intercept	0.653618	0.0296860
X variable 1	44.07482	0.0261630

Table A.53: LLOD and LLOQ results obtained for oestradiol

Injection volume (µl)	1.0	2.5	5.0	10.0	15.0	20.0
Concentration (µg/ml)	0.113	0.283	0.565	1.130	1.695	2.260
Peak area (mAU)	5.015	13.127	26.462	53.308	80.405	107.434
	5.128	13.497	26.515	53.183	79.940	107.221
	5.235	13.390	26.483	53.316	79.976	107.147
	5.159	13.185	26.672	53.221	80.522	107.544
	5.227	13.096	26.443	53.622	80.457	107.581
	5.153	13.197	26.500	53.500	80.537	107.463
Mean	5.15	13.25	26.51	53.36	80.31	107.40
SD	0.07	0.15	0.08	0.15	0.25	0.16
RSD%	1.42	1.10	0.28	0.29	0.31	0.15

Table A.54: Statistical analysis of oestradiol

Summary output		
Regression statistics		
Multiple R	0.999996	
R square	0.999993	
Adjusted R square	0.999991	
Standard error	0.122076	
Observations	6	
ANOVA	df	SS
Regression	1	8105.7911
Residual	4	0.0596106
Total	5	8105.8507
	Coefficients	Standard error
Intercept	-0.29452	0.0819286
X variable 1	47.59691	0.0645376

Table A.55: LLOD and LLOQ results obtained for progesterone

Injection volume (µl)	1.0	2.5	5.0	10.0	15.0	20.0
Concentration (µg/ml)	0.107	0.268	0.535	1.070	1.605	2.140
Peak area (mAU)	6.830	16.656	32.840	66.148	98.694	131.702
	6.940	16.675	33.034	65.581	98.882	131.614
	7.020	16.718	32.790	65.896	98.765	131.888
	6.855	16.700	32.979	66.107	98.768	131.775
	6.552	16.471	33.054	65.673	98.662	132.150
	6.822	16.517	33.184	65.941	98.912	131.858
Mean	6.84	16.62	32.98	65.89	98.78	131.66
SD	0.14	0.09	0.13	0.21	0.09	0.25
RSD%	2.12	0.57	0.40	0.32	0.09	0.19

Table A.56: Statistical analysis of progesterone

Summary output		
Regression statistics		
Multiple R	0.999999	
R square	0.999999	
Adjusted R square	0.999999	
Standard error	0.055341	
Observations	6	
ANOVA	df	SS
Regression	1	12102.902
Residual	4	0.0122505
Total	5	12102.914
	Coefficients	Standard error
Intercept	0.193765	0.0371407
X variable 1	61.42151	0.0308974

Tables A.51, A.53 and A.55 demonstrate the regression analysis data, which was attained by utilising the data showed in Tables A.52, A.54 and A.56. The standard error (σ) and X variable 1 (S) was obtained and could be applied in the equations to determine the LLOD and LLOQ of each female hormone.

Table A.57: LLOD ($\mu\text{g/ml}$) and LLOQ ($\mu\text{g/ml}$) of female hormones as determined by the linear curves procedure

Hormone	Oestriol	Oestradiol	Progesterone
LLOD: DL (detection limit) = $3.3 \times \sigma/S$	0.0022	0.0057	0.0020
LLOQ: QL (quantification limit) = $10 \times \sigma/S$	0.0067	0.0172	0.0060

A.57 presents the LLOD and LLOQ determined for each female hormone respectively with the help of Equation A.2 (LLOQ) and Equation A.3 (LLOD).

A.5 Conclusion

The HPLC method for female hormones (oestriol, oestradiol and progesterone) was successfully validated and found to be sensitive and reliable for the identification and quantification of female hormones in topical formulations. The method further complied with the acceptance criteria and could therefore be utilised to quantify female hormones during stability testing and *in vitro* skin diffusion studies.

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APPENDIX B:

PRE-FORMULATION AND CHARACTERISATION OF O/W EMULSIONS CONTAINING FEMALE HORMONES FOR TOPICAL DELIVERY

B.1 Introduction

Oestrogen deficiency is an important endogenous cause of skin ageing in women (Irrera *et al.*, 2017:622; Raine-Fenning *et al.*, 2003:1). Decreased serum oestrogen levels, brought about by menopause, alters skin characteristics and functions, ultimately contributing to the progressive loss of skin integrity characterised by fine wrinkling, skin atrophy and dryness (Farage *et al.*, 2013:5; Piérard *et al.*, 2013; Rzepecki *et al.*, 2019:85). The diverse receptor expression in human skin allows for interaction with various hormone types, thus topically applied hormones can exert their effect on ageing skin by binding to the suitable receptors (Zouboulis, 2004:10). Numerous studies have shown that the topical application of female hormones mimic multiple biological effects in human skin, facilitating structural integrity and limiting the ageing process (Kanda & Watanabe, 2005:6; Sator *et al.*, 2004:39; Silva *et al.*, 2017:845).

The increasing desire of today's population to maintain a youthful appearance has resulted in a corresponding increase in topical anti-ageing products, universally referred to as cosmeceuticals, which describes cosmetic preparations with pharmaceutical properties (Fulse *et al.*, 2012:233; Merriam-Webster, 2019; Rivers, 2008:5). Cosmeceuticals are intended to be used on a regular basis by the consumer, therefore it is essential the formulations are cosmetically acceptable to ensure consumer compliance. Consequently, a cosmetically acceptable formulation can be defined as (Barry, 2007:595; Ramchandani & Toddywala, 1997:542; Williams, 2013:685):

- a formulation that is easily transferable from the container to the skin;
- with a pleasant texture, fragrance and skin feeling after application;
- that leaves no unwanted, visible residue behind on the skin, and
- has good adhering properties at the application site.

Although topical hormone products have gained popularity in beauty care practices, it is not without risk (Draeos, 2005:291; Takeda *et al.*, 2012:19). Masuda *et al.* (2013:312) proposed that the topical application of oestrogen could express systemic effects in addition to the local effects induced at the application site. Elevated circulating concentrations of endogenous sex hormones, especially oestrogen, are linked to an increased risk for the development of breast cancer in postmenopausal women (Kaaks *et al.*, 2005:1071; Key *et al.*, 2011:709; Missmer *et al.*, 2004:1862).

Since topical drug delivery offers numerous advantages, it can be utilised to overcome unwanted systemic effects associated with oral hormone therapy (Kaaks *et al.*, 2005:1072; Masuda *et al.*, 2013:2013; Pike *et al.*, 1993:17; Shang, 2007:277). These advantages include:

- sustainable drug delivery;
- reduced side-effects;
- avoidance of hepatic first-pass metabolism;
- reduced administration frequency, and
- proven patient compliance (Leite-Silva *et al.*, 2012:384).

The one unavoidable disadvantage is the skin, which functions as a protective barrier against free radicals, chemicals and UV rays (Menon, 2002:4; Naik *et al.*, 2000:319). The barrier function has been attributed to the stratum corneum, which is selectively permeable due to its biochemical composition and structure (Ng, 2018:51; Ruela *et al.*, 2016:527). The stratum corneum regulates API flux and acts as a rate-limiting step during the absorption and permeation of actives through the skin (Baroni *et al.*, 2012:259; Hendriks *et al.*, 2006:259; Williams, 2003:28). Successful topical delivery depends on the drug's ability to overcome the stratum corneum to reach the viable epidermis (Petrilli & Lopez, 2018:1).

The penetration and absorption of hormones through the skin is dependent on multiple factors, i.e., the properties of the skin, the physiochemical properties of each respective hormone, formulation strategies to limit activity within the epidermis and characteristics of the selected drug delivery system (Chang *et al.*, 2013:41; Wiechers & Watkinson, 2008:63). In general, small and moderately lipophilic actives (i.e. MW < 500 g/mol and log P of 1 – 4) are expected to penetrate the skin well (Ng, 2018:51), and so the physiochemical properties of each respective hormone were thoroughly investigated to formulate a preparation that is safe to use and ideal for topical delivery (Williams, 2013:685). Additionally, it is important to select an acceptable delivery system that is non-irritating and compatible with the selected hormones (Weiss, 2011:471).

For the purpose of this study, an emulsion has been chosen as a possible topical delivery system. Emulsions are often used to incorporate lipophilic actives with poor aqueous solubility into the oil phase, which could help improve the absorption and permeation of actives through the skin. Lipophilic actives, such as female hormones, can therefore be delivered topically by utilising emulsions as a delivery system (Kan *et al.*, 1999:117; Kumpugdee-Vollrath *et al.*, 2014:574).

B.2 The purpose and selection of a delivery system

The intended purpose of a novel formulation should be taken into consideration during the formulation process. The aim of this study was to investigate the topical delivery of selected female hormones. Although each selected hormone presented with an ideal low MW (< 500 g/mol) (Naik *et al.*, 2000:319), other physiochemical properties, such as poor solubility, could hinder topical delivery.

In topical delivery, the selection of an appropriate vehicle as drug carrier is essential, as it should guarantee non-toxicity, stability and permeation efficacy for the intended actives (Cho *et al.*, 2014:423). Apart from accommodating the actives, the vehicle should be cosmetically acceptable and non-irritating (Weiss, 2011:471). The effectiveness of topical delivery can be described as the effective delivery of an active through the epidermis, which retains in the dermis (Kaur *et al.*, 2016:6353). The effective release of actives, along with numerous other factors, can influence drug flux and topical delivery (Barry, 2002:508), therefore selecting the correct vehicle for delivery is important, as it is responsible for bringing the active in contact with the skin (Weiss, 2011:472). After the effective release of the active from the vehicle, it moves through the skin layers, creating more challenges in the delivery process.

To summarise, the steps involved in successful topical drug delivery are:

- establishing a concentration gradient (movement of actives across the skin);
- the effective release of actives from the formulation (partition coefficient), and
- successful diffusion across the skin layers (diffusion coefficient) (Panwar *et al.*, 2011:334).

All things considered, o/w emulsions were pre-formulated and characterised to act as precursor for the formulas prepared in Appendix C; this decision was based on its ability of solubilising both lipophilic and hydrophilic compounds (Buchmann, 2001:149). Moreover, emulsions are versatile formulations that exhibit good skin spreadability with a pleasant skin feel when topically applied (Buchmann, 2001:151).

B.3 Excipients used to formulate emulsions

Good formulation design is based on simplicity; the shorter the ingredient list, the better (Chang *et al.*, 2013:44). The core components of a basic emulsion are oils (lipophilic compounds), water (hydrophilic compounds) and surfactants (Buchmann, 2001:151). Additional excipients, such as thickening agents, emollients and humectants are used in topical formulations to obtain the desired properties and performance characteristics for the formulation (Chang *et al.*, 2013:43). It is important that the excipients used to formulate the emulsion are compatible with

each another, particularly where the oil is concerned (Tamilvanan *et al.*, 2010:17), as the selected oil constituent can influence the topical delivery of actives in both o/w and w/o emulsions (Zhang *et al.*, 2011:702). Surfactants are surface active agents that play an important role in formulating emulsions as they reduce the interfacial tension between two immiscible phases (oil and water), therefore stabilising the emulsion (Lin, 2017:668; Vaidya & Ganguli, 2019:1). The type of emulsion formulated is mainly determined by the intended active, since it will be added to the core component, either being the oil or water phase (Chime *et al.*, 2014:77; Kela & Kaur, 2013:9203). During this study, the objective was to formulate three o/w emulsions each containing 2.0% (w/v) oestriol, 0.2% (w/v) oestradiol and 2.5% (w/v) progesterone. Different formulas were used to prepare each formulation, thereafter the optimal formulation was selected accordingly. The formula for each o/w emulsion was adapted from previous dissertations; note only the excipients used to formulate the optimal o/w emulsion will be discussed in Appendix C. Tables B.1 – B.3 list the excipients used to formulate each emulsion, respectively. The pre-formulated o/w emulsions will be referred to as follows:

- **(OE1)** (o/w emulsion formula 1) (Botes, 2012);
- **(OE2)** (o/w emulsion formula 2) (Burger, 2014), and
- **(OE3)** (o/w emulsion formula 3) (Van Zyl, 2012).

Table B.1: Excipients used in the formulation of **(OE1)** with their function, supplier and batch number

Excipient	Function	Supplier	Batch number
Oestriol	Active	Thonson Technology Ltd	20181001
Oestradiol		Iffect Chemphar Co., Ltd	IF-ES-181202
Progesterone		Hubei Gedian Humanwell Pharmaceutical Co., Ltd	HTT181101
Cetyl stearyl alcohol	Thickening agent	Merck	S5122073835
Cremophor® A6	Emulsifier	BASF	65753075L0
Cremophor® A25	Emulsifier	BASF	12903975L0
Liquid paraffin	Oil phase	Merck	1035428
Propyl paraben	Preservative	Merck	K41917527
Propylene glycol	Co-solvent	Merck	S5470588207
Milli-Q® water	Water phase and solvent	In-Lab	Direct Pure UP

Table B.2: Excipients used in the formulation of **(OEW2)** with their function, supplier and batch number

Excipient	Function	Supplier	Batch number
Oestriol	Active	Thonson Technology Ltd	20181001
Oestradiol		Iffect Chemphar Co., Ltd	IF-ES-181202
Progesterone		Hubei Gedian Humanwell Pharmaceutical Co., Ltd	HTT181101
Stearic acid	Thickening agent	Saarchem	5872120
Cetyl alcohol	Thickening agent	Merck	S5428004951
Crodafos™ MCK	Emulsifier	Croda	498133
Span® 60	Lipophilic surfactant	Sigma	SLNT7599
Tween® 80	Hydrophilic surfactant	Sigma	BCBL1768V
Glycerine	Humectant	Sigma	2002895
Veegum® HV	Thickening agent	R.T Vanderbilt Co, Inc.	12199370
PEG ^a 400	Oil phase and humectant	Saarchem	1027497
Dimethyl isosorbide	Penetration enhancer	Sigma-Aldrich	STBD7240V
Milli-Q® water	Water phase and solvent	In-Lab	Direct Pure UP

a) Polyethylene glycol

Table B.3: Excipients used in the formulation of **(OEW3)** with their function, supplier and batch number

Excipient	Function	Supplier	Batch number
Oestriol	Active	Thonson Technology Ltd	20181001
Oestradiol		Iffect Chemphar Co., Ltd	IF-ES-181202
Progesterone		Hubei Gedian Humanwell Pharmaceutical Co., Ltd	HTT181101
Cetyl alcohol	Thickening agent	Merck	S5428004951
Span® 60	Lipophilic surfactant	Sigma	SLBT7599
Tween® 80	Hydrophilic surfactant	Sigma	BCBL1768V
Liquid paraffin	Oil phase	Merck	1035428
Methanol	Co-solvent	Merck	I0980007846
Milli-Q® water	Water phase and solvent	In-Lab	Direct Pure UP

B.4 Formulation of emulsions

During the formulation process, a trial-and-error approach was utilised to select an acceptable formula, whereby the emulsion demonstrated proper characteristics. Emulsions are colloidal systems composed of two or more immiscible liquids, generally water and oil (Smith, 2003:262). In the case of o/w emulsions, the internal lipid phase is dispersed within the continuous water phase (Buchmann, 2001:148). Emulsions are thermodynamically unstable systems that may

experience phase separation through flocculation, coalescence or creaming. (Goodarzi & Zendehboudi, 2018:284; Ngan *et al.*, 2014:1). The method of preparation is an important aspect, as it greatly influences the stability of the emulsion (Li & Xiang, 2019:2). During the formulation of conventional macromolecule emulsions, high-speed stirring is capable of creating droplets in the micrometre range. To create droplets in the submicron range, and consequently increase stability, a high-energy input is required (Hu *et al.*, 2017:17). High-pressure homogenisation (high-energy) is frequently used to prepare o/w emulsions (Li & Xiang, 2019:2), by which the formulation is forced to pass through a small outlet at an operating pressure ranging between 500 and 5000 psi (Chime *et al.*, 2014:94). Attaining a small average particle size will result in a more elegant and stable emulsion (Engman *et al.*, 1998). De Araujo Mantovania *et al.* (2011:933) stated that the incorporation of high-pressure homogenisation during the formulation process produces kinetically stable emulsions. Although high-energy methods tend to be expensive, it allows great control of particle size (Peng *et al.*, 2015:828); it was therefore decided that high-pressure homogenisation would be applied in addition to high-speed stirring during formulation. Stable dispersions were achieved by homogenising the formulations as stated in each method, respectively.

O/w emulsions were obtained using a homogeniser with a probe (Heidolph Diax 600 Laboratory stirrer 240VAC, Heidolph Instruments GmbH & CO KG, Germany) (see Figure B.1). A coarse o/w emulsion was formulated containing 2.0% (w/v) oestriol, 0.2% (w/v) oestradiol and 2.5% (w/v) progesterone by means of continuous stirring, where after a finer emulsion was produced by means of high-pressure homogenisation. Three previously tested formulas were adapted and investigated. The optimised formula was selected for further investigation regarding the penetration enhancing effect of different natural oils chosen to serve as the oil phase in topical o/w emulsions (discussed in Appendix C).



Figure B.1: High-energy emulsification method homogeniser (Heidolph Diax 600 Laboratory stirrer 240VAC)

B.4.1 Formulation of o/w emulsions

Numerous methods can be utilised when preparing an o/w emulsion (Hu *et al.*, 2017:17). Regardless of the considerable variations between these methods, the primary objective remains the same, which is to prepare a stable emulsion with ideal physiochemical properties for topical delivery. Emulsions are colloidal two-phase systems composed of a mixture of water and oil, along with an appropriate stabilising surfactant (Buchmann, 2001:151). The selected surfactant(s) determines the lowest achievable particle size, surface characteristics of the particle and emulsion stability. By alternating the preparation methods and surfactant(s) concentration used during formulation, emulsions could possess different chemical and physical characteristics and meet the requirements for various applications in numerous fields (Hu *et al.*, 2017:17; Iyer *et al.*, 2015:1854). As mentioned in Section B.4, it was decided that high-pressure homogenisation would be used to prepare stable o/w emulsions. The formula for each o/w emulsion is listed in Tables B.4 – B.6.

Various methods were used during the formulation of o/w emulsions. Firstly, simple high-speed stirring was utilised to prepare a coarse o/w emulsion, which was then homogenised (high-energy emulsification) to achieve a more stable o/w emulsion with a smaller average particle size. This multi-step method (as seen in Figures B.2 – B.3) applied for all three manufactured formulations.

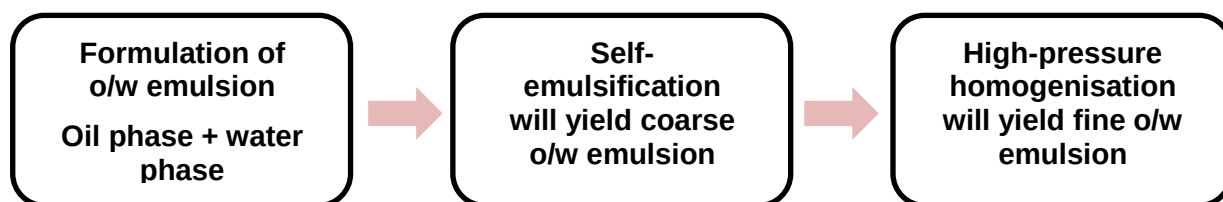


Figure B.2: Formulation process of emulsions as a diagrammatic representation

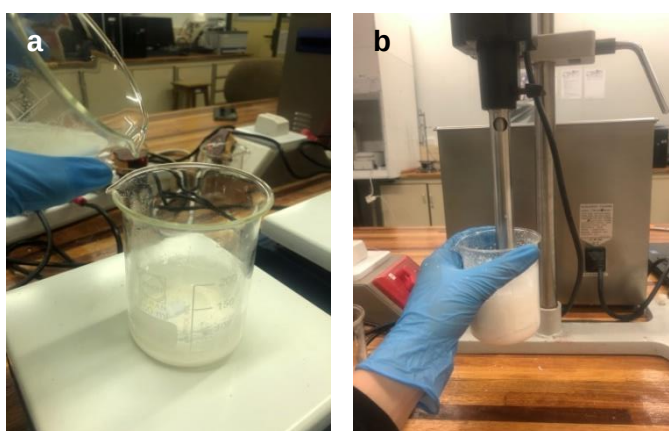


Figure B.3: The two-step formulation process: a) high-speed stirring on magnetic plate, and b) high-pressure homogenisation

B.4.1.1 Formulation method of (OEW1)

The following procedure was followed to formulate (OEW1):

- All the ingredients listed in Table B.4 were accurately weighed.
- All the excipients of phase A were added to a 100 ml beaker, which was placed on a hot plate with a magnetic stirrer to ensure continuous stirring. The mixture was heated to 80°C (temperature regulated with a thermometer).
- All the excipients of phase B were added to a 100 ml beaker, which was heated and continuously stirred until the female hormones were dissolved entirely.
- Phase C was added to a 100 ml beaker and heated to 80°C.
- Phase A was added drop wise to phase C while stirring to produce a coarse o/w emulsion.
- Phase B was added to the formed emulsion while stirring to form a cream base.
- The cream was homogenised at 200 rpm until it reached room temperature (25°C).

Table B.4: Formula of (OEW1) (100 ml)

Ingredient		%w/v	Function
A	Cetyl stearyl alcohol	8.0%	Thickening agent
	Cremophor® A6	1.5%	Emulsifier
	Cremophor® A25	1.5%	Emulsifier
	Propyl paraben	0.3%	Preservative
	Liquid paraffin	12.0%	Oil phase
B	Propylene glycol	8.0%	Co-solvent
	Oestriol	2.0%	Active
	Oestradiol	0.2%	
	Progesterone	2.5%	
C	Milli-Q® water	64.0%	Solvent

B.4.1.2 Formulation method of (OEW2)

The following procedure was followed to formulate (OEW2):

- All the ingredients listed in Table B.5 were accurately weighed.
- All the excipients of phase C were added to a 100 ml beaker, which was placed on a hot plate with a magnetic stirrer to ensure continuous stirring. The phase was heated to 70 to 75°C (temperature regulated with a thermometer).

- All the excipients of phase A were added to a 100 ml beaker, which was heated to 70 to 75°C with continuous stirring to ensure all the excipients were dissolved entirely.
- All the excipients of phase B were added to a 100 ml beaker, which was heated to 70 to 75°C with continuous stirring.
- Phase B was added drop wise to phase C while stirring to produce a coarse o/w emulsion.
- The formed emulsion was added to phase A while stirring to form a cream base.
- The cream was homogenised at 9 500 rpm for 5 min, where after it was slowly stirred until it reached room temperature.

Table B.5: Formula of **(OEW2)** (100 ml)

	Ingredient	%w/v	Function
A	Stearic acid	4.0%	Thickening agent
	Cetyl alcohol	0.5%	Thickening agent
	Crodafos™ MCK	1.7%	Emulsifier
	Span® 60	0.4%	Emulsifier
B	Dimethyl isosorbide	13.0%	Penetration enhancer
	PEG 400	5.0%	Oil phase and humectant
	Oestriol	2.0%	Active
	Oestradiol	0.2%	
	Progesterone	2.5%	
C	Glycerine	10.0%	Humectant
	Tween® 80	3.2%	Hydrophilic surfactant
	Veegum HV	0.5%	Thickening agent
	Milli-Q® water	62.0%	Solvent

B.4.1.3 Formulation method of (OEW3)

The following procedure was followed to formulate **(OEW3)**:

- All the ingredients listed in Table B.6 were accurately weighed.
- All the excipients of phase A were added to a 100 ml beaker, which was placed on a hot plate with a magnetic stirrer to ensure continuous stirring. The phase was heated to 70°C (temperature regulated with a thermometer).
- All the excipients of phase C were added to a 100 ml beaker, which was heated to 70°C with continuous stirring.

- The female hormones (phase B) were dissolved entirely in the methanol and added to phase A.
- Phase A was added drop wise to phase C while stirring to produce a coarse o/w emulsion.
- The o/w emulsion was homogenised at 500 rpm until it reached room temperature.

Table B.6: Formula of **(OEW3)** (100 ml)

Ingredient		%w/v	Function
A	Cetyl alcohol	10.0%	Thickening agent
	Span [®] 60	1.5%	Emulsifier
	Liquid paraffin	12.0%	Oil phase
B	Methanol	6.3%	Co-solvent
	Oestriol	2.0%	Active
	Oestradiol	0.2%	
	Progesterone	2.5%	
C	Tween [®] 80	1.5%	Emulsifier
	Milli-Q [®] water	64.0%	Solvent

B.4.2 Outcome

All three formulations (**(OEW1)**, **(OEW2)** and **(OEW3)**) were formulated successfully without the presence of any aggregation or sedimentation. Since no oil droplets were visible, it was evident that the effective dispersion of oil droplets within the continuous water phase was accomplished. As seen in Figure B.4.a and c, both **(OEW1)** and **(OEW3)** presented as creamy (milky) viscous semi-solids, whereas **(OEW2)** (Figure B.4.b) presented as a translucent-like (less milky) thinner formulation. Whilst **(OEW1)** and **(OEW3)** had a pleasant skin feel after application, **(OEW2)** displayed better spreadability.

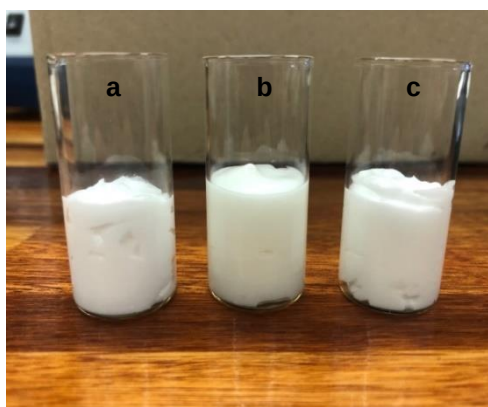


Figure B.4: The formulated o/w emulsions: a) **(OEW1)**, b) **(OEW2)** and c) **(OEW3)**

B.5 Characterisation of the pre-formulated o/w emulsions

The characterisation of o/w emulsions provides useful information to the formulator to determine which formula possesses the ideal physiochemical properties to serve as the optimised formulation, acceptable stability, performance, appearance and the ability to be mass-produced (Chaurasia, 2016:2313). The results of the characterisation tests were compared to each other, after which the optimised formula was selected for further research studies. The characterisation tests that were performed during the study include:

- pH;
- zeta-potential;
- particle size and distribution, and
- viscosity.

B.5.1 pH

To ensure topical formulations are safe to use on the skin and non-irritating in nature, it is essential to measure the pH values of the dispersions (Baser *et al.*, 2015:1881; Nair *et al.*, 2013:425). The pH value of **(OEW1)**, **(OEW2)** and **(OEW3)** was measured using a Mettler Toledo[®] pH meter (Mettler Toledo, United States of America), equipped with a Mettler Toledo[®] InLab[®] 410 electrode (Mettler Toledo, United States of America) (Figure B.5). The electrode was calibrated prior to measurements by means of Mettler Toledo[®] pH buffer solutions at multiple pH values (4, 7 and 10). Measurements were taken in triplicate to determine the average pH value for each emulsion. The pH reading provides necessary information regarding the degree of acidity or basicity of the dispersion (Bahadori & Smith, 2016:318). The concentration hydrogen ions [H⁺] to hydroxide ions [OH⁻] is expressed in terms of pH. In a stable dispersion, these concentrations remain constant over a period of time (Hach Company, 2010:6).



Figure B.5: A Mettler Toledo[®] pH meter with a Mettler Toledo[®] InLab[®] 410 electrode

It is important to note that pH is the most important factor that influences the zeta-potential of a formulation, which can be attributed to the change in the surface charge of the dispersion (Joseph & Singhvi, 2019:99). Therefore, it is essential to determine the pH value before determining the zeta-potential.

Table B.7: Average pH value of the respective o/w emulsions

O/w emulsion	pH			
	Reading 1	Reading 2	Reading 3	Average
(OWE1)	4.456	4.481	4.758	4.565 ± 0.1369
(OWE2)	5.549	5.544	5.535	5.543 ± 0.0058
(OWE3)	5.305	5.191	5.011	5.169 ± 0.1210

The skin seems to be slightly acidic, with an average pH value of 5 (Lambers *et al.*, 2006:359). Topical formulations with a pH value that do not fall within the 3 – 9 range could potentially influence the integrity and permeability of the targeted skin (Barry, 2002:512). From Table B.7 it can be observed that **(OWE1)**, **(OWE2)** and **(OWE3)** obtained an average pH value within the required range for topical application (Barry, 2002:512), therefore the application of any of the emulsions should not cause any skin irritation or sensitivity. The highest pH values were obtained by **(OWE2)** and **(OWE3)**, whereas the slight difference in the pH value between the emulsions can be attributed to the incorporation of methanol within **(OWE3)**. Elfiyani *et al.* (2017:2) stated that alcohol could easily undergo oxidation and produce carboxylic acid within an emulsion, consequently reducing the pH. Accordingly, **(OWE1)** could be considered more acidic than **(OWE2)** and **(OWE3)**, which could possibly be due to the use of propylene glycol in the formula. Propylene glycol is prone to thermal oxidative reactions, which produce carbon-based acids with a consequential decrease in the pH of the formulation (Clifton *et al.*, 1985:77).

B.5.2 Zeta-potential

Zeta-potential is the measurement of the electric surface charge of a dispersion (Krstić *et al.*, 2018:494; Selvamani, 2019:425). The zeta-potential of an emulsion is an indicator of the electrostatic charges (attraction or repulsion) present between particles, which are typically caused by attractive Van der Waals forces and repulsive electrical double layer forces (Arnold, 1995:913; Gqebe *et al.*, 2016:576). Photon correlation spectroscopy (PCS) is a technique that can be used to determine zeta-potential by measuring the fluctuations in scattered light intensity between dispersed particles caused by Brownian motion (Gaur *et al.*, 2014:40; Yennawar *et al.*, 2016:438).

The short- and long-term stability of an emulsion is greatly related to zeta-potential (Gqebe *et al.*, 2016:580). It is evident that higher zeta-potential values (positive or negative) are indicative

of higher colloidal system stability (Sun *et al.*, 2016:8525). Emulsions with a zeta-potential greater than 30 mV or less than - 30 mV are considered to be stable due to the strong repulsion and weak attraction forces that hinder aggregation (Da Costa *et al.*, 2014:5; De Castro *et al.*, 2017:918; Gqebe *et al.*, 2016:575; Larsson *et al.*, 2012:210). Contrarily, an emulsion destabilises when the attraction forces exceed the repulsion forces (Shammas & Wang, 2015:421). It has been noted that optimal flocculation occurs when the zeta-potential is reduced to around 0 mV (Mehta, 1999:29; Narong & James, 2006:130).

Zeta-potential is a key parameter that influences the diffusion of actives through the skin (Gillet *et al.*, 2011:223). It is proposed that negatively charged molecules could enhance diffusion and subsequently improve API flux, since most cell membranes are negatively charged (Clogston & Patri, 2011:63; Sinico *et al.*, 2005:129). According to Fonte *et al.* (2012:302), zeta-potentials between - 10 mV and 10 mV are considered approximately neutral, whereas those that fall outside the range of - 30 mV to 30 mV are regarded as strongly anionic and cationic, respectively.

For the purpose of this study, the Malvern Zetasizer Nano ZS (Malvern Instruments, United Kingdom) (Figure B.6.a) was used to measure the zeta-potential of **(OWE1)**, **(OWE2)** and **(OWE3)** by means of PCS (Gaur *et al.*, 2014:40). Samples of **(OWE1)**, **(OWE2)** and **(OWE3)** were prepared respectively by adding 20 mg of each formulation to a glass beaker containing 30 ml Milli-Q[®] water. Thereafter, each glass beaker was sealed with a piece of Parafilm[®] and placed in an ultrasonic bath for 15 min to ensure proper dissolution. A syringe was used to inject 2 ml of each dilution into a clear disposable zeta-cell (DTS1070 folded capillary cell) (Figure B.6.b) for analysis. Measurement was done in triplicate and performed on the day of sample formulation.

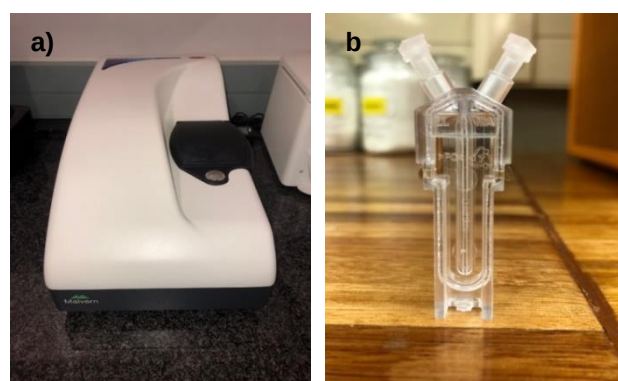


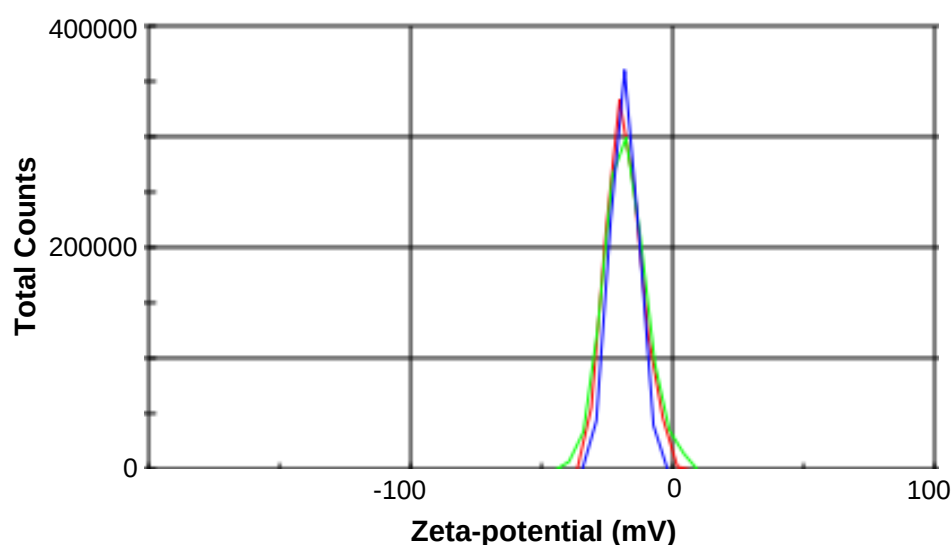
Figure B.6: a) Malvern Zetasizer Nano ZS, and b) a clear disposable DTS1070 folded capillary zeta-cell

Table B.8: Average zeta-potential (mV) of the respective o/w emulsions

O/w emulsion	Zeta-potential (mV)			
	Reading 1	Reading 2	Reading 3	Average
(OWE1)	- 18.5	- 18.2	- 18.5	- 18.40 ± 0.1414
(OWE2)	- 57.1	- 54.3	- 56.4	- 55.90 ± 1.8980
(OWE3)	- 14.0	- 14.2	- 13.7	- 13.97 ± 0.2055

From Table B.8, it is observed that **(OWE1)**, **(OWE2)** and **(OWE3)** presented with negative zeta-potential values. Both **(OWE1)** and **(OWE3)** had an average zeta-potential value in the range between 0 mV and - 30 mV; therefore it can be stated that unstable emulsions were formed with strong attraction forces between the particles with a greater probability of flocculation and sedimentation (Narong & James, 2006:130; Shammass & Wang, 2015:421). The average zeta-potential value of **(OWE2)** was well within the required criteria ($\leq - 30$ mV), which indicated that the repulsion forces between the particles exceeded that of the attraction, consequently forming a stable emulsion with a lesser probability of aggregation (Silva *et al.*, 2012:860; Sun *et al.*, 2016:8525).

Histological studies have revealed that negatively charged molecules diffuse through the stratum corneum considerably faster than positively charged molecules at the initial time stage after topical application (Ogiso *et al.*, 2001:49). Concerning the effect of the surface charge of each respective emulsion on drug penetration, **(OWE2)** would penetrate the skin rapidly and contribute to the increased permeation of actives, whereas **(OWE1)** and **(OWE3)** would possibly present with poor skin permeation (Silva *et al.*, 2012:860).

**Figure B.7:** Average zeta-potential (mV) of **(OWE1)**

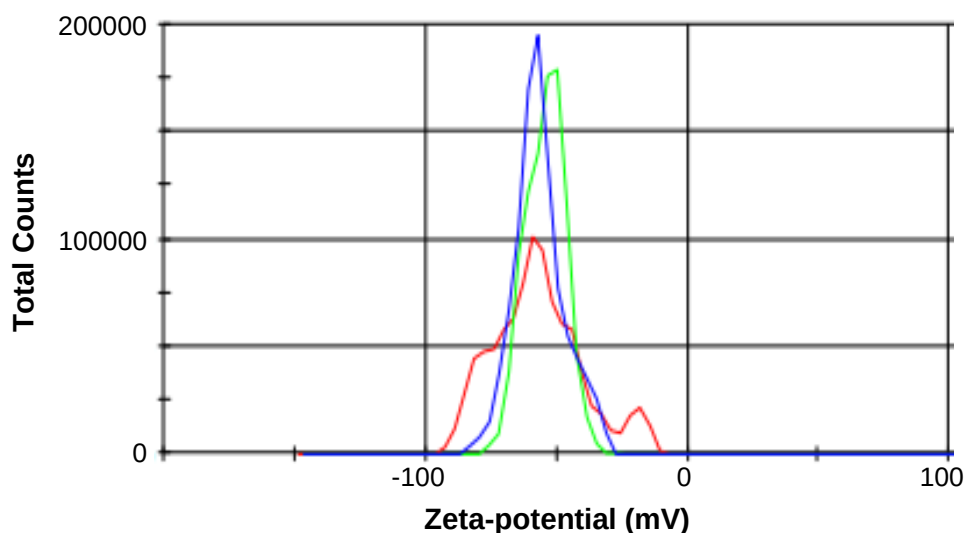


Figure B.8: Average zeta-potential (mV) of **(OWE2)**

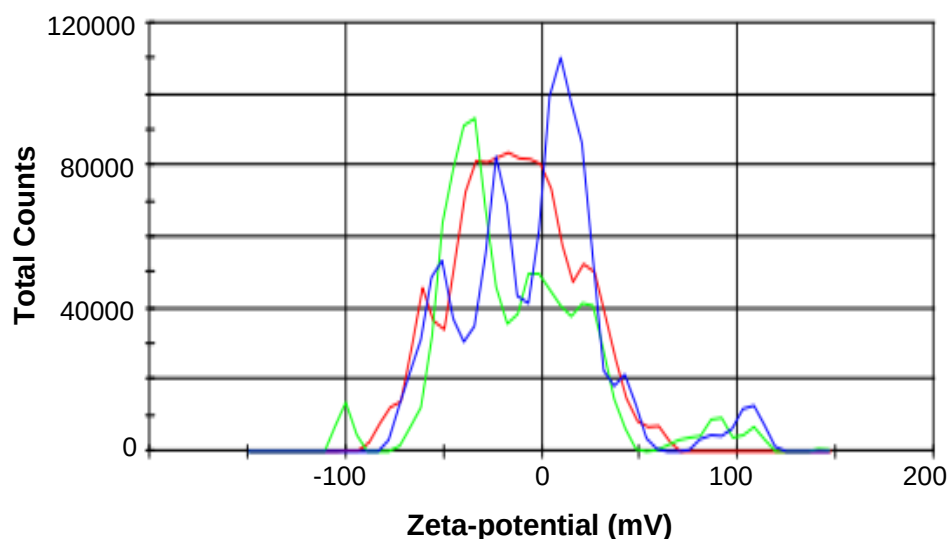


Figure B.9: Average zeta-potential (mV) of **(OWE3)**

Figures B.7– B.9 demonstrate the average zeta-potential values for the respective o/w emulsions (**(OWE1)**, **(OWE2)** and **(OWE3)**). Table B.8 represents the triplicate zeta-potential results obtained, as well as the average zeta-potential value for each of the individual o/w emulsion. As mentioned before, only **(OWE2)** presented with an average zeta-potential within the acceptance range (≤ -30 mV), proposing stability and favourable skin permeation, whereas **(OWE1)** and **(OWE3)** obtained values closer to 0 mV. It can therefore be stated that **(OWE1)** and **(OWE3)** did not fall within the required range for dispersion stability and enhanced skin permeation.

Different distribution curves can be observed for each respective emulsion in Figures B.7 – B.9. The symmetrical curve presented by **(OWE1)** is indicative of a narrow data distribution, whereas

the asymmetrical curve obtained for **(OWE3)** shows large deviation between the triplicate measurements, hence a poor distribution of data. As for **(OWE2)**, the distribution of data appears not to differ much between two measurements (Skoglund *et al.*, 2017:16). Despite the broader distribution of data, it can be stated that **(OWE2)** presented as more ideal compared to **(OWE1)** and **(OWE2)**.

B.5.3 Particle size and distribution

Particle size has a direct effect on the physiochemical properties of an emulsion, including the viscosity, stability, texture and feel, appearance, dissolution rate and efficacy of delivery to the target area (Malvern Instruments Limited, 2015a). Ideal physiochemical properties are typically associated with small, uniform particle size (Ramin *et al.*, 2009:11). Various factors could influence the particle size of dispersions; these include the properties of the oil and aqueous phase, emulsifier type and concentration, and homogenisation pressure and temperature (Lin, 2009:28). Laser diffraction (LD) is a popular technique used to determine particle size by measuring the intensity of scattered light, detected over a range of angles, as a laser beam passes through a dispersed particulate sample (Gupta & Yan, 2016:33; Osborne *et al.*, 2019:102). LD is able to measure particles within the size range of 1 – 600 µm (Buchmann, 2001:166). Large angle scattering is characteristic of small particles, whereas small angle scattering is characteristic of large particles. The data was analysed to determine the size of the particle in the sample responsible for producing the scattering pattern (Malvern Instruments Limited, 2015a). Mastersizer 2000 Ver. 5.60 software was used to compile the data.

Span is a measurement of the distribution width based on the 10, 50 and 90% standard percentile (Lee & Choo, 2015:4380) and expresses the polydispersity index (Pdl) of particle in an emulsion (Horiba Scientific, 2017; Lee & Choo, 2015:4382). Equation B.1 is used to calculate the span (Dyankova *et al.*, 2016:5):

$$\text{Span} = \frac{d(x, 0.9) - d(x, 0.1)}{d(x, 0.5)} \quad \text{Equation B.1}$$

Where:

$d(x, 0.9)$ = 90% of the sample lies below this particle size

$d(x, 0.5)$ = median, 50% of the sample lies below this particle size

$d(x, 0.1)$ = 10% of the sample lies below this particle size

The span value indicates the dispersity of the particles through the continuous water phase; therefore how uniform particles are in the emulsion (Alloid *et al.*, 2018:78). Lower span values are indicative of narrower particle size distribution and more monodispersed emulsions (De Sousa Lobato *et al.*, 2013:3908; Dyankova *et al.*, 2016:5).

For the purpose of this study, the Malvern Mastersizer 2000 Particle Size Analyser (Malvern Instruments, United Kingdom), equipped with a Hydro 2000SM Dispersion Unit (Malvern Instruments, United Kingdom) (Figure B.10), was used to measure the average particle size of **(OE1)**, **(OE2)** and **(OE3)**. For the formulations to be classified as coarse emulsion, the particle size is required to be higher than 500 nm (Buchmann, 2001:150). A sample of each emulsion was prepared by adding 10 ml Milli-Q® water into a polytop with 1 ml of freshly prepared formulation, followed by thorough mixing. A pipette was used to transfer small volumes of the sample into the Hydro 2000SM Dispersion Unit until the degree of obscuration was within the required range (10 – 20%) for testing (Malvern Instruments, 2007). A refractive index of 1.658 was used for the emulsions. The pump speed was set at 1 200 rpm and measurements were run in triplicate to determine the average particle size for each emulsion.



Figure B.10: a) Malvern Mastersizer 2000 Particle Size Analyser with b) a Hydro 2000SM Dispersion Unit

Table B.9: Average particle size (µm) of the respective o/w emulsions

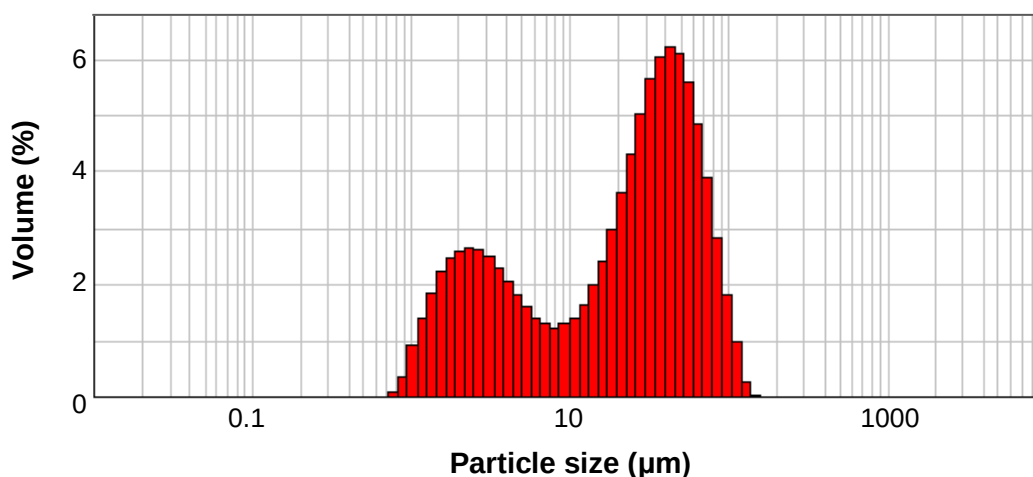
O/w emulsion	Particle size (µm)			
	Reading 1	Reading 2	Reading 3	Average
(OE1)	29.851	25.112	20.658	25.207 ± 3.7536
(OE2)	47.582	47.611	46.486	47.226 ± 0.5236
(OE3)	9.301	8.757	8.434	8.800 ± 0.3923

Table B.10: Average span value of the respective o/w emulsions

O/w emulsion	Span			
	Reading 1	Reading 2	Reading 3	Average
(OWE1)	2.226	2.671	3.207	2.701 ± 0.401
(OWE2)	1.847	1.881	1.932	1.887 ± 0.035
(OWE3)	4.917	3.786	5.323	4.675 ± 1.000

As demonstrated by Table B.9, **(OWE1)**, **(OWE2)** and **(OWE3)** had a particle size within the acceptable range to be classified as coarse dispersions (> 500 nm). The smallest particle size was presented by **(OWE3)**. Smaller particle size enhances the rate and depth of skin penetration, which will result in an increased amount of active found in deeper layers of the skin (Adib *et al.*, 2016:32). Increased stability and viscosity is also associated with small particle size (Engman *et al.*, 1998; Goodarzi & Zendehboudi, 2019:282; Ramin *et al.*, 2009:11). The influence of particle size on emulsion viscosity will be discussed in Section B.5.4.

As observed in Table B.10, the emulsions presented with considerably different span values. The lower span value of **(OWE2)** displayed narrow particle size distribution and is indicative of a dispersion that is more monodispersed compared to the other dispersions. The higher span values of **(OWE1)** and **(OWE3)** displayed broader particle size distribution and are more polydispersed. Emulsions with lower span values are typically more stable against sedimentation and aggregation over time (Ramin *et al.*, 2009:11). O/w emulsions are characterised as two-phase systems and will never be entirely monodispersed with regard to particle size (Mohos, 2010:146).

**Figure B.11:** Average particle size distribution for **(OWE1)**

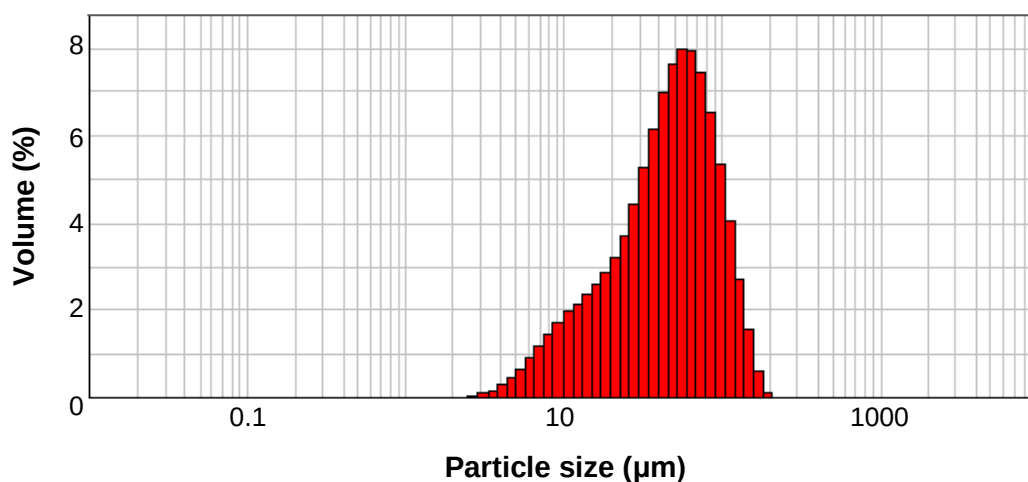


Figure B.12: Average particle size distribution for **(OEW2)**

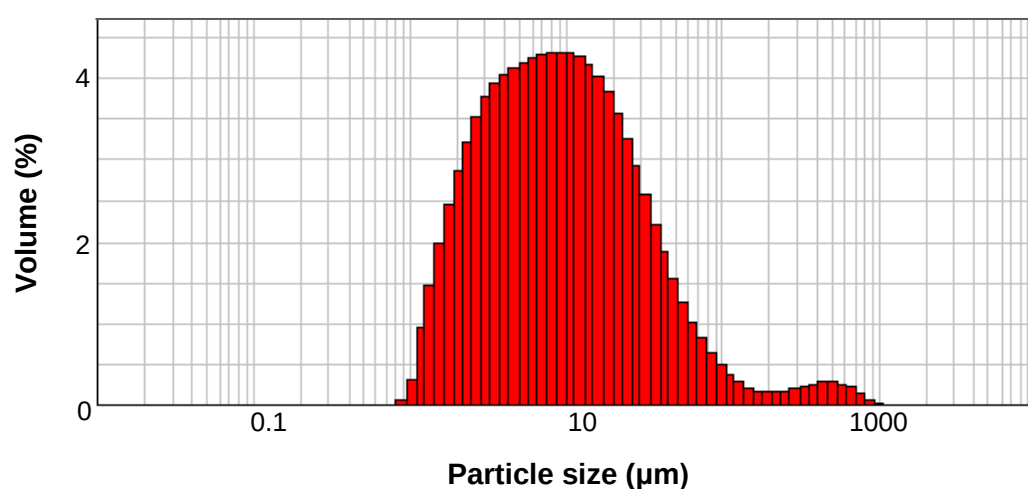


Figure B.13: Average particle size distribution for **(OEW3)**

Figures B.11 – B.13 demonstrate the distribution curves obtained during the determination of particle size of each respective emulsion. For **(OEW1)** and **(OEW3)**, both emulsions displayed bimodal volume-based size distribution, which indicates the presence of two modes (Rámirez *et al.*, 2002:311). The mode represents the particle size with the highest frequency in the distribution (Ahmed & Ahmed, 2008:151). High-pressure homogenisation decreases particle size, but increases the standard deviation of particle size distribution (volume-based), rendering wide-ranging bimodal distribution (Gohtani & Yoshii, 2018:113). **(OEW1)** exhibited unimodal volume-based size distribution and negative skewness, which indicates an asymmetrical distribution towards more negative values (Dias, 2014:40), signifying more fine particles are present in the dispersion compared to coarse particles (Devi *et al.*, 2015:1). Eliot *et al.* (2003:235) proposed that unimodal particle size distribution indicates a stable emulsion, whereas bimodal distribution indicates an instable emulsion. From Figures B11 – B.13 it can be seen that all the dispersions were polydispersed, which is expected from o/w emulsions (Mohos, 2010:146).

B.5.4 Viscosity

Viscosity describes a fluid's resistance to flow (Buchmann, 2001:162; Zhang, 2016:128), which is an important characteristic that affects the drug release and stability of formulations (Nastiti *et al.*, 2017:44). Fluids that flow easily are indicated by low viscosity values (Zhang, 2016:128). The viscosity of an emulsion is a function of the excipients; therefore, emulsions can vary significantly as they can be formulated as semi-solids or liquids (Das & Chakraborty, 2016:285). The degree of viscosity of an o/w emulsion is determined by numerous factors, which include (Buchmann, 2001:153):

- Ratio of dispersed to continuous phase: increasing the lipid concentration results in a corresponding increase in viscosity, but not definitely in creams.
- Lipid type: the use of high melting point lipophilic excipients could result in soft o/w creams.
- Incorporation of thickenings agents in the lipid phase: generally results in hard o/w creams.
- Incorporation of thickening agents in the water phase: the ultimate approach to increase the viscosity of thin o/w emulsions.

The viscosity of a dispersion can be influenced by changing the particle characteristics (Crowley, 2006:768). Generally, emulsions with small average particle size and a narrow distribution of data present with increased viscosity and stability (Engman *et al.*, 1998; Goodarzi & Zendehboudi, 2019:282). Reducing the particle size, increases the amount of particles present in the constant volume fraction, which in turn, increases the interactions among the particles, especially particles in the submicron range. The surface charge and surrounded adsorption layers (hydration) of these particles significantly increase their hydrodynamic size, which increases the emulsion viscosity by increasing the volume fraction for a fixed particle loading (Malvern Instruments Limited, 2015b). Furthermore, an increase in the dispersed phase volume fraction results in an increase in emulsion viscosity. A higher dispersed phase volume fraction presents with closely packed particles that cannot generate much movement, causing the emulsion to display viscoelastic properties (Lin, 2009:31).

To determine the viscosity of **(OEW1)**, **(OEW2)** and **(OEW3)**, a Brookfield Viscometer DV2T LV Ultra (AMETEK Brookfield, United States of America), connected to a thermostatic water bath set at $\pm 25^{\circ}\text{C}$ (Figure B.14), was used to acclimatise the emulsions by placing the formulations into the bath for 60 min prior to measurements. The appropriate spindle (as seen in Table B.11) was inserted into the sample and attached to the viscometer. The viscosity of each emulsion was measured at the predetermined rotation speed (as seen in Table B.11). The viscosity readings were expressed in terms of centipoise (cP) at room temperature

($\pm 25^{\circ}\text{C}$). Rheocalc T1.2.19 software was used to measure and collect multipoint data at 10 s intervals for 2 min. These measurements were used to calculate the average viscosity value for each emulsion. The average viscosity value for **(OEW1)**, **(OEW2)** and **(OEW3)** are exhibited in Table B.12.

Table B.11: Parameters for the viscosity measurement of the respective o/w emulsions

O/w emulsions	Spindle	Speed (rpm)
(OEW1)	T-F	0.3
(OEW2)	T-E	30.0
(OEW3)	T-F	0.5



Figure B.14: a) Brookfield Viscometer DV2T LV Ultra, connected to b) a thermostatic water bath

Table B.12: Average viscosity (cP) and torque (%) measurements of the respective o/w emulsions

O/w emulsions	Viscosity (cP)	Torque (%)
(OEW1)	1 710 333.0 $\pm$ 158 899.0	55.20 $\pm$ 5.0871
(OEW2)	9 471.2 $\pm$ 251.6	60.51 $\pm$ 1.4739
(OEW3)	975 233.3 $\pm$ 150 494.9	52.01 $\pm$ 8.0227

As demonstrated in Table B.12, the emulsions presented with considerably different viscosity measurements. **(OEW1)** presented with the highest viscosity, whereas **(OEW2)** presented the lowest. The low viscosity of **(OEW2)** can be attributed to its larger water phase fraction when compared to the other two emulsions: **(OEW2)** 75.7% > **(OEW3)** 65.5% > **(OEW1)** 65.0%. Since the continuous aqueous phase is the largest component in o/w emulsions, increasing the water phase will lower the emulsion viscosity (Chime *et al.*, 2014:97). The high viscosity of **(OEW1)** can be due to the incorporation of cetyl stearyl alcohol into the lipid phase, which facilitates the formation of a hard semi-solid (Buchmann, 2001:153). As seen in Table B.12

(OEW3) has the smallest average particle size, resulting in an increase in viscosity. As stated above, smaller average particle size increases the viscosity of an emulsion due to an increase in the volume fraction for a specified particle loading (Yapici *et al.*, 2018:585). Accordingly, **(OEW2)** presented with the largest particle size and lowest viscosity. The torque ranged between 52.01 – 60.61%.

Rheopectic fluids display increasing viscosities when subject to constant shear rate (rpm), while thixotropic fluids display decreasing viscosities; rheopexy and thixotropy can also occur in combination (Hurst *et al.*, 2015:940). Figure B.15 illustrates the change in viscosity as a function of time for each emulsion. Both **(OEW1)** and **(OEW3)** experienced an initial increase in viscosity followed by a constant decrease at 20 s and 50 s respectively. Hence, both dispersions initially exhibited rheopectic characteristics followed by thixotropic characteristics as time progressed; note the viscosity of both dispersions increased again at 110 s. As displayed in Figure B.15.b, **(OEW2)** experienced a constant decrease in viscosity and can therefore be characterised as a thixotropic fluid, as it became less viscous over time when stirred (Hurst *et al.*, 2015:940).

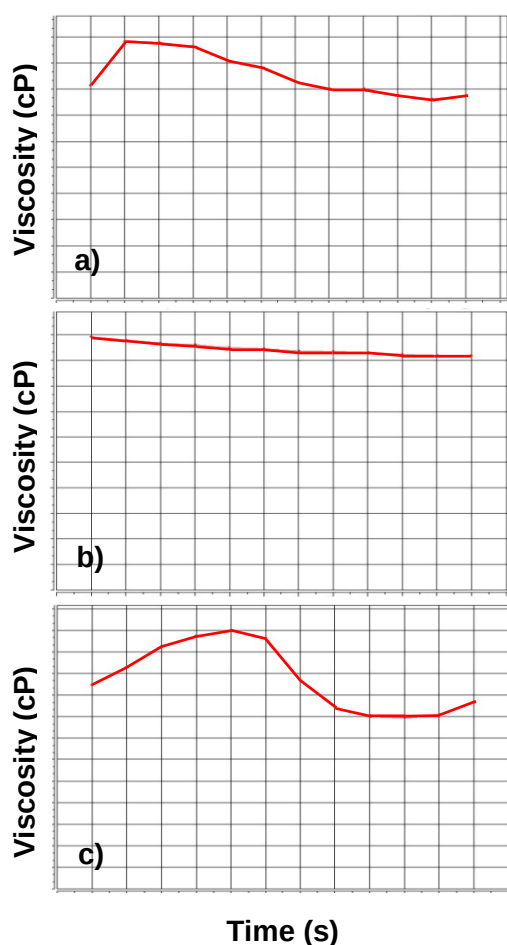


Figure B.15: Viscosity data graph obtained for a) **(OEW1)**, b) **(OEW2)** and c) **(OEW3)**

B.6 Decision on final formula to be used

Pre-formulation studies form a fundamental part of formulation design (Patel, 2019:1). The pre-formulation process includes identifying the intended purpose of the formulation, selecting the drug delivery system, assessing the physiochemical properties of the excipients and evaluating the characteristics of the formulation (Barry, 2007:596; York, 2007:5). During this study various tests were performed to analyse and characterise the o/w emulsions ((**OEW1**), (**OEW2**) and (**OEW3**)), each containing 2.0% (w/v) oestriol, 0.2% (w/v) oestradiol and 2.5% (w/v) progesterone. To determine the optimal formula, the results obtained during the characterisation tests (Section B.5) were evaluated and compared. A summary of the characteristics are listed in Table B.13.

Table B.13: Summary of the characteristics of the pre-formulated o/w emulsions

Formula	pH	Zeta-potential (mV)	Particle size (µm)	Viscosity (cP)
(OEW1)	4.565 (± 0.1369)	- 18.40 ± 0.1414	25.207 (± 3.7536)	1 710 333.0 (± 158 899.0)
(OEW2)	5.543 (± 0.0058)	- 55.90 ± 1.8980	47.226 (± 0.5236)	9471.2 (± 251.6)
(OEW3)	5.169 (± 0.1210)	- 13.97 ± 0.2055	8.800 (± 0.3923)	975 233.3 (± 150494.9)

Measuring the pH of emulsions intended for topical use is important, as pH outside of the required range (3 – 9) could possibly cause skin irritation or damage (Barry, 2002:512; Barry, 2007:576). All three emulsions exhibited values safe for topical use, ranging between 4 and 6; therefore, they should not cause any skin irritation when applied topically.

Zeta-potential values greater than 30 mV and lesser than - 30 mV are indicative of stability (Da Costa *et al.*, 2014:5; Gqebe *et al.*, 2016:575). From Table B.13, it can be observed that (**OEW2**) was the only formulation that obtained a zeta-potential within the required range, therefore it was considered to be more stable. Furthermore, highly negative values imply increased skin permeation (Silva *et al.*, 2012:860). While all three emulsions obtained negative values, (**OEW2**) would exhibit more favourable skin permeability ((**OEW2**) > (**OEW1**) > (**OEW3**)). For (**OEW1**) and (**OEW3**), both displayed negative values between 0 mV and - 30 mV, therefore both emulsions can be considered as unstable with poor skin permeability.

Particle size is an important characteristic that influences numerous physiochemical properties of an emulsion, which include the stability, viscosity, appearance and effective drug delivery (Malvern Instruments Limited, 2015a). Determining the average particle size of each emulsion is essential in order to select the optimal formula for further investigation. The small particle

size of **(OEW3)** indicates efficient skin penetration and stability. However, the larger particles of **(OEW2)** are more favourable if the purpose is to formulate a topical hormone emulsion with limited skin penetration characteristics. The lower span value obtained by **(OEW2)** is representative of a monodispersed emulsion, hence particles are uniformly dispersed throughout the aqueous phase. Generally, monodispersed emulsions display greater stability against aggregation and sedimentation (Ramin *et al.*, 2009:11). The narrow particle size distribution (monodispersed) displayed by **(OEW2)** provides better control of uniform drug distribution over the topically applied surface (Buchmann, 2001:159; Liu *et al.*, 2010:75), which ensures the delivery of therapeutically effective drug concentrations and prevents possible skin irritation (Garg *et al.*, 2002:84; Nair *et al.*, 2013:421; Saettone *et al.*, 2000:315).

The viscosity of the respective emulsions varied considerably. The extremely high viscosity showed by **(OEW1)** is due to the incorporation of cetyl stearyl alcohol, which is a thickening agent used to form hard semi-solids. The low viscosity of **(OEW2)** is attributed to the large water phase fraction. Spreadability is an essential characteristic of topical formulations, since it is responsible for multiple formulation properties:

- extrudability for the container;
- consumer preference;
- accurate dosage transfer to application site, and
- easy application to the skin (Garg *et al.*, 2002:84).

The increased viscosity of both **(OEW1)** and **(OEW3)** will increase the retention time of the emulsions on the skin, but will decrease their spreadability (Garg *et al.*, 2002:86). Furthermore, the lower viscosity of **(OEW2)** will, in theory, exhibit better spreadability. Welin-Berger *et al.* (2001:309) stated that permeability increases as viscosity decreases. If the purpose is to formulate an emulsion that exhibits limited permeation through the skin (viable epidermis) to prevent the adverse effect of systemic delivery (i.e. female hormones), this is an important factor to consider. The high viscosity of **(OEW1)** and **(OEW3)** will therefore decrease skin permeability.

Considering all the characteristics, it was apparent that **(OEW2)** presented with the most optimal properties and displayed no visible aggregation or sedimentation, therefore **(OEW2)** was selected as the optimal formula for further investigation concerning the penetration enhancing effects of selected natural oils that will be discussed in Appendix C.

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APPENDIX C:

FORMULATION AND CHARACTERISATION OF O/W EMULSIONS SEPARATELY CONTAINING SELECTED NATURAL OILS

C.1 Introduction

During pre-formulation, the o/w emulsions were characterised and compared, thereafter the optimised formula (**OEW2**) was selected as it revealed the most favourable physicochemical properties. Emulsions present with numerous advantages, including the ability of solubilising both hydrophilic and lipophilic actives, which consequently improves skin absorption and permeation (Buchmann, 2001:149). The use of high-pressure homogenisation during the formulation process produces emulsions that are homogenous and have smaller average particle sizes, which will result in more elegant and kinetically stable dispersions (De Araujo Mantovana *et al.*, 2011:933; Engman *et al.*, 1998; Hebishy *et al.*, 2013:166; Intarasirisawat *et al.*, 2016:1).

Natural oils are frequently used in topical formulations as stabilisers, emulsifiers or penetration enhancers (Čižinauskas *et al.*, 2017:1536; Lin *et al.*, 2018:70), as they are considered safe and non-toxic due to their natural origin (Dreger & Wielgus, 2013:150), along with being readily available and relatively inexpensive (Vaughn *et al.*, 2017:106). The ideal characteristics of penetration enhancers include the following (Charoo *et al.*, 2008:301; Fox *et al.*, 2011:10509):

- pharmacologically inactive;
- cosmetically acceptable with a pleasant skin feel;
- compatible with the API and other excipients;
- physically and chemically stable;
- release the API from the preparation, and
- its effect should be reversible (the normal barrier properties of the skin should return following the removal of the enhancer).

It is of utmost importance that the chosen natural oil possesses the ability to solubilise the intended actives and facilitates the formation of the emulsion (Zhao *et al.*, 2013:3772). As a result of the adequate solubility of actives within the oil phase, maximum drug loading can be attained (Gurram *et al.*, 2015). It is proposed that the fatty acids present in natural oils increase skin permeation by disrupting the densely packed intercellular lipid region within the stratum corneum, subsequently increasing the partitioning and diffusion of both hydrophilic and lipophilic actives across the skin barrier (Pham *et al.*, 2016:180; Wang *et al.*, 2005:25). Conversely,

Viljoen *et al.* (2015:2052) proposed that the incorporation of certain natural oils could influence the penetration ability of the active by decreasing API flux as a result of epidermis-dermis entrapment.

The physical state of the dispersed oil phase in an o/w emulsion can influence several of its physicochemical and organoleptic properties, which includes the appearance, aroma, rheology and stability (Meybodi *et al.*, 2014:69). Increasing the oil phase volume would result in a corresponding increase in emulsion viscosity (Meybodi *et al.*, 2014:69), which plays an important role in the topical delivery of actives; as viscosity increases, permeability usually decreases (Welin-Berger *et al.*, 2001:309). The aim was to formulate cost-effective and stable optimised emulsions that deliver adequate amounts of oestriol, oestradiol and progesterone to the skin to induce a therapeutic effect without systemic absorption. For this study, three natural oils were separately selected to replace dimethyl isosorbide as the penetration enhancer and oil phase in the optimised formula. The fatty acid composition of the respective oils varies greatly, which could potentially influence the penetration depth and quantity of the topically applied hormones (Viljoen *et al.*, 2015:2052). The modified optimal formula will be further referred to as **(NOE)** (natural oil emulsions).

C.2 Intended purpose of the formulation

The increased risk of breast cancer that is associated with elevated hormone concentrations poses the greatest limitation with the clinical application of topical hormone formulations. Moreover, the physicochemical characteristics of the hormones, i.e. adequate lipophilicity and low MW, facilitate easy permeation through the skin (Polonini *et al.*, 2014:332). Idexis aimed to formulate a topical hormone delivery system that would not diffuse through the skin into the systemic circulation, but still initiate a cutaneous anti-ageing effect. However, due to the classified nature of this study, the vehicle and excipients used during formulation of the respective topical products **((FS), (EC) and (MC))** were unknown to the student. Therefore, the purpose of this study was to formulate three simple emulsions by utilising the selected optimised formula (as discussed in Appendix B) to investigate the penetration depth and quantity of the respective hormone incorporate within the formulation by performing *in vitro* diffusion studies (as discussed in Appendix E). Ultimately, the *in vitro* diffusion study results obtained for the simple formulations were compared to that of the complex Idexis products to evaluate the efficacy of the polymers and vehicle used in the Idexis products to inhibit: 1) skin penetration beyond the epidermis and, consequently, 2) adverse systemic effects.

C.3 Excipients used to formulate emulsions

The aim of this study was to formulate three o/w emulsions, each containing 2.0% (w/v) oestriol, 0.2% (w/v) oestradiol, 2.5% (w/v) progesterone and 13.0% (w/v) of the respective natural oil.

Tables C.1 – C.2 illustrate the excipients used to formulate the **(NOE)**. The **(NOE)** will be referred to as follows:

- **(CoOE)** (o/w emulsion containing coconut oil);
- **(CaOE)** (o/w emulsion containing castor oil), and
- **(EOE)** (o/w emulsion containing emu oil).

Table C.1: Excipients used in the formulation of **(NOE)** with their function, supplier and batch number

Excipient	Function	Supplier	Batch number
Oestriol	Active	Thonson Technology Ltd	20181001
Oestradiol		Ifect Chemphar Co., Ltd	IF-ES-181202
Progesterone		Hubei Gedian Humanwell Pharmaceutical Co., Ltd	HTT181101
Stearic acid	Thickening agent	Saarchem	5872120
Cetyl alcohol	Thickening agent	Merck	S5428004951
Crodafos™ MCK	Emulsifier	Croda	498133
Span® 60	Lipophilic surfactant	Sigma	SLNT7599
Tween® 80	Hydrophilic surfactant	Sigma	BCBL1768V
Glycerine	Humectant	Sigma	2002895
Veegum® HV	Thickening agent	R.T Vanderbilt Co, Inc.	12199370
PEG ^a 400	Oil phase and humectant	Saarchem	1027497
Milli-Q® water	Water phase and solvent	In-lab	Direct Pure UP

a) Polyethylene glycol

Table C.2: Natural oils used in the formulation of **(NOE)**

Oil	Supplier	Batch number	Toxicological information
Coconut oil	ENCO Fuels CC	15-7-2010	Proposed to be non-irritating to most skin types ^a , however prolonged exposure may cause irritation in sensitive skin ^b
Castor oil	Allied Drug Company Ltd.	BV323	Evidence of mild skin irritation ^{c, d}
Emu oil	Emuphoria	549-08-02-10	Proposed to be non-irritating to most skin types ^{e, f}

a) Varma *et al.*, 2019:5; b) MSDS, 2012; c) Verheyden *et al.*, 2017:1; d) MSDS, 2014; e) Jacobs *et al.*, 2017:75; f) MSDS, 2008

C.3.1 Female hormones

From Table C.3, it can be observed that all the selected hormones (oestriol, oestradiol and progesterone) present with high log P values, therefore indicating a lipid character and higher affinity for the oil phase (Bhal, 2007:1; Bhatia *et al.*, 2014:2). Lipid-soluble actives are generally

incorporated into inert lipid carriers such as o/w emulsions (Wadhwa *et al.*, 2012:179). Therefore, in this study, the selected hormones were formulated within the oil phase of the (NOE) for adequate drug delivery. The concentration of each respective hormone was kept constant throughout the (NOE).

Table C.3: Physicochemical characteristics of selected female hormones

	Oestriol	Oestradiol	Progesterone
Chemical formula	C ₁₈ H ₂₄ O ₃ ^a	C ₁₈ H ₂₄ O ₂ ^a	C ₂₁ H ₃₀ O ₂ ^b
MW (g/mol)	288.4 ^a	272.4 ^a	314.5 ^b
Melting point (°C)	280 ^a	173 – 179 ^a	121 ^b
Boiling point (°C)	No data	No data	394.13 ^d
Aqueous solubility	Practically insoluble ^a	Practically insoluble ^a	Practically insoluble ^b
Log P	2.4 ^a	4.0 ^a	3.9 ^b
pK_a value	10.4 ^a	10.7 ^c	18.9 ^c
Solubility	Soluble in ethanol, acetone and chloroform ^a	Soluble in ethanol, acetone and chloroform ^a	Soluble in ethanol and chloroform ^b

a) Moffat *et al.*, 2004:1350-1351; b) Moffat *et al.*, 2004:1964; c) Marques *et al.*, 2015:610; d) Drugbank, 2005a

C.3.2 Thickening agents

Thickening agents play an essential role in cosmetic and skin care products, incorporated to not only regulate the viscosity of the formulation, but also improve the rheological properties of feel and flow, improve physical stability and promote the formation of aesthetically pleasing products that are more favourable to the consumer (Karsheva *et al.*, 2007:187; Riemer & Russo, 2017:43). Furthermore, thickening agents delay skin penetration based on diffusional resistance in the preparation, when a thick layer is applied to the skin. Contrarily, when the formulation is thinly applied, thickening agents endorse penetration through greater stratum corneum diffusivity as a result of increased hydration (Cross *et al.*, 2001:147). Certain thickeners function as humectants, since they have the ability to retain water in the skin, while others possess emulsifying properties (Riemer & Russo, 2017:29). Guillot *et al.* (1982:53) established that the incorporation of thickeners into topical formulations are safe and present no hazard for the skin. With regard to the dispensing system, the properties of the container should be matched with the formulation viscosity and the use of thickening agents to create the desirable flow. Properties of the container that may affect dispensing include the orifice opening, delivery rate, bottle flexibility and dispensing pressure (Riemer & Russo, 2017:37). In summary, thickeners improve the overall efficacy and acceptability of topical products, which include optimal mechanical properties (firmness and spreadability), ideal rheological behaviour (pseudoplastic behaviour with thixotropy) and appropriate adhesion at the application site (Estanqueiro *et al.*, 2016:389). During this study, stearic acid, cetyl alcohol and Veegum[®] were

selected as thickening agents. The physicochemical characteristics of stearic acid and cetyl alcohol are outlined in Table C.4. No data was found on Veegum[®] except for its insolubility in cold water (MSDS, 2005).

Table C.4: Physicochemical characteristics of selected thickening agents

	Stearic acid	Cetyl alcohol
Chemical formula	C ₁₈ H ₃₆ O ₂ ^a	C ₁₆ H ₃₄ O ^b
MW (g/mol)	284.47 ^a	242.44 ^b
Melting point (°C)	69 – 70 ^a	49 ^b
Boiling point (°C)	383 ^a	344 ^b
Aqueous solubility	Practically insoluble ^a	Practically insoluble ^b
Log P	8.20 ^a	6.83 ^d
pK_a value	4.95 ^c	16.20 ^d
Solubility	Freely soluble in chloroform and ether; soluble in ethanol (95%) and propylene glycol ^a	Freely soluble in ethanol (95%) and ether; solubility increases with increasing temperature ^b

a) Rowe *et al.*, 2009:155; b) Rowe *et al.*, 2009:697; c) Drugbank, 2005b; d) Pubchem, 2004

C.3.2.1 Stearic acid

Stearic acid, commonly known as octadecanoic acid, is a mixture of approximately equal quantities of stearic and palmitic acid, which is widely used in o/w emulsions to function as a lipophilic thickening agent and stabiliser (Manchanda *et al.*, 2018:811; Rubino, 2006:330). Furthermore, the incorporation of stearic acid into topical formulations contributes to emollient properties, which softens the skin by creating an occlusive oil film over the stratum corneum and subsequently decreases TEWL and increases epidermal hydration (Mueller, 2008:553; Sidbury, 2018:1471; Sousa *et al.*, 2018:7). Stearic acid occurs as a white and slightly glossy crystalline solid with a mild odour that presents with better solubility in oils and organic solvents (Liebert, 1987:325; MSDS, 2018). According to Rowe *et al.* (2009:697), stearic acid is safe to use in topical formulations, since it is considered a non-toxic and non-irritating compound to most skin types.

C.3.2.2 Cetyl alcohol

Cetyl alcohol, also referred to as 1-hexadecanol, is a fatty alcohol naturally derived from coconut oil, which is frequently used in o/w emulsions to increase viscosity and stability (Fukushima & Yamaguchi, 2001:2). Besides functioning as a thickening agent, cetyl alcohol also serves as an emulsifier and emollient (Ash & Ash, 2004:665) and presents as a waxy white solid at room temperature with a faint soapy odour. Although cetyl alcohol is a well-documented contact allergen (Blondeel *et al.*, 1978:270; Goossens, 2011:3; Hannuksela, 1988:9), when taking into account the high dilution factor in formulations (EFSA, 2012:28), this compound has

little to no effect on the skin, as proven by Liebert (1988:361) who found no evidence of skin irritation or sensitisation. The melting point of cetyl alcohol (as seen in Table C.4) is higher than that of the average skin surface temperature (33.2 °C), thus it is useful in formulating cosmetic products that are easily softened and moulded when warmed by the skin (Encyclopaedia Britannica, 2011; Karlsbad & Kopp, 1991:225).

C.3.2.3 Veegum®

Veegum®, also referred to as magnesium aluminium silicate, is a naturally occurring anionic mineral derived from smectite clay that is used essentially as a stabilising, thickening and suspending agent in cosmetic products (Vanderbilt Minerals, 2016). One of the most useful characteristics of Veegum® is its capability to stabilise an o/w emulsion at low concentration levels (Adebisi *et al.*, 2015:120). Since the structure is not influenced by heat, Veegum® lessens the tendency of an emulsion to weaken and break at raised temperatures (Vanderbilt Minerals, 2016; Wood *et al.*, 1963:354). Furthermore, Veegum® improves rheological properties, which include the formulation of emulsions with favourable spreadability, controlled thixotropy, enhanced skin feel and easy dispensing (pump or pour) without losing stability (Pongjanyakul & Puttipatkhachorn, 2009:346). Lastly, Veegum® possesses the ability to modify drug release from formulations, which is a useful characteristic to use in the attempt to limit systemic absorption of topically applied female hormones (Adebisi *et al.*, 2015:122).

C.3.3 Emulsifiers

Emulsifiers are more commonly referred to as surfactants or surface acting agents (Setya *et al.*, 2014:2218). Miscellaneous characteristics of the indicated application area will determine the requirements regarding the emulsifiers. As a result of the amphipathic nature of surfactants, the solubility of a surfactant molecule in water or oil is one of the key properties to determine in order to utilise the emulsifiers in the appropriate area (Nagy *et al.*, 2015:580). Due to the two-phase system of an emulsion (oil and water phase), the incorporation of a lipophilic surfactant in conjunction with a hydrophilic surfactant will be required for successful formulation (Magdassi & Garti, 1999:156). Moreover, Setya *et al.* (2014:2219) proposed that the stability of an emulsion would increase through the combination of surfactants. The hydrophilic-lipophilic balance (HLB) describes the balance between the strength and size of the hydrophilic (head) and lipophilic (tail) moieties of the surfactant molecule (Lu, 2016:25; Zheng *et al.*, 2015:252). The total HLB value of both surfactants used during formulation should range within 9 – 18, so as to facilitate the formation of an o/w emulsion (Eid *et al.*, 2014:4). A stable emulsion is best formulated when the HLB value of the selected oil is close to the combination HLB value of the surfactants used in the formulation (Fernandes *et al.*, 2013:108). Furthermore, surfactants play an essential role in stability by preventing droplet coalescence through o/w interface adsorption and increased

repulsive forces among droplets (Karsheva *et al.*, 2007:188), which subsequently causes a decrease in the interface tension present between the two phases of the o/w emulsion (Setya *et al.*, 2014:2218). Surfactants are also added to emulsions to encourage the emulsification process during formulation (Baibhav *et al.*, 2011:68; Hyma *et al.*, 2014:4). During this study, Span[®] 60 and Tween[®] 80 were selected as the lipophilic and hydrophilic surfactants, respectively.

Table C.5: Physicochemical characteristics of selected emulsifiers

	Span [®] 60	Tween [®] 80	Crodafos [™] MCK
Chemical formula	C ₂₄ H ₄₆ O ₆ ^a	C ₆₄ H ₁₂₄ O ₂₆ ^b	C ₁₆ H ₃₄ O ₄ P K ^d
MW (g/mol)	431.00 ^a	1310.00 ^b	360.40 ^d
Melting point (°C)	50.00 – 60.00 ^a	- 20.56 ^c	161.00 – 166.00 ^d
Boiling point (°C)	No data	100 ^c	No data
Aqueous solubility	Insoluble, but generally dispersible ^a	Soluble ^b	Easily soluble in water ^d
Log P	No data	No data	No data
Solubility	Soluble and dispersible in oils; mostly soluble in organic solvents ^a	Soluble in ethanol (95%); insoluble in mineral and vegetable oil ^b	Soluble in fats and oils ^d

a) Rowe *et al.*, 2009:675-677; b) Rowe *et al.*, 2009:549-551; c) Drugbank, 2015a; d) MSDS, 2011

C.3.3.1 Span[®] 60

Span[®] 60, commonly known as sorbitan monostearate, is a non-ionic surfactant possessing the ability to function as a suspending, emulsifying, dispersing and wetting agent (Zhang, 2009b:675). It has been proposed that the addition of Span[®] 60 increases the creaming stability of a formulation over time (Joshi & Barhate, 2011:81). The HLB value can be utilised to estimate emulsifier properties (Setiati *et al.*, 2018:2). Literature states that an emulsifier with HLB value below 10 can be classified as lipophilic (Zhang, 2009b:678). Therefore, Span[®] 60, with a HLB value of 4.7, will be added to the oil phase of the **(NOE)** (Zhang, 2009b:678). Attributable to its lipophilic character, it will present with favourable solubility in oils and organic solvents (Durán *et al.*, 2011:78; Zhang, 2009b:678).

C.3.3.2 Tween[®] 80

Tween[®] 80, also referred to as polysorbate 80, is a non-ionic surfactant characterised as a polyoxyethylene 20 sorbitan fatty acid, which is commonly used in the formulation of cosmetics and skin care products to serve as an emulsifier, stabiliser and to promote the formation of o/w emulsions (Prieto & Calvo, 2013:8; Zhang, 2009a:549-550). Tween[®] 80 presents with an HLB value of 15, which is indicative of its hydrophilic character, and will consequently be added to the continuous water phase (Tao *et al.*, 2018:2; Zhang, 2009a:551). As stated before,

surfactants contribute to the stability of the emulsion, therefore adding a lipophilic surfactant together with a hydrophilic surfactant to a formulation will reduce the amount of energy needed to form an o/w emulsion (Chime *et al.*, 2014:91).

C.3.3.3 Crodafos™ MCK

Crodafos™ MCK, commonly known as potassium cetyl phosphate (PCP), is a water-soluble emulsifier that enables the incorporation of sun protection into multipurpose cosmetic and skin care products, without affecting the formulation's texture (Janssen & Klock, 2017:13). Crodafos™ MCK occurs as a fine white powder with a slight fatty odour that is non-irritating and non-sensitising to the skin (MSDS, 2011). A study conducted by Mendrok-Edinger *et al.* (2015:3) demonstrated that the charge distribution of PCP molecules leads to repulsive forces among droplets, which is the main reason for the stabilisation of an emulsion. The incorporation of Crodafos™ MCK into emulsions provides good rheological properties and a powdery after feel to the skin, which helps formulate a cosmetically acceptable emulsion that meets consumer needs with the added boost of a SPF (Croda, 2019).

C.3.4 Humectant

Humectants are hygroscopic compounds that play an essential role in emulsions and the hydration of skin by attracting water into the stratum corneum from two main sources: the dermis and humid environments (Purnamawati *et al.*, 2017:75; Sethi *et al.*, 2016:280). The stratum corneum is composed of dead keratin-filled corneocytes that contain an endogenous water-soluble fraction of low MW humectants termed natural moisturising factors (NMF) (Dayan, 2005:104; Lodén, 2003:773; Rawlings & Harding, 2004:43). Topically applied humectants mimic the NMF found in skin, improving hydration, desquamation, plasticity and barrier homeostasis, which subsequently improve the symptoms of dry and ageing skin (Harding & Rawlings, 2016:204; Jemec & Na, 2002:322; Robinson *et al.*, 2010:13). Furthermore, humectants exhibit other benefits as well, which include fixative and bacteriostatic activities (Mitsui, 1997:134). During the formulation of **(NOE)** both glycerine and PEG 400 were added to not only improve skin hydration, but also to maintain the stability and moisture content of the **(NOE)** (Cowley, 2012:139).

Table C.6: Physicochemical characteristics of selected humectants

	Glycerine	PEG 400
Chemical formula	$C_3H_8O_3^a$	$HOCH_2(CH_2OCH_2)_mCH_2OH$ where m signifies the average number of oxyethylene groups ^e
MW (g/mol)	92.05 ^a	280.00 – 420.00 ^d
Melting point (°C)	17.8 ^b	4.0 – 6.0 ^d
Boiling point (°C)	290 ^b	> 250 ^c
Aqueous solubility	Soluble ^b	Soluble ^c
Log P	- 1.9 ^a	No data
pK_a value	13.61 ^a	No data
Solubility	Practically insoluble in oils; soluble in ethanol (95%) and methanol ^b	Soluble in acetone; alcohols; benzene and glycerine ^e

a) Drugbank, 2015b; b) Rowe *et al.*, 2009:283; c) Drugbank, 2015c; d) MSDS, 2015; e) Rowe *et al.*, 2009:519

C.3.4.1 Glycerine

Glycerine, also referred to as glycerol, is an endogenous humectant often used in topical formulations and cosmetic products to increase cutaneous elasticity, skin moisture, and epidermal barrier repair (Angerhofer *et al.*, 2009:240; Fluhr *et al.*, 2008:23; Rowe *et al.*, 2009:283). In addition to its humectant properties, glycerine possesses the ability to function as an emollient, plasticiser, co-solvent and an antimicrobial preservative (Rowe *et al.*, 2009:283). The use of glycerine prolongs the shelf life of products by maintaining the moisture content and texture of the formulation (Pagliaro & Rossi, 2008:8). Glycerine contains three hydrophilic alcoholic hydroxyl groups that are the main reason for its hygroscopic character and solubility in water (Pagliaro & Rossi, 2008:2). Glycerine occurs as a clear, viscous and odourless liquid at room temperature (SDA, 1990:1). Lodén *et al.* (2002:45) found no evidence of skin irritation caused by glycerine. Multiple studies proposed that glycerine has deeper effects than merely hydrating the skin, which included preventing stratum corneum lipid phase transition in dry conditions (Froebe *et al.*, 1990:51), stabilising skin collagen (Na, 1986:967) and influencing the skin's protective function against irritation and the penetration of compounds through the stratum corneum (Bettinger *et al.*, 1998:18). During this study, glycerine was incorporated within the water phase of the (NOE).

C.3.4.2 Polyethylene glycol 400

PEG is a condensation polymer with numerous degrees of polymerisation (D'souza & Shegokar, 2015:1257; Mekala *et al.*, 2015:2; Mitsui, 1997:134-137). PEG 400 is characterised as a water soluble, non-volatile, inert and thermally stable compound broadly used in cosmetic and skin care products to serve as a humectant (Fruijtier-Pöllöth, 2005:5; Garg *et al.*, 2015:975), as it is considered safe for dermatological use, with no evidence of skin irritation

(MSDS, 2015; Value, 2012:252). Although mild cases of instant hypersensitivity were reported, PEG 400 did not demonstrate a sensitising potential in further testing (Basketter *et al.*, 1997:846; Fruijtier-Pölloth, 2005:20). Liquid PEG 400 occurs as a clear viscous liquid with a mild odour at room temperature (MSDS, 2015). A study led by Valia *et al.* (2008:951) demonstrated that PEG 400 does not affect the barrier properties of the stratum corneum, although it has been shown to influence the aqueous solubility, permeability coefficient and skin/formulation partition coefficient of female hormones. Despite the hydrophilic character of PEG 400, it does not readily penetrate intact skin and can be removed from the application surface simply by washing (Fruijtier-Pölloth, 2005:1; Rowe *et al.*, 2009:517). Tsai *et al.* (2001:302) proposed that the degree of enhancement relative to skin penetration increased with molecular weight and TEWL of the skin. During this study, PEG 400 was used due to its penetration abilities and humectant properties.

C.3.5 Water

The continuous water or hydrophilic phase of an o/w emulsion is composed of an aqueous material, such as water or alcohol (Hyma *et al.*, 2014:4). The aqueous phase plays an important role during the formulation of an o/w emulsion (Silva *et al.*, 2012:857), as it forms the largest part of the dispersion. Lovelyn & Attama (2011:626) state that increasing the aqueous phase volume lowers the viscosity of an emulsion. In this study, Milli-Q® water was used during the formulation of the **(NOE)** to serve as the continuous aqueous phase.

C.3.6 Natural oils

A number of oils, ranging from synthetic or natural lipids, fatty acids, fatty alcohols and medium (6 – 12 carbons) or long (> 12 carbons) chain triglycerides can be used to serve as the oil phase (Abolmaali *et al.*, 2011:140; Pawar & Babu, 2014:429). Natural oils are multifaceted mixtures of compounds consisting primarily of SFA and UFA (Montoya *et al.*, 2019:403). Cultures around the world have used plant-based oils for thousands of years to preserve skin health and beauty (González-Minero & Bravo-Díaz, 2018:50; Vaughn *et al.*, 2017:105). The topical application of natural oils have been shown to increase stratum corneum hydration and enhance corneocyte adhesion, keeping the stratum corneum elastic and smooth (Vaughn *et al.*, 2017:105). Occlusion of the skin limits the escape of water, consequently increasing the stratum corneum water content (Vaughn *et al.*, 2017:105). Natural oils support the lipid layer of the skin, consequently restoring lipid composition and barrier function (Correa *et al.*, 2014:39; Lautenschläger, 2004:46; Lin *et al.*, 2018:70). These oils are commonly used in cosmetic and skin care products as they are considered non-toxic and safe due to their natural origin (Dreger & Wielgus, 2013:150). A study conducted by Viljoen *et al.* (2015:2045), proposed that natural oils containing predominantly mono-unsaturated oleic acid presented with an increased release

of the active when compared to oils comprised of an even mixture of monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). Furthermore, oils containing high concentrations of stearic acid presented with low flux-values due to epidermis-dermis entrapment (Viljoen *et al.*, 2015:2052). For this study, coconut, castor and emu oil were separately chosen to act as the penetration enhancer and oil phase in the **(NOE)**. The fatty acid concentrations present in the selected natural oils are listed in Table C.7. These concentrations are expressed as a gram of fatty acid for each 100 g of oil (Cowley, 2014:35).

Table C.7: Fatty acid composition of natural oils

	Lauric	Myristic	Palmitic	Stearic	Palmitoleic	Oleic	Linoleic	Linolenic
	SFA				MUFA		PUFA	
Oil	12:0	14:0	16:0	18:0	16:1	18:1	18:2	18:3
Coconut ^a	46.6	18.6	9.5	2.7		7.0	1.9	
Castor ^b			1.3	1.1		3.6	5.6	0.5
Emu ^c			24.6	9.4	5.2	49.9	10.0	0.9

a) Petrauskaitė *et al.*, 2000:582; b) Maia *et al.*, 2012:2351; c) Shimizu & Nakano, 2003:58

Table C.8: Physicochemical characteristics of selected natural oils

	Coconut oil	Castor oil	Emu oil
Melting point (°C)	23 – 26 ^a	- 12 ^d	No data
Boiling point (°C)	> 450 ^a	313 ^c	> 300 ^e
Aqueous solubility	Practically insoluble ^a	Practically insoluble ^d	Practically insoluble ^e
Log P	12.2 ^b (atom-additive method)	17.8 ^c (atom-additive method)	No data
pK _a value	No data	No data	No data
Solubility	Soluble in chloroform and ether; soluble at 60 °C in 2 parts of ethanol (95%), less soluble at lower temperatures ^a	Soluble in ethanol (95%), miscible with chloroform, ethanol and diethyl ether; practically insoluble in mineral oil unless mixed with another vegetable oil ^d	No data

a) Rowe *et al.*, 2009:184; b) Pubchem, 2016; c) Pubchem, 2007; d) Rowe *et al.*, 2009:126; e) MSDS, 2017

C.3.6.1 Coconut oil

Coconut oil, also referred to as copra oil, is composed of polyphenols, vitamin E and medium chain triglycerides, especially lauric acid (Kumar *et al.*, 2018:624; Pengon *et al.*, 2018:410). The colour of coconut oil varies from pale yellow to brownish yellow (O'Brien, 2004:42). Over 90% of the fatty acids in coconut oil are saturated, while only 9% are unsaturated (Baoteng *et al.*, 2016:192), making this oil the richest source of medium chain fatty acids (C₆ to C₁₀) (Gervajio, 2005:4). Due to the high concentrations of lauric acid, coconut oil will pass abruptly from a brittle solid to a liquid, within a small temperature range (Aziz *et al.*, 2012:666). Coconut oil is rich in monolaurin, an antimicrobial compound, which exerts its antimicrobial activity by disrupting the lipid membranes of microbial organism (Nakatsuji *et al.*, 2009:2484; Vaughn *et al.*, 2018:110). The topical application of coconut oil has been shown to increase skin surface lipids and improve hydration without altering the pH and TEWL significantly (Vaughn *et al.*, 2018:112). The fatty acid composition of oils is important in relation to oxidative stability, where the oxidation reaction process increases with an increase in the amount of UFA. Therefore, the oxidative stability of coconut oil is due to the small concentrations of UFAs compared to SFAs (Maszewska *et al.*, 2018:1746).

C.3.6.2 Castor oil

Castor oil is composed of various fatty acids, including up to 90% ricinoleic, 4% linoleic, 3% oleic, 1% stearic and not more than 1% linolenic fatty acid (Patel *et al.*, 2016:3). Observational studies have shown that the topical use of ricinoleic acid, which is the main component in castor oil, exerts significant anti-inflammatory and antioxidant effects (Martini *et al.*, 2018:391; Vieira *et al.*, 2000:223). Castor oil can be characterised as a natural oil polyol due to the hydroxyl functionality of ricinoleic acid, which provides oxidative stability by inhibiting peroxide formation (Patel *et al.*, 2016:3; Vyas *et al.*, 2016:2314). Castor oil is a non-volatile and non-drying viscous oil, which is pale yellow in colour (Patel *et al.*, 2016:3). A study led by Alaayedi *et al.* (2018:140) found that the incorporation of castor oil in a formulation can increase the permeability of lipophilic APIs when applied topically by changing the barrier characteristics of the stratum corneum.

C.3.6.3 Emu oil

Crude emu oil is a creamy semi-solid mass at room temperature, which turns into a clear yellow liquid after being processed and refined (Patel *et al.*, 2015:87). The majority of the fatty acids present in emu oil appear to be oleic and palmitic acid (Shimizu & Nakano, 2003:58). Emu oil also contains a high concentration of stearic acid compared to other natural oils, which has been reported to decrease API flux during topical delivery (Viljoen *et al.*, 2015:2052). A study conducted by Bennett *et al.* (2008:1345) established that emu oil has antioxidant properties, as

well as a protective role against oxidative damage, which can be attributed to the minor components in the non-triglyceride fraction of the oil and the greater ratio of UFA and SFA, respectively. Compared to the other oils, emu oil is the richest in linoleic acid (Table C.7). Formulations enrich with linoleic acid have been linked to an increase in the brightness and softness of skin (Sousa *et al.*, 2018:7).

C.4 Formulation of emulsions

Through pre-formulation studies, an optimised formula and method was selected (as discussed in Appendix B). The aim of this study was to formulate **(NOE)** containing the female hormones, and to determine if the female hormones could be delivered topically. In this study, the optimised formula of **(OEW2)** (as selected in Appendix B) was utilised to formulate the **(NOE)**, each containing a respective natural oil. The formula of the **(NOE)** is outlined in Table C.9. Note that dimethyl isosorbide is substituted by the natural oils, respectively.

Table C.9: Formula of the **(NOE)** (100 ml)

	Ingredient	%w/v	Function
A	Stearic acid	4.0%	Thickening agent
	Cetyl alcohol	0.5%	Thickening agent
	Crodafos™ MCK	1.7%	Emulsifier
	Span® 60	0.4%	Emulsifier
B	Natural oil*	13.0%	Penetration enhancer and oil phase
	PEG 400	5.0%	Humectant
	Oestriol	2.0%	Active
	Oestradiol	0.2%	
	Progesterone	2.5%	
C	Glycerine	10.0%	Humectant
	Tween® 80	3.2%	Hydrophilic surfactant
	Veegum®	0.5%	Thickening agent
	dH ₂ O	60.0%	Water phase and solvent

* Coconut-/castor-/emu oil

A step-wise demonstration of the method used to formulate the **(NOE)** is shown in Figure C.1. The full method, as described in Section B.4.1.2, was utilised for the preparation of **(CoOE)**, **(CaOE)** and **(EOE)**.

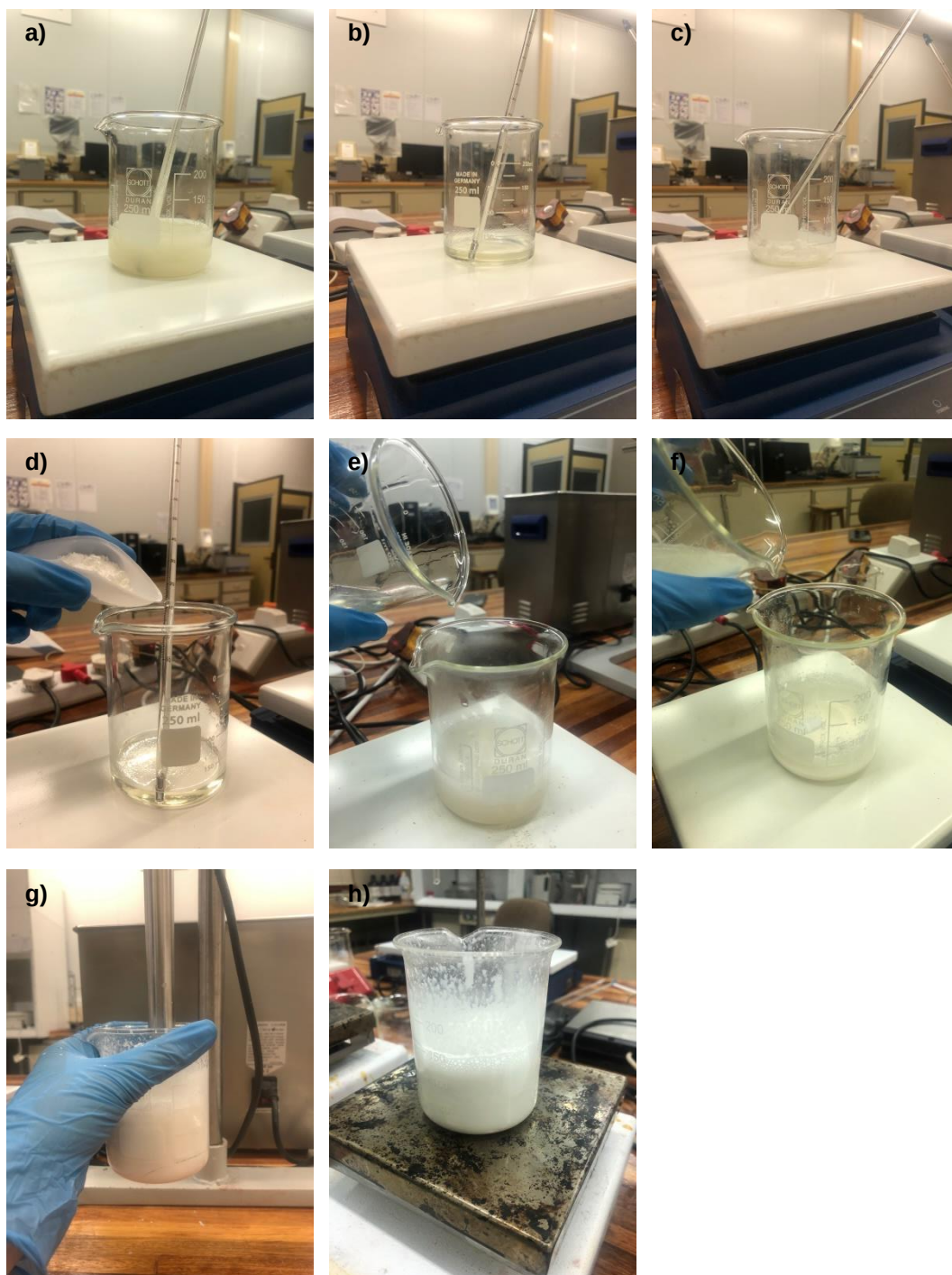


Figure C.1: Formulation method of the optimised emulsion: a) Phase C preheated; b) Phase B preheated; c) Phase A preheated; d) addition of female hormones to preheated Phase B; e) Phase B added drop wise to Phase C to form o/w emulsion; f) emulsions added to Phase A to form cream base; g) homogenised at 9 500 rpm for 5 min; h) slowly stirred till formulation reaches room temperature

C.5 Outcome

The formulation design for the **(NOE)** was greatly influenced by two major aspects, i.e. the purpose of the formulations and the desired site of action. The aim was to formulate simple semi-solids containing female hormones. Consequently, a cream was formulated for each of the selected natural oils by utilising the optimised formula selected in Appendix B. The **(NOE)** resulted in smooth homogenous creams that were white in colour (Figure C.2). From visual examination of the flow properties, **(CaOE)** presented with a lower viscosity compared to **(CoOE)** and **(EOE)**. The **(NOE)** were characterised to establish whether the formulations were suitable for topical delivery; these characteristics were then compared to evaluate the influence of the various oils on the overall properties of the formulation, as well as on the penetration ability of the female hormones.

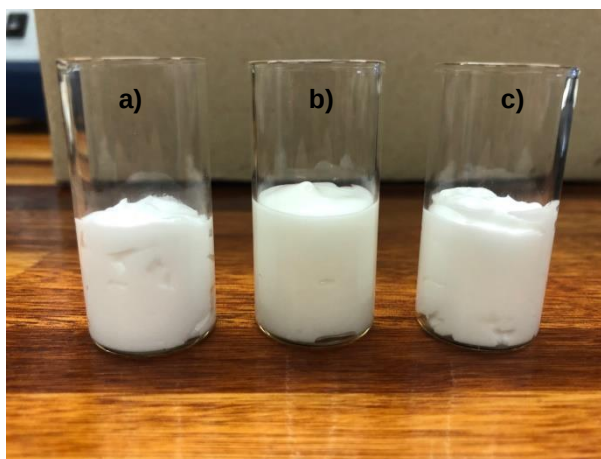


Figure C.2: The formulated **(NOE)**: a) **(CoOE)**; b) **(CaOE)** and c) **(EOE)**

C.6 Characterisation of emulsions

As previously mentioned in Section C.4, the optimised formula (selected in Appendix B) was used to formulate emulsions for each of the selected natural oils. Characterisation had to be performed on these emulsions (**(CoOE)**, **(CaOE)** and **(EOE)**) in terms of:

- pH;
- zeta-potential;
- particle size and distribution, and
- viscosity.

Hung *et al.* (2007) stated that the oil component could significantly influence the physicochemical properties of an emulsion and should therefore be investigated. These physicochemical characteristics, along with others (i.e. the partition coefficient of the active, the

interfacial tension between the two phases and the formulation rheology), can have a significant influence on the effective release of actives (Valentine, 2014:153).

C.6.1 pH

The average pH values obtained for the **(NOE)** were within the required range for topical application (Barry, 2002:512). A Mettler Toledo® pH meter (Mettler Toledo, United States of America) equipped with a Mettler Toledo® InLab® 410 electrode (Mettler Toledo, United States of America) (Figure B.5) was used for the determination. Prior to measuring the pH, the electrode was calibrated using Mettler Toledo® pH buffer solutions at several pH values (4, 7 and 10). Measurements were performed in triplicate to determine the average pH value. Yang *et al.* (2007:285) stated that a higher pH provides more stability to emulsions. Although the stability of the emulsions was not investigated during this study, it is worth mentioning that the emulsions could experience a decrease in pH due to the hydrolysis of fatty acids esters (Bernardi *et al.*, 2011:47).

Table C.10: Average pH values of the respective **(NOE)**

O/w emulsion	pH			
	Reading 1	Reading 2	Reading 3	Average
(CoOE)	7.00	7.00	6.24	6.75 ± 0.358
(CaOE)	7.01	6.34	6.38	6.58 ± 0.307
(EOE)	6.30	6.29	6.31	6.30 ± 0.008

The average pH value of the optimised formula selected in Appendix B, increased considerably when formulated with natural oils. Both plant-derived oils (**(CoOE)** and **(CaOE)**) presented with slightly higher pH values compared to the oil derived from animal fat (**(EOE)**). Emu oil is high in PUFA, which are more susceptible to oxidation, consequently decreasing the pH (Shimizu & Nakano, 2003:58; Teh & Mah, 2018:1385).

C.6.2 Zeta-potential

The electrokinetic potential in colloidal systems can be defined as the zeta-potential, which measures the effective electric charge on the surface of particles (Gumustas *et al.*, 2017:86; Selvamani, 2019:438). Higher zeta-potential values (positive or negative) are associated with higher stability (Sun *et al.*, 2016:8525). Numerous studies reported the following outcomes in relation to zeta-potential and the stability of an emulsion (Mahbubul, 2019:77):

- Values above + 60 mV and below - 60 mV are indicative of excellent stability;
- values above + 30 mV and below - 30 mV are indicative of physical stability;

- values above + 20 mV and below - 20 mV are indicative of limited stability, and
- values lower than 5 mV are indicative of agglomeration.

A Malvern Zetasizer Nano ZS (Malvern Instruments, United Kingdom) (Figure B.6) was utilised to determine the zeta-potential of each **(NOE)** by using PCS, which measures the scattered light fluctuation between dispersed particles (Gaur *et al.*, 2014:40). Samples of each **(NOE)** were prepared by diluting 20 mg of the formulation in a glass beaker with 30 ml Milli-Q[®] water. To ensure proper dissolution, the glass beakers were individually wrapped with a piece of Parafilm[®] and placed in an ultrasonic bath for 15 min. Thereafter, 2 ml of each dilution was injected into a clear disposable zeta-cell (DTS1070 folded capillary cell) with a syringe. The measurement was taken in triplicate on the day of formulation.

Table C.11: Average zeta-potential (mV) of the respective **(NOE)**

O/w emulsion	Zeta-potential (mV)			
	Reading 1	Reading 2	Reading 3	Average
(CoOE)	- 100.0	- 96.6	- 98.0	- 98.2 ± 1.71
(CaOE)	- 90.6	- 91.9	- 90.3	- 90.9 ± 0.85
(EOE)	- 78.7	- 75.4	- 78.5	- 77.5 ± 1.85

From Table C.11, it can be observed that all three emulsions presented with highly negative zeta-potential values below - 60 mV. Therefore, these emulsions are considered to be extremely stable with greater repulsion forces between the particles compared to that of attraction, consequently hindering aggregation (Silva *et al.*, 2012:860; Sun *et al.*, 2016:8525). It can be stated that the incorporation of natural oils into the oil phase increased the stability of the optimised formula selected in Appendix B. **(CoOE)** is considered to be the most stable emulsion, which can be explained by the small concentrations of UFAs compared to SFAs present in coconut oil (Maszewska *et al.*, 2018:1746). Ogiso *et al.* (2001:49) stated that API flux and diffusion improve with negatively charged molecules, therefore all three **(NOE)** will display enhanced skin permeation (Duangjit *et al.*, 2011:6). Due to the lower zeta-potential, **(EOE)** should penetrate the skin to a lesser extent.

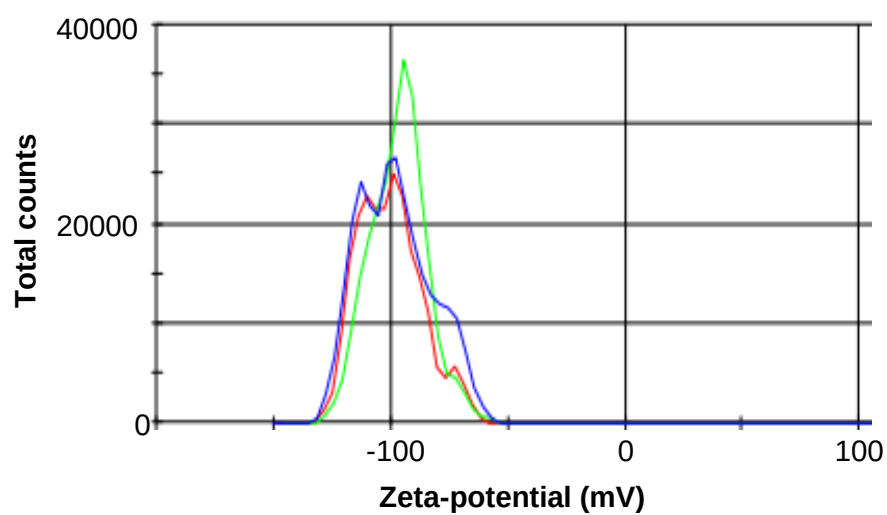


Figure C.3: Average zeta-potential (mV) of **(CoOE)**

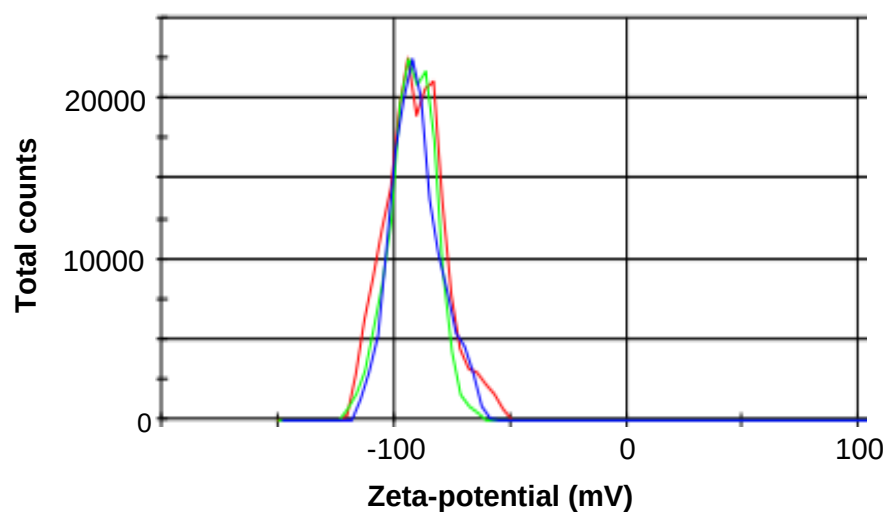


Figure C.4: Average zeta-potential (mV) of **(CaOE)**

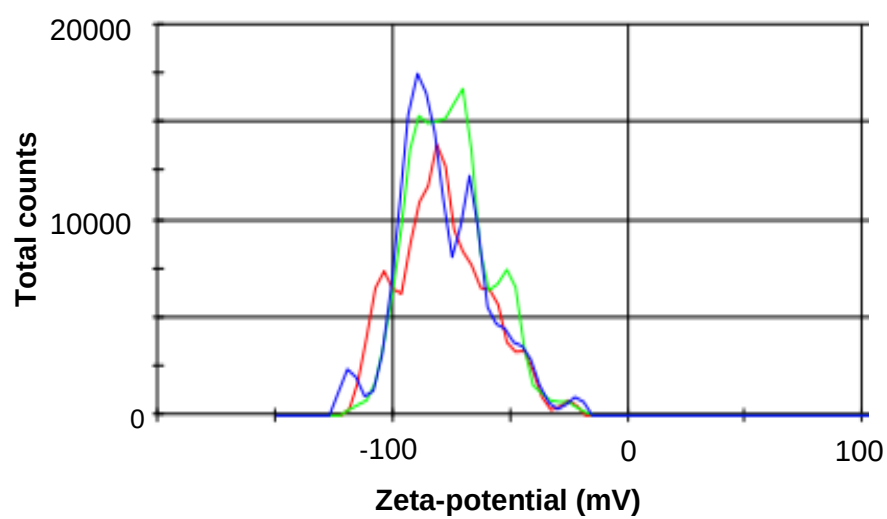


Figure C.5: Average zeta-potential (mV) of **(EOE)**

Figures C.3 – C.5 demonstrate the distribution curves for each **(NOE)**. As previously stated, all the **(NOE)** displayed an average zeta-potential value below - 60 mV, which indicates excellent stability and favourable skin permeation. The symmetrical curve displayed by **(CaOE)** indicates a narrow data distribution, whereas the asymmetrical curve displayed by both **(CoOE)** and **(EOE)** shows a poor distribution of data due to the deviation between the triplicate measurements. Regardless of the broader distribution of data, **(EOE)** appeared to be more ideal than **(CoOE)** and **(CaOE)** due to the decreased skin permeation when applied topically, since elevated circulating concentrations of female hormones have been shown to increase the occurrence of breast cancer in postmenopausal women (Kaaks *et al.*, 2005:1071; (Key *et al.*, 2011:709; Missmer *et al.*, 2004:1862).

C.6.3 Particle size and distribution

LD is a non-aerodynamic optical method used to determine particle size and distribution of dispersed particulate samples (Farina, 2010:270). During this characterisation test, the particles are illuminated in a laser beam, creating scattered light patterns (ATA Scientific Instruments, 2019). As a general rule, smaller particles will create wide angle scattering (ATA Scientific Instruments, 2019; Malvern Instruments Limited, 2015). Therefore, LD was used to determine the particle size and distribution of the **(NOE)** by utilising a Malvern Mastersizer 2000 Particle Size Analyser (Malvern Instruments, United Kingdom) connected to a Hydro 2000SM Dispersion Unit (Malvern Instruments, United Kingdom) (Figure B.10).

Particle size provides useful information on various dispersion characteristics, which includes the dissolution rate, viscosity and stability (Malvern Instruments Limited, 2015). The average particle size of coarse (macromolecule) dispersions is above 500 nm (Buchmann, 2001:150). According to Buchmann (2001:161), dispersions that present with small particle size with similar electrical charge are more kinetically stable and resistant to flocculation. The span value expresses the dispersity of the particles within the aqueous phase, therefore how uniform the particle sizes are distributed in the emulsion (Alloid *et al.*, 2018:78). Monodispersed emulsions are characterised by lower span values, whereas polydispersed emulsions are characterised by higher span values (De Sousa Lobato *et al.*, 2013:3908; Dyankova *et al.*, 2016:5). A dilution was prepared for each of the respective emulsions (**(CoOE)**, **(CaOE)** and **(EOE)**) as described in Section B.5.3. Small volumes of the sample were added to the Hydro 2000SM Dispersion Unit until the obscuration was within the acceptable range (10 – 20%) (Malvern Instruments, 2007). The refractive index and pump speed were set at 1.658 and 1 200 rpm, respectively. Triplicate measurements were performed to determine the average particle size for each emulsion. Tables C.12 and C.13 display the average particle size and span value obtained for each **(NOE)**.

Table C.12: Average particle size (μm) of the respective **(NOE)**

(NOE)	Particle size (μm)			
	Reading 1	Reading 2	Reading 3	Average
(CoOE)	14.400	14.239	14.064	14.23 ± 0.137
(CaOE)	12.500	9.948	8.641	10.36 ± 1.603
(EOE)	14.074	13.642	13.650	13.79 ± 0.202

Table C.13: Average span value of the respective **(NOE)**

(NOE)	Span			
	Reading 1	Reading 2	Reading 3	Average
(CoOE)	2.285	2.299	2.275	2.286 ± 0.010
(CaOE)	3.964	4.678	5.288	4.643 ± 0.541
(EOE)	2.804	2.704	2.724	2.744 ± 0.043

From Table C.12 it can be observed that all three **(NOE)** obtained an average particle size above 500 nm and can therefore be classified as coarse dispersions (Buchmann, 2001:150). Compared to the results obtained by **(OE2)**, the incorporation of natural oils resulted in a significant decrease in the average particle size ($47.226 \pm 0.5236 \mu\text{m} > 14.23 \pm 0.137 \mu\text{m}$), therefore preparing a more stable emulsion (Ramin *et al.*, 2009:11). A study conducted by Luo *et al.* (2011:42) found that the use of castor oil in dispersions decreased the particle size. Another study found that a stable emulsion with small average particle size could be formulated using coconut oil in combination with a surfactant (Pengon *et al.*, 2018:409). Smaller particle size is associated with increased skin penetration (Adib *et al.*, 2016:32). Therefore, the higher average particle size of **(CoOE)** is more favourable when decreased skin permeation and systemic absorption is desired. Compared to **(OE2)**, the span value increased, indicating that a more polydispersed emulsion was formulated with the use of natural oils. The high span value obtained by **(CaOE)** is indicative of a polydispersed emulsion, which is evident in the bimodal distribution displayed in Figure C.7. Both **(CoOE)** and **(EOE)** obtained moderately low span values, which represented more monodispersed emulsions with higher stability (Ramin *et al.*, 2009:11).

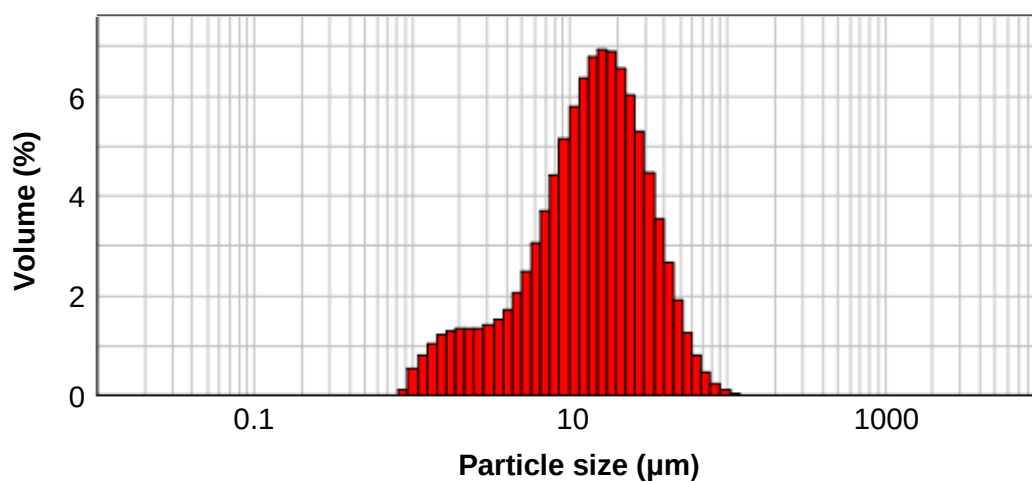


Figure C.6: Average particle size distribution for **(CoOE)**

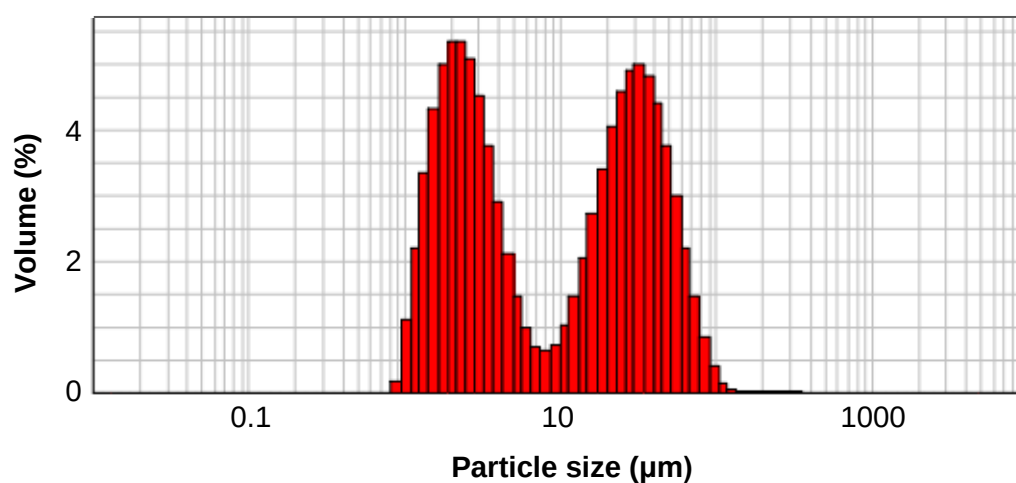


Figure C.7: Average particle size distribution for **(CaOE)**

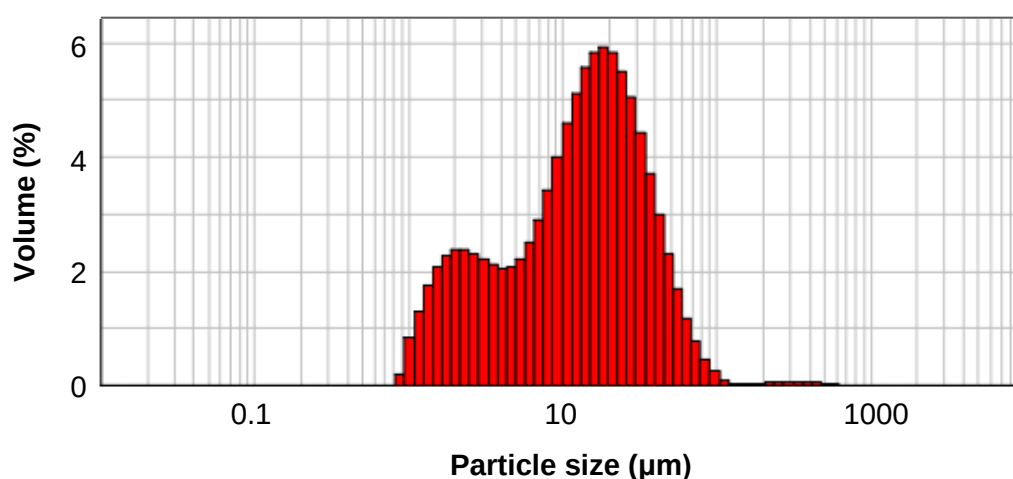


Figure C.8: Average particle size distribution for **(EOE)**

Figures C.6 – C.8 display the distribution curves obtained for each **(NOE)**. Various physicochemical properties (i.e. rheology, optical characteristics and stability) depend on the

particle size distribution of a formulation (Khor *et al.*, 2014:9190). All the **(NOE)** displayed bimodal particle size distributions, which is indicative of two modes (Rámirez *et al.*, 2002:311). The mode describes the particle size most frequently found in the distribution (Dyankova *et al.*, 2016:5). Bimodal particle size distribution represents unstable emulsions (Eliot *et al.*, 2003:235). As shown in Figures C.6 – C.8, all the **(NOE)** were polydispersed, which is an expected characteristic for o/w emulsions. In conclusion, **(CaOE)** presented with the smallest average particle size, which is more favourable for topical drug delivery. However, it also displayed the highest span value, which is indicative of an unstable emulsion; maintaining physicochemical integrity and suitable stability within the formulation could be challenging.

C.6.4 Viscosity

Viscosity indicates a fluid's resistance to flow, which is influenced by temperature, as well as the characteristics and concentration of the solutes (Katyal & Morrison, 2007:515). During this study, various natural oils were substituted with dimethyl isosorbide into the oil phase of the optimised formula selected in Appendix B. A Brookfield Viscometer DV2T LV Ultra (Middleboro, United States of America) (Figure B.14), coupled to a thermostatic water bath set at a constant temperature of $\pm 25\text{ }^{\circ}\text{C}$, was utilised to determine the viscosity of each emulsion. A sample of each **(NOE)** was placed in the water bath to acclimatise 60 min prior to testing. T-bar spindles, in cooperation with the Helipath Stand accessory, enable the determination of viscosity of formulations that are slow-flowing or non-flowing, i.e. gels, pastas and creams. The results are considered “apparent” as the unique geometry of the T-bar spindle excludes the calculation of shear stress or shear rate (Brookfield Engineering Laboratories, 2014:5). For this study, a T-E spindle was immersed directly into the sample and fitted to the viscometer. The viscosity of **(CoOE)**, **(CaOE)** and **(EOE)** was then measured at a spindle rotation speed of 30 rpm. Rheocalc T1.2.19 software was pre-programed to read and collect multipoint data at 10 s intervals for 2 min.

The full scale range (FSR) describes the maximum viscosity that can be measured by the spindle/speed combination (Brookfield Engineering Laboratories, 2016:2). Equation C.1 can be used to determine the full scale viscosity range (cP) of T-bar spindles (Brookfield Engineering Laboratories, 2018:3):

$$FSR\text{ (cP)} = \frac{\text{Spindle coefficient}}{\text{Spindle speed}} \quad \text{Equation C.1}$$

where the (FSR) (cP) of the selected T-E spindle on a LV Series at 30 rpm is:

$$FSR\text{ (cP)} = \frac{468\,000}{30} = 15600\text{ cP}$$

The minimum viscosity range (MVR) describes 10% of the FSR. Equation C.2 can be used to determine the MVR of the same spindle/speed combination.

$$MVR (cP) = \frac{\text{Full scale range}}{10} \quad \text{Equation C.2}$$

where the MVR (cP) of a T-E spindle on a LV Series at 30 rpm is:

$$MVR (cP) = \frac{15\,600}{10} = 1560 \text{ cP}$$

By utilising Equations C.1 and C.2, it was determined that the T-E spindle used to determine the viscosity of the **(NOE)** obtained values within the fixed range. The average viscosity value for each **(NOE)** is listed in Table C.14.

Table C.14: Average viscosity (cP) and torque (%) measurements of the respective **(NOE)**

O/w emulsion	Viscosity (cP)	Torque (%)
(CoOE)	3734.42 ± 11.975	23.96 ± 0.076
(CaOE)	2438.83 ± 61.227	15.61 ± 0.393
(EOE)	3334.58 ± 84.150	21.34 ± 0.539

From Table C.14 it is clear that the incorporation of natural oils resulted in a notable decrease in the viscosity of the optimised formula **(OEW2)** (9471.2 ± 251.6 cP). It can also be noted that while the same quantity (13 g) of natural oil was incorporated into each respective **(NOE)**, the viscosity measurements varied considerably. Yalcin *et al.* (2012:183) proposed a correlation between viscosity and the concentration of MUFAs and PUFAs present in oil, where the viscosity decreases with an increasing amount of PUFAs and a decreasing amount of MUFAs. The high viscosity value demonstrated by **(EOE)** can be attributed to the high amount of oleic acid present in emu oil. A study conducted by Nouredini *et al.* (1992:1190) reported that oleic acid demonstrated higher viscosity values at various temperatures compared to myristic, lauric, palmitic and stearic acid. Fatty acids present in natural oils each contributes to the total viscosity of the emulsion, where long chain SFA present with higher viscosity compared to short chain SFA or long chain UFA (Devi *et al.*, 2016:143). From Table C.7 it can be observed that coconut oil contains the highest concentration of long chain SFA compared to castor and emu oil. Due to the fatty acid composition of coconut oil, **(CoOE)** displayed the highest viscosity. The low viscosity of **(CaOE)** can be attributed to the fatty acid composition of castor oil, which contains extremely low concentrations of long chain SFA and oleic acid (Maia *et al.*, 2012:2351). The torque ranged between 15.61 – 23.96%.

Figures C.9.a – C.9.c show the change in viscosity as a function of time for each **(NOE)**. Both **(CaOE)** and **(EOE)** displayed thixotropic properties, since the viscosities decreased under

constant shear rate (rpm) (Brookfield Engineering laboratories, 2018:16; Hurst *et al.*, 2015:940), whereas **(CoOE)** displayed constant viscosity throughout the duration of the test.

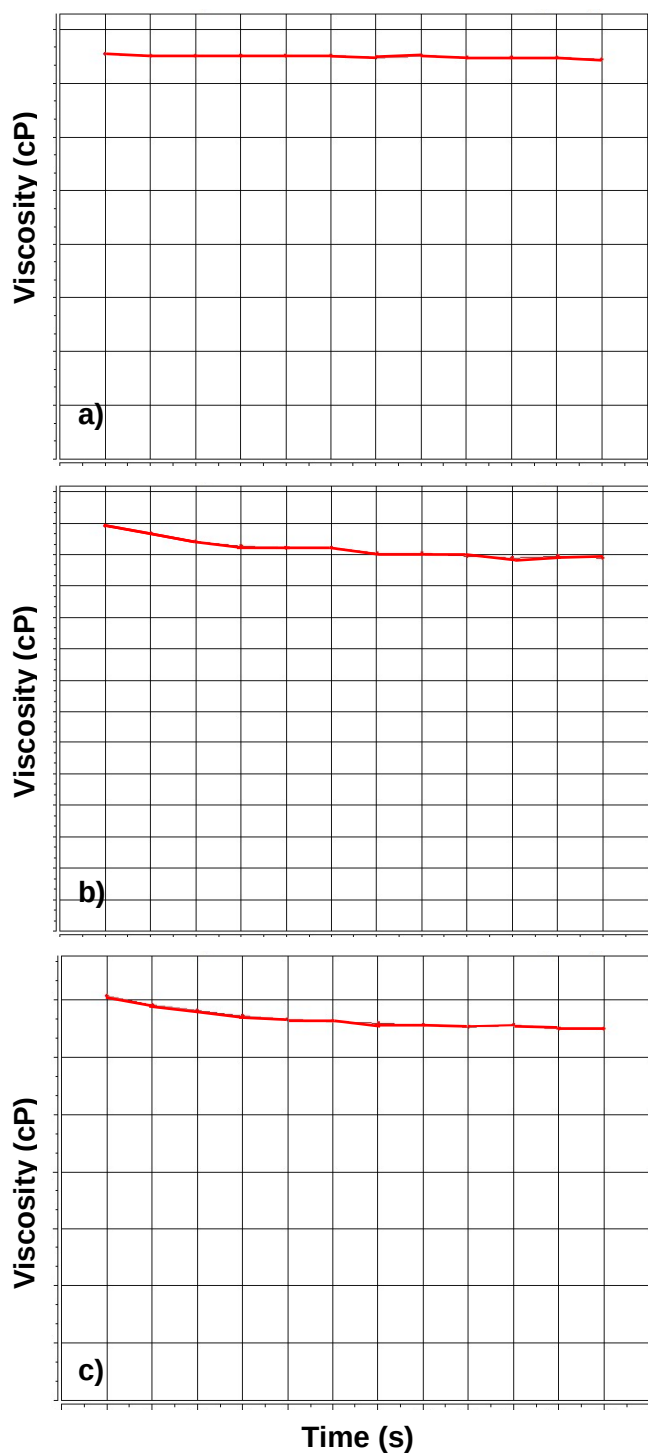


Figure C.9: Viscosity data graph obtained for: a) **(CoOE)**, b) **(CaOE)** and c) **(EOE)**

C.7 Conclusion

During this study, various tests were performed to analyse and characterise the **(NOE)**, each containing 2.0% (w/v) oestriol, 0.2% (w/v) oestradiol and 2.5% (w/v) progesterone. A summary of the characteristics are tabulated in Table C.15.

Table C.15: Summary of the characteristics of **(NOE)**

Formula	pH	Zeta-potential (mV)	Particle size (µm)	Viscosity (cP)
(CoOE)	6.75 (± 0.358)	- 98.2 (± 1.71)	14.23 (± 0.137)	3734.42 (± 11.975)
(CaOE)	6.58 (± 0.307)	- 90.9 (± 0.85)	10.36 (± 1.603)	2438.83 (± 61.227)
(EOE)	6.30 (± 0.008)	- 77.5 (± 1.85)	13.79 (± 0.202)	3334.58 (± 84.150)

From Table C.15 it can be observed that all the **(NOE)** displayed pH values that were within the required range for topical use (3 – 9). It is essential to determine the pH of topical formulations, since values outside the range could cause skin irritation or damage (Barry, 2002:512; Barry, 2007:576). Generally, higher pH contributes more stability to a formulation (Yang *et al.*, 2007:285). Therefore, **(CoOE)** is considered to be more stable compared to the other emulsions.

The incorporation of selected natural oils produced stable emulsions, which is indicated by the highly negative zeta-potential values (Mahbubul, 2019:77; Silva *et al.*, 2012:860). All three emulsions displayed values below - 60 mV, which indicate excellent stability (Mahbubul, 2019:77). Negatively charged formulations are also associated with increased skin permeability (Duangjit *et al.*, 2011:6). When comparing the zeta-potential values between the emulsions, **(CoOE)** exhibits more favourable skin permeability and stability due to the highly negative value. If postmenopausal women (prone to procure breast cancer) were to apply the **(NOE)**, the lower zeta-potential of **(EOE)** would be more favourable as it would not penetrate the skin as rapidly and efficiently as **(CoOE)**, although **(EOE)** still displays excellent stability (> - 60 mV).

Particle size influences various physicochemical properties of an emulsion, including the viscosity, effective drug delivery, appearance and stability (Malvern Instruments Limited, 2015). The smaller average particle size of **(CaOE)** is indicative of efficient skin permeation and stability. Conversely, the larger average particles of **(CoOE)** will be more favourable for limited skin penetration. The lower span values of **(CoOE)** and **(EOE)** represent more monodispersed formulations compared to **(CaOE)**, which provides better stability against aggregation, as well

as uniform drug distribution when applied topically (Buchmann, 2001:159; Liu *et al.*, 2010:75; Ramin *et al.*, 2009:11). Uniform drug distribution is essential in topical formulations as it ensures constant and even delivery of therapeutically effective drug concentrations (Garg *et al.*, 2002:84; Nair *et al.*, 2013:421; Saettone *et al.*, 2000:315).

Yalcin *et al.* (2012:181) reported a correlation between viscosity and the MUFA and PUFA composition of oils. Due to the varied fatty acid composition of the selected natural oils (Table C.7), it was expected to obtain different viscosity results for each **(NOE)**. The viscosity of a formulation plays an important role in skin permeation; as viscosity decreases, permeability increases (Welin-Berger *et al.*, 2001:309). Thus, if the focus was to formulate an emulsion that did not increase skin permeability of the female hormones (due to breast cancer), the **(NOE)** with the higher viscosities (**(CoOE)** and **(EOE)**) would be more favourable.

Considering all the characteristics, it was apparent that **(EOE)** exhibited more ideal properties with no visible aggregation or sedimentation. *In vitro* diffusion studies were performed on all three **(NOE)** to compare the simple formulations formulated by the student with the complex Idexis formulations with regard to the topical delivery of the respective hormones. The amount of oestriol, oestradiol and progesterone released from the formulations, along with its penetration depth within the skin layers were investigated (as discussed in Appendix E).

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APPENDIX D:

STABILITY TESTING OF TOPICAL PRODUCTS CONTAINING SELECTED FEMALE HORMONES AND ANTIOXIDANTS

D.1 Introduction

Stability testing forms an essential part of the testing programme for cosmetic and pharmaceutical products, as the instability of formulations modify the three primary specifications, i.e. efficacy, quality and safety (Baby *et al.*, 2007:406; Bilia *et al.*, 2001:200). The characteristics of formulations can be influenced by various environmental factors, including pH, light, temperature and humidity, which can result in phase separation, sedimentation, colour, odour and viscosity changes, microbial growth and loss of activity (Bouranen, 2017:19; Dao *et al.*, 2017:74; Stephane, 2015:1; Rodrigues Ueoka & Moraes, 2018:26). The principle of stability studies is to provide data on how the quality of the product, in the selected marketing container, varies with time under the influence of a variety of environmental factors, for example humidity and temperature (ICH, 2003:1; Niazi, 2004:7). Furthermore, stability studies are used to demonstrate the stability limits of a product, to test the estimated shelf-life and determine the ideal storage conditions at which the product meets the chemical and physical quality standards (ANVISA, 2004:21; COLIPA, 2004:1; Patel & LoBrutto, 2007:716; WHO, 1996:68). Therefore, the evaluation of product stability is essential to ensure the efficacy, quality and safety of the products with potential for economic success (Bouranen, 2017:19).

Shelf-life can be defined as the period of time during which a product, if stored under the recommended conditions, is expected to display no decline in quality or acceptability (Dube, 2015:13; WHO, 1996:67). Real time (long-term) stability testing involves storing the products under climatic conditions that mimic those it is expected to encounter in the proposed market (Dube, 2015:13; WHO, 1996:67). The data is used to determine the shelf-life, confirm the estimated shelf-life and recommend storage conditions (WHO, 1996:67). While shelf-life determination is practical for short shelf-life products, it demands an unreasonable amount of time for long shelf-life products (Dube, 2015:13). Therefore, accelerated stability testing is performed to overcome this problem, and is designed to accelerate the chemical degradation and physical changes of a product by using elevated storage conditions (i.e. temperature and humidity). The data is compared to the real time stability results to evaluate the effect of short term excursions outside the ideal storage conditions, as it might occur during transport and use (WHO, 1996:66).

The packaging of a pharmaceutical or cosmetic product must ensure adequate stability of the formulation throughout the shelf-life by providing protection against various climatic conditions

(i.e. temperature and humidity) and physical, chemical and biological hazards (Briasco *et al.*, 2016:32; Kunal *et al.*, 2012:1282). In the pharmaceutical and cosmetic packaging field, the most commonly used plastic material is polyethylene, a thermoplastic resin produced through the polymerisation of ethylene (Briasco *et al.*, 2016:32; Encyclopaedia Britannica, 2017). While polyethylene exhibits excellent characteristics as packaging material, the plastic, as well as additives used in the manufacturing process, can migrate from the container into the product over time due to an increase in mechanical stress, temperature or ageing (Briasco *et al.*, 2016:33; WHO, 2018:323). Therefore, it is essential that stability studies include the intended container, since it can directly influence product stability due to interactions that can occur between the product, the container and the environment (COLIPA, 2004:4).

Standard stability tests cannot be applied to cosmetic products due to the wide range and inherent complexity of each product. However, according to COLIPA (2004:2), the stability test used for real time or accelerated stability must ensure the assessment of:

- stability and physical integrity of products under ambient conditions of transport, storage and use;
- microbiological stability;
- chemical stability, and
- the compatibility between the product and the proposed container.

According to the ICH (2003:9), a significant change in a product is defined as a 5% change in the API assay from its initial value, any degradation products greater than the acceptable criteria, failure to meet the acceptable criteria for physical attributes (i.e. phase separation, hardness and caking) and appearance (i.e. colour), and failure to meet the acceptable criteria for pH. For the purpose of this study, the stability of the provided Idexis products were evaluated by means of accelerated stability studies, which is a universally recognised test for the accurate prediction of product shelf-life in numerous industries (COLIPA, 2004:3).

D.2 Antioxidants used in Idexis products

Oxidative stress contributes significantly to skin ageing, especially extrinsic skin ageing (Gianeti & Maia Campos, 2014:18269; Rinnerthaler *et al.*, 2015:545), which is characterised by atypical pigmentation and wrinkles (Masaki, 2010:85). ROS are responsible for this phenomenon, while long term exposure to UV light reduces the antioxidant activity of the skin (Gianeti & Maia Campos, 2014:18269; Xu *et al.*, 2017:69). Antioxidants can be defined as molecules capable of reducing or inhibiting the oxidation of other molecules (Saljoughian, 2008) and can be divided into the following classes (CPMP, 2003:8; Srivastava, 2006:202):

- True antioxidant: These compounds react with free radicals, preventing the chain reaction.
- Reducing agents: These compounds have lower redox potentials and are more readily oxidised than the API they are protecting (Loftsson, 2014:87).
- Antioxidant synergists: These compounds enhance the activity of antioxidants.

Once a product is opened, it is susceptible to contamination caused by exposure to fluctuating temperatures (stored in consumer's bathroom) and consumer use (repeated dipping of non-sterile fingers into the product). Micro-organisms can easily proliferate and cause unpleasant odour and discolouration, as well as degrade the API in the product, leading to product instability. Skin care products are expected to resist microbial growth within their proposed period of use and shelf-life (Kerdudo *et al.*, 2016:1078), therefore, antimicrobial agents are required to prevent product degeneration and consequently infections caused by harmful microbes (CPMP, 2003:8; Halla *et al.*, 2018:1571; Kerdudo *et al.*, 2016:1078). Multiple studies have proposed that selected antioxidants exhibit antimicrobial activity (Hwang & Lim, 2015:10034; Reygaert, 2014:434).

Antioxidants are frequently added to prepare multifunctional skin care products that not only improve skin appearance, but also prevent skin damage caused by UV-induced oxidative stress by scavenging ROS (Bouranen, 2017:5; Gianeti & Maia Campos, 2014:18269; Ribeiro *et al.*, 2018:175). Furthermore, the addition of antioxidants can be used to extend the shelf-life of cosmetic and pharmaceutical products by hindering oxidation and reducing microbial proliferation (CPMP, 2003:10). The efficacy of an antioxidant depends on its characteristics, the stage at which the antioxidant is formulated into the final product, the type of container and the formulation (CPMP, 2003:8). The antioxidants incorporated within each Idexis formulation are shown in Table A.2.

D.2.1 Vitamin E acetate

Vitamin E, also referred to as α -tocopherol, is a lipid-soluble antioxidant that functions as the major chain-breaking factor controlling the oxidation of low-density lipoprotein (Allemann & Baumann, 2008:6; Princen *et al.*, 1995:325). There are eight active isoforms of vitamin E that are grouped into tocopherols (α -, β -, γ -, and σ) and the related tocotrienols, whereas α -tocopherol is the most abundant vitamin E derivative found in human skin and sera (Allemann & Baumann, 2008:6; Keen & Hassan, 2016:311; Konger, 2006:1448). Various experimental studies propose that topical vitamin E has photoprotective, antitumorigenic and skin barrier stabilising properties (Keen & Hassan, 2016:311; Thiele & Ekanayake-Mudiyanselage, 2007:646). Vitamin E acetate (VEA), which is the acetic ester of α -tocopherol and the most stable synthetic form of vitamin E, is widely used in many dermatological and cosmetic products

since it protects the skin from numerous unfavourable effects caused by solar radiation by acting as a potent free radical scavenger (Bidossi *et al.*, 2017:2; Cheng *et al.*, 2016:681; Keen & Hassan, 2016:311). A study led by Teo *et al.* (2010:7) found that VEA has stabilising properties in formulations. VEA containing formulations demonstrated greater stability compared to vitamin E (Nada *et al.*, 2012:1). Furthermore, Bidossi *et al.* (2017:1) stated that VEA displayed antimicrobial activity; however, the activity of vitamin E phosphate was greater.

D.2.2 Alpha lipoic acid

Alpha lipoic acid (ALA), also commonly referred to as thioctic acid, is a naturally occurring compound widely used in dermatological and cosmetic products due to its many favourable effects on the skin (Gomes & Negrato, 2014:80; Pagano *et al.*, 2019:35). Topically applied ALA penetrates the stratum corneum rapidly and can be found in the dermis and hypodermis within 4 h. The presence of ALA in the skin has been shown to provide antioxidant protection against UV-generated free radicals (Pagano *et al.*, 2019:35; Perricone, 2000:219). A study conducted by El-Komy *et al.* (2017:358) reported that the topical application of ALA is a safe and effective modality for improving facial skin ageing. ALA is both lipid and water soluble and can therefore move into all portions of the cell to neutralise free radicals (Perricone, 2000:219). Contrarily, a study conducted by Pagano *et al.* (2019:42) observed that the poor water solubility of ALA limited its incorporation within hydrophilic formulations. Furthermore, its dispersion within a hydrophilic base is not an appropriate formulation strategy, since the crystallinity degree experiences modifications at high relative humidity (RH) conditions. It is safe to presume that a similar modification should occur when free ALA crystals are dispersed within a hydrophilic base, which subsequently decreases product shelf-life.

D.2.3 Co-enzyme Q10

Co-enzyme Q10 (CoQ10), also known as ubiquinone, is an endogenous lipid soluble antioxidant that is essential for cellular energy production (Allemann & Baumann, 2008:6; Knott *et al.*, 2015:383; Liu *et al.*, 2016:85). Ubiquinone is referred to as “co-enzyme” due to its ability to partake in chemical reactions while still remaining at steady-state levels (Varela-López *et al.*, 2015:450). However, these levels decline with age and under the influence of external stressors, i.e. pollution, smoking and UVR (Clatici *et al.*, 2017:192; Knott *et al.*, 2015:383). Results obtained from a study led by Knott *et al.* (2015:383) proved that the topical treatment of CoQ10 has a beneficial effect on stressed skin by reducing free radicals and increasing antioxidant capacity. Furthermore, CoQ10 can exert its antioxidant activity indirectly by effectively regenerating vitamin E (Alkholy *et al.*, 2019:225; Varela-López *et al.*, 2016:375). Multiple studies have demonstrated the anti-ageing effects of CoQ10 at skin level (Blatt & Littaru, 2011:381; Prah *et al.*, 2008:245). There is a paucity of literature regarding the influence

of CoQ10 on product stability; however, according to Kulkarni & Shaw (2016:49), CoQ10 is added to protect formulation ingredients against oxidation.

D.2.4 Caffeine liposome

Caffeine is becoming an increasingly popular ingredient used in cosmetic products due to its high pharmacological activity and ability to penetrate the stratum corneum (Herman & Herman, 2013:8). Caffeine and its catabolic products, xanthine and theobromine, are fundamental components of coffee and tea (Azam *et al.*, 2003:325), which have been associated with numerous anti-ageing effects, such as limiting photodamage, decreasing skin roughness, reducing wrinkle formation and the appearance of crow's feet (Arantes *et al.*, 2018:7556; Rodriques *et al.*, 2016a:496). Multiple studies suggest that caffeine possesses antioxidant properties, which help protect the skin against UVR and decelerates the photoageing process by inhibiting oxidative reactions that cause damage (Choi *et al.*, 2010:283; Herman & Herman, 2013:8; Petrucci *et al.*, 2018:1782). Coffee silverskin, a coffee by-product produced during the roasting process, has a caffeine concentration that is 3.5 times lower than conventional coffee (Bessada *et al.*, 2018:5; Bresciani *et al.*, 2014:196). A study by Rodriques *et al.* (2016b:99) investigated the stability of coffee silverskin-containing hand cream and found that the formulation displayed stable physical characteristics independently of the climatic conditions; microbiological stability was also validated (Rodriques *et al.*, 2016b:99).

D.2.5 Green tea extract

Green tea is a popular beverage made from the leaves of the *Camellia sinensis* plant (Allemann & Baumann, 2008:7; Reygaert, 2014:1). There are four main polyphenolic catechins found in green tea, of which epigallocatechin-3-gallate is the most abundant and exhibits the highest antioxidant activity (Allemann & Baumann, 2008:7; Singh *et al.*, 2011:1807). Moreover, green tea polyphenols act as anti-inflammatory, antibacterial and anticarcinogenic agents (Allemann & Baumann, 2008:7; Chu *et al.*, 2017:1; Singhal *et al.*, 2017:108). Topically applied green tea polyphenols have been associated with anti-ageing effects by effectively scavenging ROS produced by UVR, consequently preventing photoageing, which causes skin roughness and sagging (Hsu, 2005:1055). A study led by Hong *et al.* (2013:137) investigated the effects of topically applied green tea extract (GTE) at the crow's feet of 42 Korean females for the duration of 8 weeks. The results showed that GTE has beneficial properties as an anti-ageing agent as 63.60% reported moderate improvement in the appearance of wrinkles (Hong *et al.*, 2013:137). Various studies have reported the stability of GTE formulations at different storage conditions (Mahmood & Akhtar, 2013:6; Ramadan *et al.*, 2018:66). Note that the eye cream in this study is the only product that contains GTE.

D.3 Stability studies

In the pharmaceutical industry, stability refers to the total storage time allowed before any degradation impurities in the formulation reaches a concentration that poses any risk to the consumer (Waterman & Adami, 2005:101). In accordance with the ICH Guidelines (2003:4), accelerated stability studies were performed for a minimum period of 3 months, since significant change has occurred in the Idexis products. The products tested included the **(FS)**, **(EC)** and **(MC)**. The concentrations of the female hormones and antioxidants present in each product are shown in Tables A.1 – A.2. Samples of each of the aforementioned products were weighed as follows for each storage condition:

- 3 x 20 g for stability testing each month, and
- 1 x 10 g for mass loss study (container was kept closed throughout the whole study).

The samples were weighed and packed in provided plastic containers. Thereafter, the samples were kept in Labcon incubators (Labcon Laboratory Equipment, South Africa), each at the following storage conditions (ICH, 2003:3):

- long-term (25 °C/60% RH);
- intermediate (30 °C/65% RH), and
- accelerated (40 °C/75% RH).

At predetermined time intervals (months 0, 1, 2 and 3), the samples were removed from the incubators and evaluated to determine the effect of different environmental factors on product stability. The physicochemical characteristics that were evaluated at each time interval included:

- Concentration assay;
- mass loss;
- visual appearance;
- pH;
- particle size and distribution;
- zeta-potential, and
- viscosity.

D.3.1 Concentration assay

The validated HPLC method, as described in Appendix A, was applied to analyse and quantify the concentration of each female hormone (oestriol, oestradiol and progesterone) in the

respective Idexis products during the stability testing. For the purpose of this study, an Agilent® 1200 Series HPLC system (Agilent Technologies, United States of America), equipped with an Agilent® 1200 gradient pump, autosampler injection mechanism and UV detector, was used to determine the concentration hormones in the **(FS)**, **(EC)** and **(MC)** at each predetermined time interval. The chromatographic conditions used during testing are listed in Section A.2.

Approximately 1 g of each sample, representative of each storage condition, was weighed into a 100 ml volumetric flask and made up to volume with methanol. This was done in triplicate for each sample. The flasks were placed into an ultrasonic bath for 10 min to ensure proper dissolution; thereafter, each sample was filtered through a 0.45 µm PTFE filter into amber auto sampler vials for HPLC analysis. A standard linear regression curve, obtained by injecting a standard solution (as described in Section A.3.1) at different injection volumes, was used in combination with the linear regression equation (Equation A.1) to analyse the data. The data was used to calculate the percentage concentration changes (relative to the initial assay results) in all the samples at all the storage conditions to assess the stability of the products against humidity and temperature over 3 months. The Idexis products used to determine stability were provided with limited information regarding the ingredients and concentrations used (as listed in Table A.1) due to the classified nature of this study. Keeping possible human error in mind at the compounding facility during formulation, the concentration of each hormone present in the various formulations was merely the estimated percentage and not specific to each individual cream/serum formulated. A slight difference in the stated concentration to the true value can influence the outcome of the concentration assay.

The percentage recovery (%) of each hormone in each product was calculated on the day of receipt and used as a reference value throughout stability testing. Polymers play a fundamental role with regards to solubilisation and crystallisation inhibition (Vaka *et al.*, 2014:138). The low initial %recovery obtained for oestriol, oestradiol and progesterone in all three products could be due to the polymer that was used, which consequently altered the solubility of the hormones and affected the initial drug recovery.

According to ICH Guidelines (ICH, 2003:9), none of the female hormones complied with the acceptable 5% variation limit over the 3-month period. From Tables D.1 – D.6 and Figures D.3 – D.11, it can be observed that the assay results displayed significant variations, which could have been due to numerous influences. Overall, there was an increase in the concentrations of oestriol, oestradiol and progesterone in the **(FS)**, **(EC)** and **(MC)** at all three storage conditions over the 3-month period. Bajaj *et al.* (2012:130) proposed that the loss of moisture/vehicle during storage could result in an increase in the API concentration. This could

possibly explain the concentration increase exhibited by the Idexis products, since mass loss was confirmed in all three products at all three storage conditions in Section D.3.2.

Moreover, challenges were encountered during HPLC analysis. During method validation, the provided products had completely dissolved in the selected solvent (methanol). However, during the testing of month 3, the samples failed to dissolve completely despite vigorous shaking and sonication for 3 h. Each prepared sample solution remained milky with visible lumps of the product settling at the bottom of the flask. Due to poor product solubility, the active ingredients were not fully dissolved, altering assay results. No possible reason could be recognised for the sporadic change in product solubility.

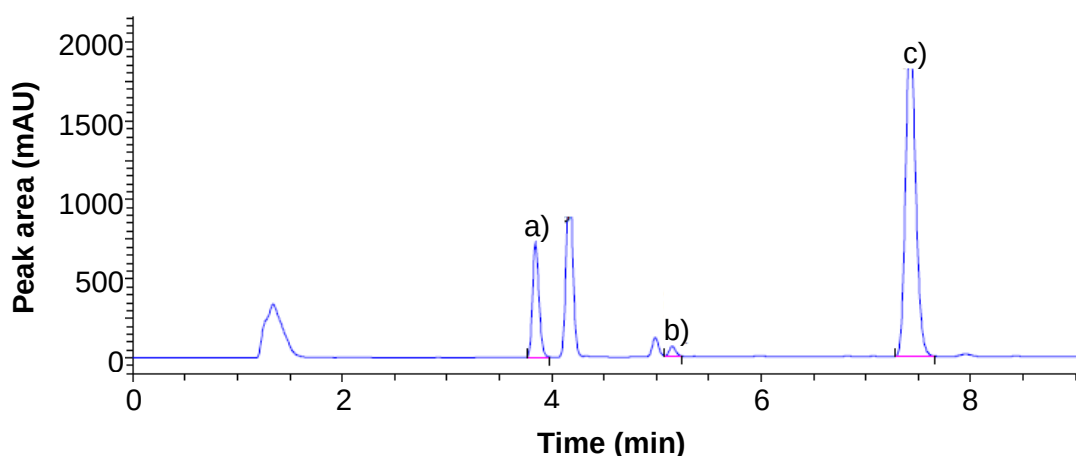


Figure D.1: Integrated chromatogram of: a) oestriol, b) oestradiol and c) progesterone following initial HPLC analysis

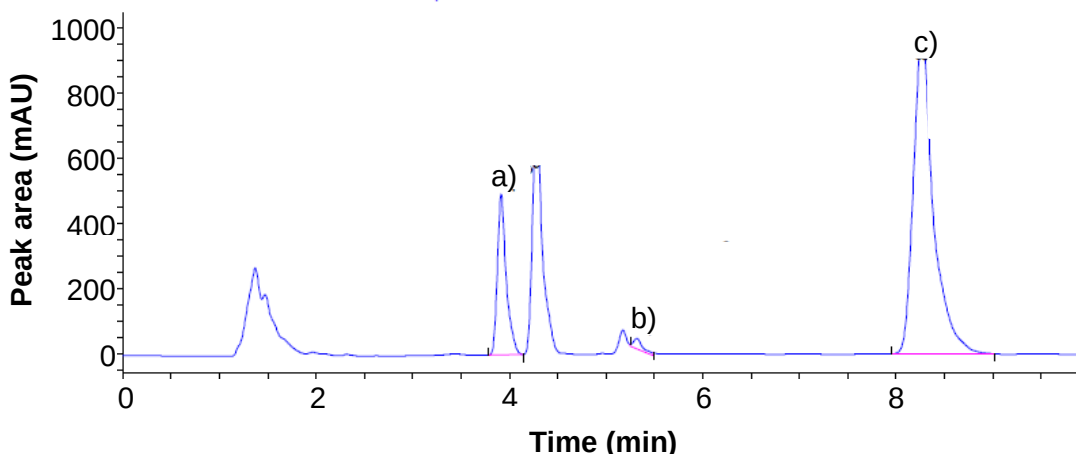


Figure D.2: Integrated chromatogram of: a) oestriol, b) oestradiol and c) progesterone following month 3 HPLC analysis

No additional peaks were detected on the chromatograms during HPLC analysis (Figure D.1). The validation elution times of all the female hormones remained unchanged during HPLC analysis. However, from month 3, the peak that eluted at 5.148 min, interfered with oestradiol, forming a split peak (Figure D.2). According to Rossi (2002:202), compound instability, whether arising from biotransformation or chemical degradation, can result in additional or split peaks. Therefore, it can be proposed that the unknown compound underwent sufficient degradation over the 3-month period, which resulted in the interference of the oestradiol peak. This made it difficult to integrate oestradiol accurately; consequently, no assay results were available for oestradiol for month 3.

More than a 5% increase of the hormone content with respect to initial concentrations was observed for **(FS)**, **(EC)** and **(MC)** after 90 days of storage. This increase in oestriol, oestradiol and progesterone concentrations of 5% and more could possibly indicate instability due to possible degradation products and impurities along with moisture loss. In some pharmaceutical preparations, there is little to no degradation over a certain period, and then it proceeds at a rapid rate. Lag time behaviour is often caused by reaction inhibitors (i.e. antioxidants), which effectively prevent the degradation of active ingredients until it is consumed, at which point oxidation can proceed (Waterman, 2008:124). Therefore, the increased concentrations displayed over time could be due to degradation as a result of insufficient protection from the numerous antioxidants used in the respective products (Table A.2).

D.3.1.1 Face serum

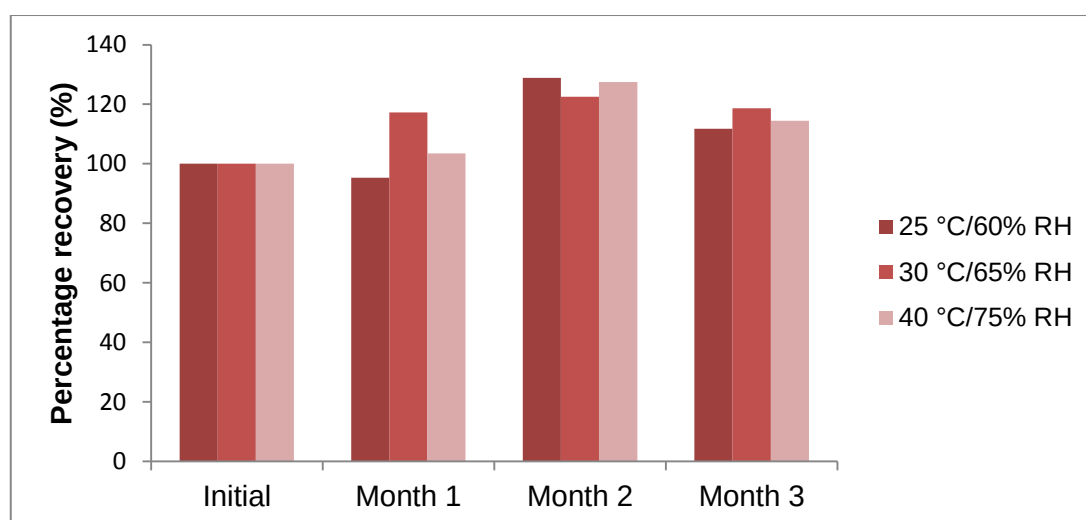
As stated by Idexis, the **(FS)** contains 2.0% oestriol, 0.2% oestradiol and 2.5% progesterone. Table D.1 displays the %recovery determined for each hormone following the initial HPLC analysis, as well as the recovery reference values (µg/ml) used throughout stability testing for the **(FS)**. Based on ICH Guidelines (2003:9), the concentrations of oestriol, oestradiol and progesterone in the **(FS)** showed significant changes at 25 °C/60% RH, 30 °C/65% RH and 40 °C/75% RH during storage. The change in percentage of each female hormone in the **(FS)** at the different storage conditions over the 3-month period is summarised in Table D.2 and illustrated in Figures D.3 – D.5. To conclude, the **(FS)** samples stored at 25 °C/60% RH, 30 °C/65% RH and 40 °C/75% RH were considered to be unstable as the assay results for oestriol, oestradiol and progesterone were outside the acceptable 5% variation limit after the 3-month period.

Table D.1: Recovery for each female hormone in (FS)

Hormone	Concentration ($\mu\text{g/ml}$)	Recovery	
		($\mu\text{g/ml}$)	%
Oestriol	212.34	74.26	34.97
Oestradiol	21.23	1.97	9.27
Progesterone	265.43	215.87	81.33

Table D.2: Percentage recovery (%) of each female hormone in the (FS) at the different storage conditions for each time interval

	Initial	Month 1	Month 2	Month 3
25 °C/60% RH				
Oestriol	100.00	95.30	128.90	111.80
Oestradiol	100.00	107.58	141.40	No data
Progesterone	100.00	78.05	105.33	112.77
30 °C/65% RH				
Oestriol	100.00	117.24	122.50	118.69
Oestradiol	100.00	134.99	133.41	No data
Progesterone	100.00	94.55	99.53	119.22
40 °C/75% RH				
Oestriol	100.00	103.50	127.43	114.43
Oestradiol	100.00	121.29	137.62	No data
Progesterone	100.00	84.52	103.21	114.26

**Figure D.3:** Percentage recovery (%) of oestriol in (FS) at different storage conditions at each time interval

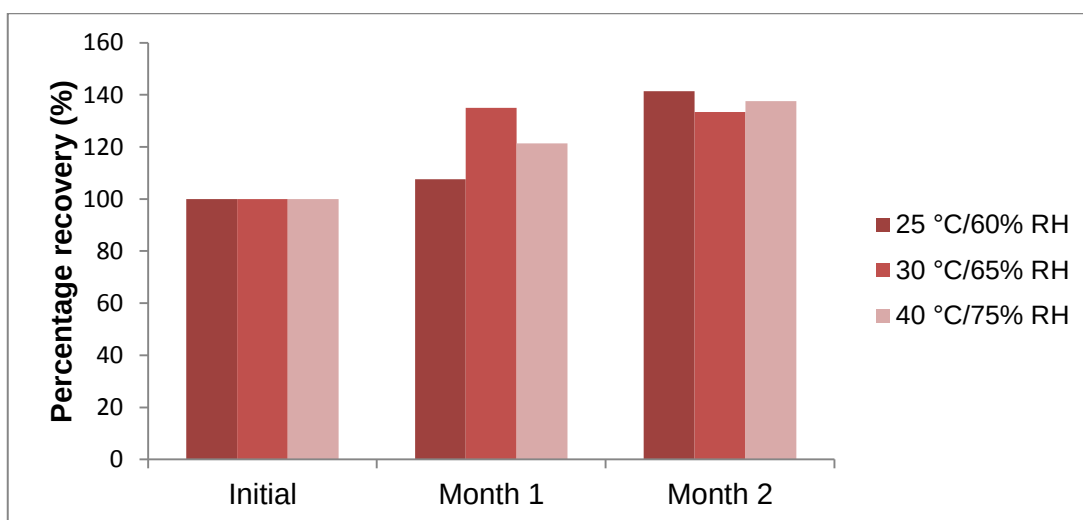


Figure D.4: Percentage recovery (%) of oestradiol in **(FS)** at different storage conditions at each time interval

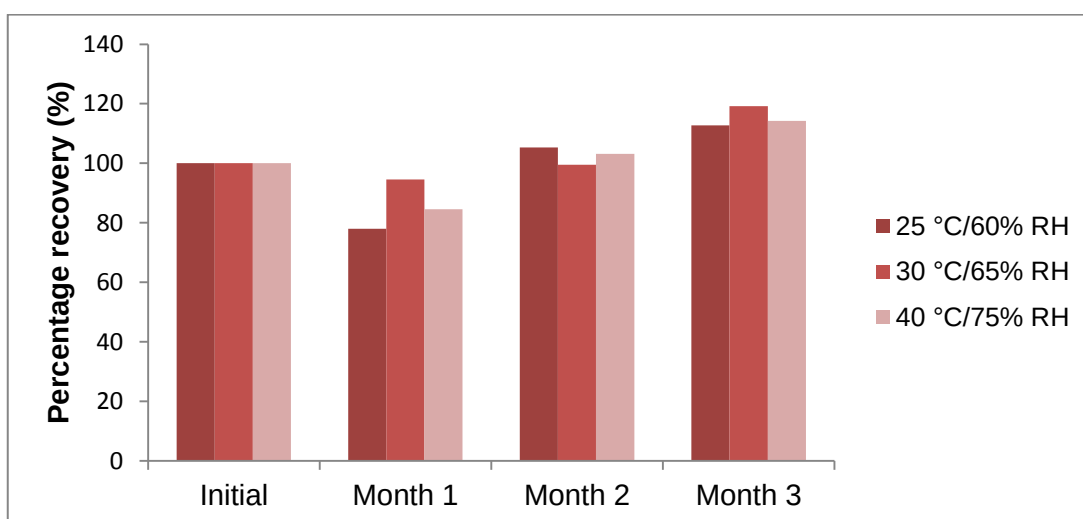


Figure D.5: Percentage recovery (%) of progesterone in **(FS)** at different storage conditions at each time interval

D.3.1.2 Eye cream

As stated by Ilexis, the **(EC)** contained 0.5% oestriol, 0.1% oestradiol and 2.0% progesterone. Table D.3 displays the %recovery determined for each hormone following the initial HPLC analysis, as well as the recovery reference values (µg/ml) used throughout stability testing for the **(EC)**. According to ICH Guidelines (2003:9), the concentrations of oestriol, oestradiol and progesterone displayed significant changes at all three storage conditions over the 3-month period. The change in percentage of each female hormone in the **(EC)** at the different storage conditions over the 3-month period is summarised in Table D.4 and illustrated in Figures D.6 – D.8. In conclusion, the **(EC)** samples stored at 25 °C/60% RH, 30 °C/65% RH and

40 °C/75% RH are considered to be unstable as the assay results for oestriol, oestradiol and progesterone were outside of the acceptable 5% variation limit after the 3-month period.

Table D.3: Recovery for each female hormone in (EC)

Hormone	Concentration (µg/ml)	Recovery	
		µg/ml	%
Oestriol	56.86	75.94	133.56
Oestradiol	11.37	2.06	18.12
Progesterone	227.43	228.80	100.60

Table D.4: Percentage recovery (%) of each female hormone in the (EC) at the different storage conditions for each time interval

	Initial	Month 1	Month 2	Month 3
25 °C/60% RH				
Oestriol	100.00	109.71	126.91	115.94
Oestradiol	100.00	105.37	104.63	No data
Progesterone	100.00	88.26	101.13	112.24
30 °C/65% RH				
Oestriol	100.00	119.69	131.49	119.41
Oestradiol	100.00	116.51	107.39	No data
Progesterone	100.00	95.98	105.06	115.20
40 °C/75% RH				
Oestriol	100.00	115.33	140.28	113.28
Oestradiol	100.00	110.40	118.56	No data
Progesterone	100.00	92.63	110.16	109.01

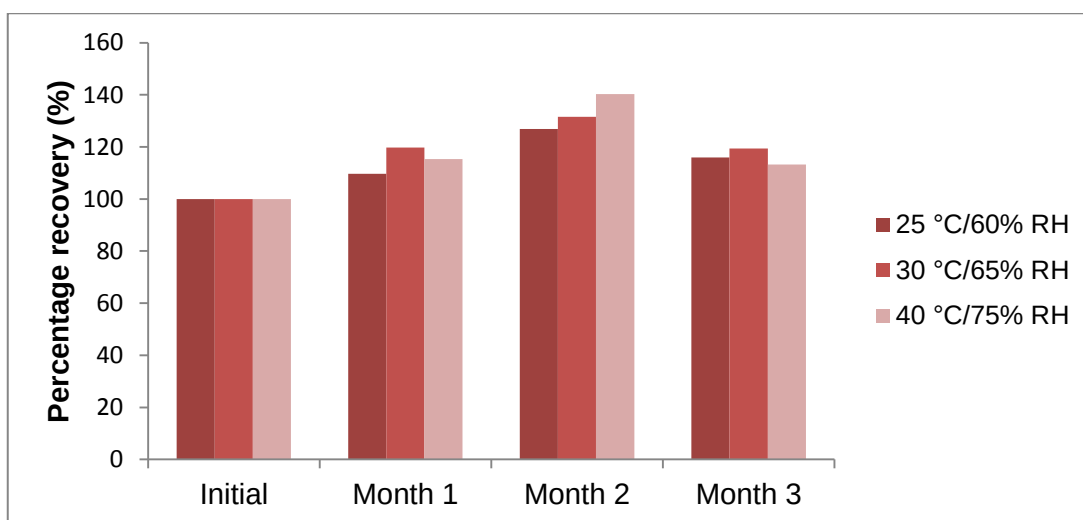


Figure D.6: Percentage recovery (%) of oestriol in **(EC)** at different storage conditions at each time interval

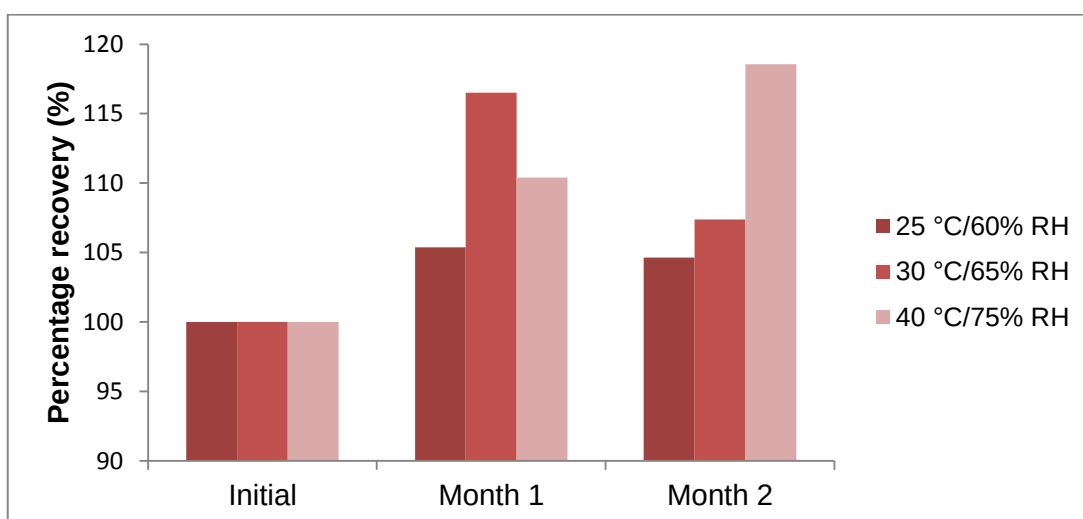


Figure D.7: Percentage recovery (%) of oestradiol in **(EC)** at different storage conditions at each time interval

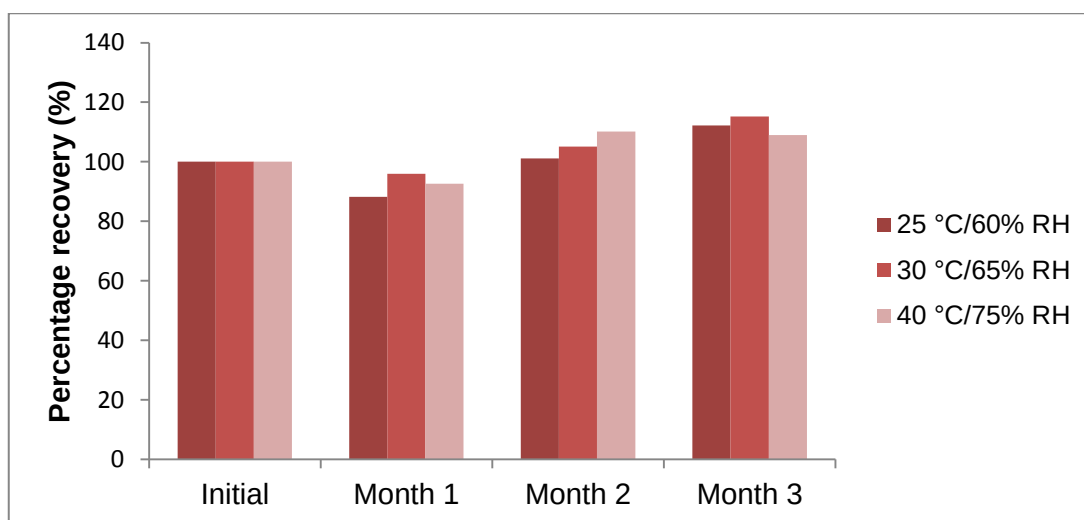


Figure D.8: Percentage recovery (%) of progesterone in **(EC)** at different storage conditions at each time interval

D.3.1.3 Male cream

As stated by Idexis, the **(MC)** contained 1.0% oestriol, 0.2% oestradiol and 2.0% progesterone. Table D.5 displays the %recovery determine for each hormone following the initial HPLC analysis, as well as the recovery reference values used throughout stability testing for the **(MC)**. During storage, significant changes were observed in the concentrations of oestriol, oestradiol and progesterone in the **(MC)** at all three storage conditions (ICH, 2003:9). The change in percentage of each female hormone in the **(MC)** at the different storage conditions over the testing period is summarised in Table D.6 and demonstrated in Figures D.9 – D.11. Ultimately, the **(MC)** samples stored at 25 °C/60% RH, 30 °C/65% RH and 40 °C/75% RH were considered to be unstable as the assay results for oestriol, oestradiol and progesterone were outside of the acceptable 5% variation limit after the 3-month period.

Table D.5: Recovery for each female hormone in **(MC)**

Hormone	Concentration (µg/ml)	Recovery	
		µg/ml	%
Oestriol	112.58	89.02	79.07
Oestradiol	22.52	2.02	8.95
Progesterone	225.15	245.35	108.97

Table D.6: Percentage recovery (%) of each female hormone in the **(MC)** at the different storage conditions for each time interval

	Initial	Month 1	Month 2	Month 3
25 °C/60% RH				
Oestriol	100.00	121.88	127.29	108.93
Oestradiol	100.00	144.30	135.64	No data
Progesterone	100.00	98.16	102.36	106.70
30 °C/65% RH				
Oestriol	100.00	120.12	113.34	126.31
Oestradiol	100.00	140.84	119.39	No data
Progesterone	100.00	97.97	92.42	124.42
40 °C/75% RH				
Oestriol	100.00	114.53	134.78	108.91
Oestradiol	100.00	135.81	112.11	No data
Progesterone	100.00	91.36	107.36	105.85

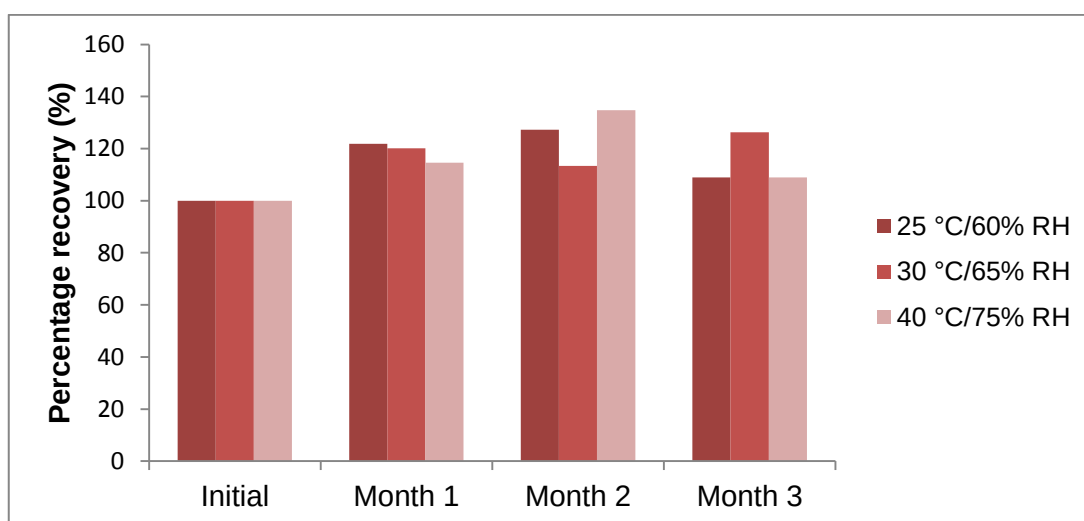


Figure D.9: Percentage recovery (%) of oestriol in **(MC)** at different storage conditions at each time interval

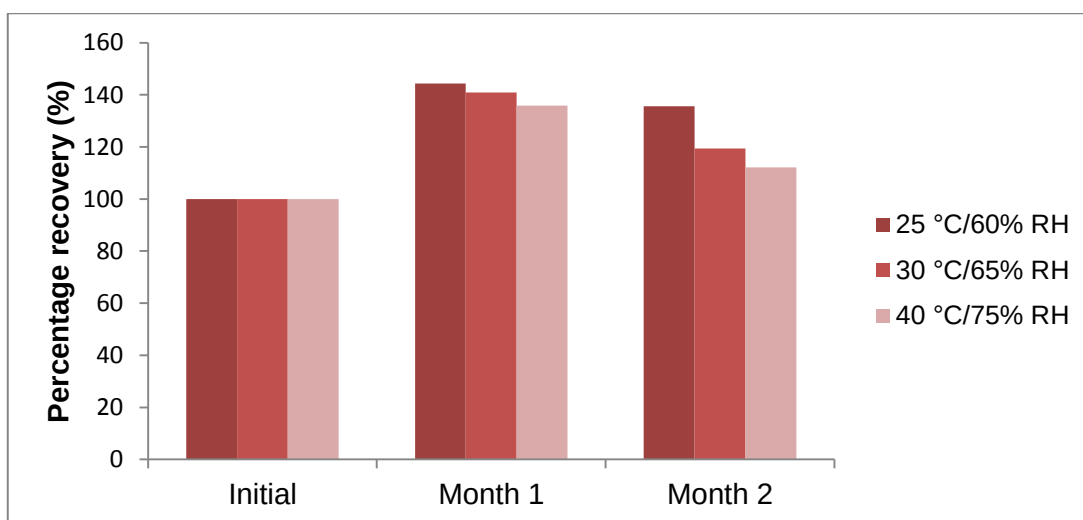


Figure D.10: Percentage recovery (%) of oestradiol in **(MC)** at different storage conditions at each time interval

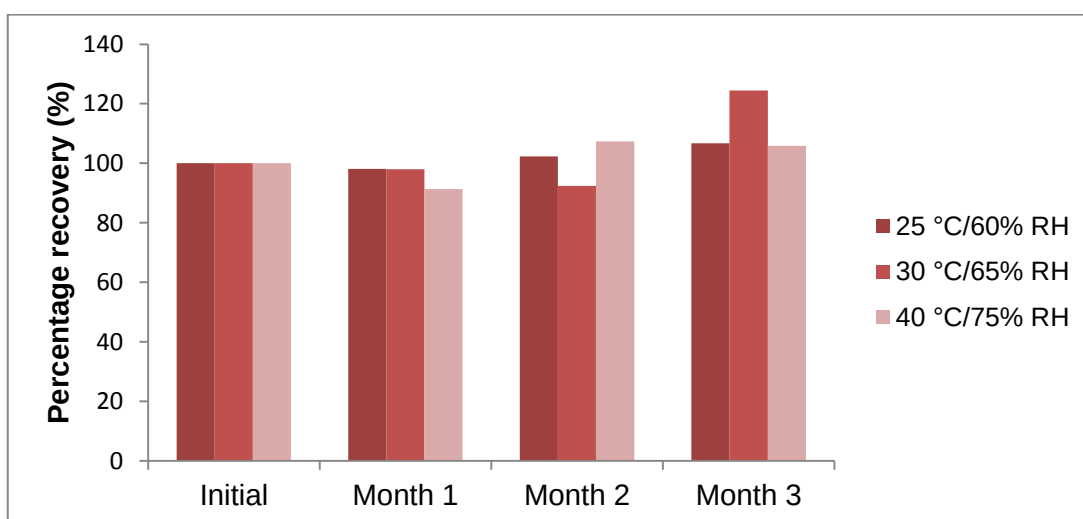


Figure D.11: Percentage recovery (%) of progesterone **(MC)** at different storage conditions at each time interval

D.3.2 Mass loss

According to Barry *et al.* (2004:262), the most common cause for a packaged product not meeting its requirements is its instability due to moisture uptake. Moisture vapour transmission rate describes the transfer of moisture into or out of a container (Waterman, 2011:934), which occurs via two main routes through the container wall, i.e. permeation and diffusion through narrow defectiveness channels (Barry *et al.*, 2004:262). Moisture ingress (into the container) and egress (out of the container) are relative to the difference in the RH between the inner and outer environments (Nelson & Huang, 2011:332; Waterman *et al.*, 2016:1572). The loss of moisture through the closure gaps or container wall must be investigated through a mass loss test on the intended container with the lid fixed firmly to 100% of the target screw at various

accelerated climatic conditions for a 3-month period (Bhavya *et al.*, 2019:728). For this study, three plastic containers were individually filled with approximately 10 g of the formulation, weighed and stored (lids on) in the different climatic conditions (initial measurement). This process was repeated for all three products. The samples stored at the different climates were weighed after months 1, 2 and 3 using a calibrated Mettler Toledo® balance (Schwarzenbach, Switzerland) (Figure D.12) to determine if any mass loss occurred due to elevated temperature and humidity. After the concluding time interval, the mass of each formulation was determined and subtracted from the initial mass to determine the total mass loss that occurred at each storage condition over the course of the accelerated stability studies. Note that each plastic container was weighed prior to adding the formulation in order to calculate the mass loss accurately.



Figure D.12: Mettler Toledo® balance

D.3.2.1 Face serum

Table D.7: Mass (g) of **(FS)** at different storage conditions for each time interval

Initial	Month 1	Month 2	Month 3	Total %mass loss
25 °C/60% RH				
10.0417	9.5875	9.2130	8.9212	11.16
30 °C/65% RH				
10.1336	9.8842	9.7257	9.5642	5.62
40 °C/75% RH				
10.0071	9.5155	9.2326	8.8891	11.17

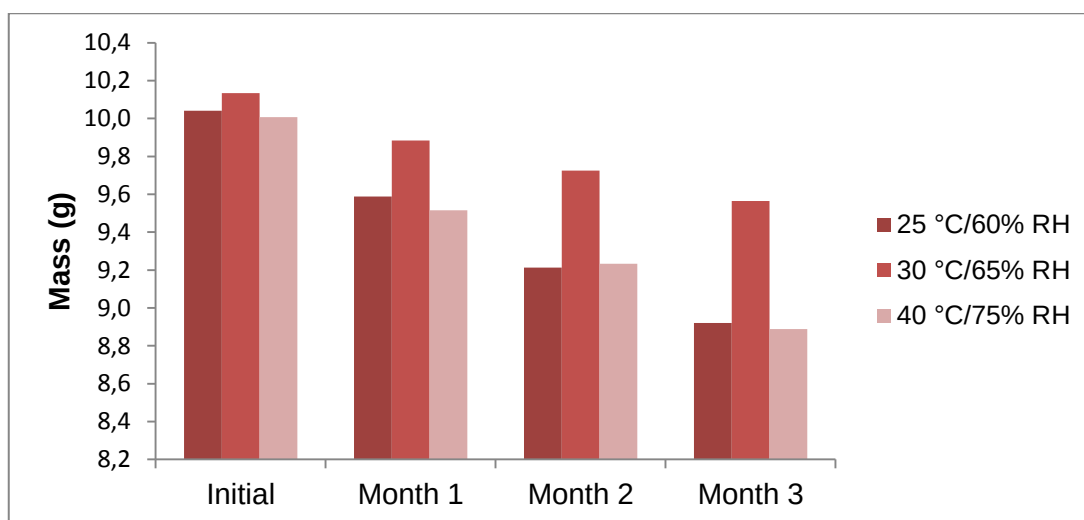


Figure D.13: Mass (g) for **(FS)** at different storage conditions for each time interval

From Table D.7, it can be observed that the loss in mass for **(FS)** did not remain stable over the 3-month period. The biggest decreases in mass were observed for both samples stored at 25 °C/60% RH and 40 °C/75% RH respectively, with a decrease of approximately 11%. The sample stored at 30 °C/65% RH displayed a total mass loss of roughly 6% over 3 months. The mass loss could be attributed to the loss of moisture through the container wall or closure gaps due to the porosity of the selected container or the container lid that did not seal sufficiently, which allows evaporation of moisture from the formulation to the surrounding environment during storage (Gerber, 2012:130). The evaporation of moisture from the final product could result in product instability by means of phase separation (Stephane, 2015:7).

D.3.2.2 Eye cream

Table D.8 Mass (g) of **(EC)** at different storage conditions for each time interval

Initial	Month 1	Month 2	Month 3	Total %mass loss
25 °C/60% RH				
10.7434	10.2531	9.7938	9.3112	13.33
30 °C/65% RH				
10.1208	9.4828	8.8772	8.2566	18.42
40 °C/75% RH				
10.1396	9.6068	9.0793	8.5531	15.65

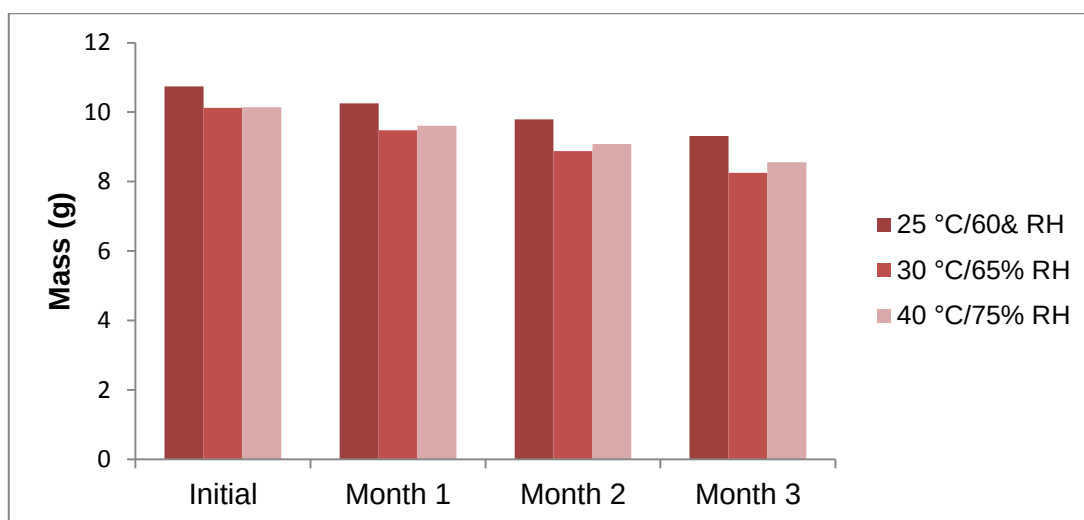


Figure D.14: Mass (g) for **(EC)** at different storage conditions for each time interval

The loss in mass of the **(EC)** did not remain stable for all three storage conditions over the 3-month period. Both samples stored at 25 °C/60% RH and 40 °C/75% RH displayed the lowest decrease in mass of approximately 13% and 16%, respectively. The biggest decrease in mass was observed in the sample stored at 30 °C/65% RH, with a decrease of roughly 18% over 3 months. This could indicate the container did not seal sufficiently, allowing evaporation during storage. The lower loss of mass observed in the sample stored at 40 °C/75% RH, compared to the samples stored at 30 °C/65% RH, could be attributed to the higher humidity and porosity of the selected container, which allowed moisture through (Gerber, 2012:130). The loss of moisture in the samples could lead to phase separation (Stephane, 2015:7).

D.3.2.3 Male cream

Table D.9: Mass (g) of **(MC)** at different storage conditions for each time interval

Initial	Month 1	Month 2	Month 3	Total %mass loss
25 °C/60% RH				
10.0760	9.3604	8.7361	8.0720	19.89
30 °C/65% RH				
10.1752	9.9108	9.6866	9.4610	7.02
40 °C/75% RH				
10.0705	9.9669	9.8785	9.7926	2.76

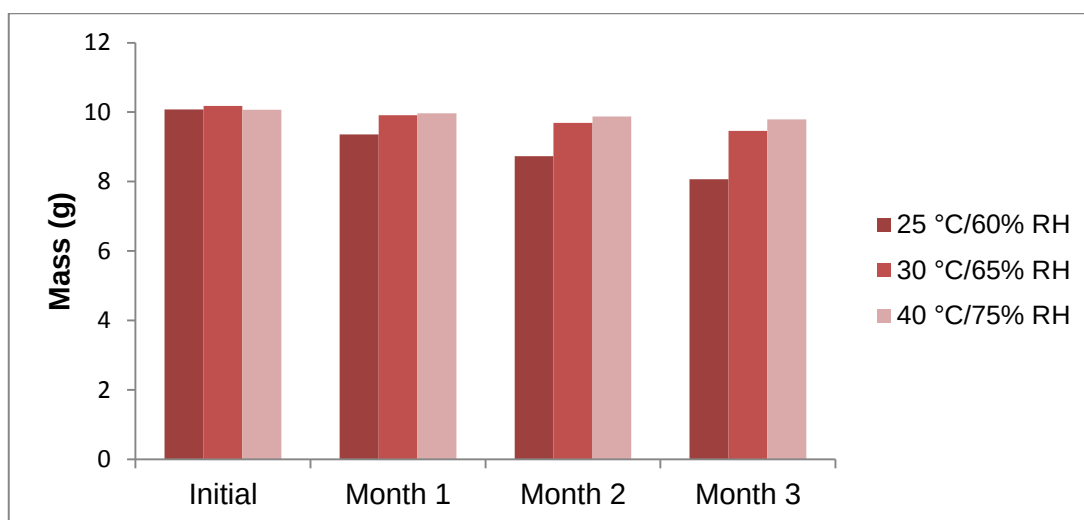


Figure D.15: Mass (g) for (MC) at different storage conditions for each time interval

From Table D.9, it can be observed that the loss in mass for both samples stored at 25 °C/60% RH and 30 °C/65% RH did not remain stable over the 3-month period, with a decrease of approximately 20% and 7%, respectively. The large increase in mass observed in the sample stored at 25 °C/60% RH possibly indicates the container did not seal adequately, allowing moisture to evaporate through the closure gaps. The biggest decrease in mass was displayed by the sample stored at 25 °C/60% RH, which could be due to the loss of moisture through the container wall or closure gaps due to the porosity of the containers or the lid that did not seal sufficiently, allowing evaporation of moisture (Gerber, 2012:130). The sample stored at 40 °C/75% RH remained stable over the 3-month period with an insignificant decrease of approximately 3%. This could be due to moisture transmission from the higher humid environment into the porous container.

D.3.3 Visual appearance

According to Naveed & Sajid (2016:81), visual evidence of degradation or instability in dosage forms includes:

- change in texture, appearance or odour;
- change in colour or discolouration;
- crystal formation;
- microbial growth;
- sample shrunk due to evaporation of moisture;
- phase separation, and
- grittiness.

Another noticeable change is the formation of air bubbles. According to Lin *et al.* (1970:323), the formation of air bubbles affects the texture, as well as the stability of the product, since trapped air bubbles can encourage microbial growth or unwanted oxidation. Air bubbles are generally integrated during the manufacturing process. In preparations with low viscosities, the incorporated air bubbles can escape quickly, whereas for more viscous preparations the rate of escape may be extremely low (Lin *et al.*, 1970:324). The formation of air bubbles in a pharmaceutical or cosmetic product can be due to the following mechanisms (Lin *et al.*, 1970:336):

- decreased solubility of the dissolved air within a product, which results in the excess air being released as air bubbles, or
- increased density, resulting in product shrinkage. At times the product hardens before the full shrinkage can occur, resulting in the gradual formation of air bubbles.

Therefore, visual assessment of each sample at each storage condition was performed at each time interval to evaluate the occurrence of any visual changes or instabilities. The visual appearance of the products on the day of receipt were used as the visual stability standard. Photographs of the various samples at different storage conditions were taken at months 0, 1, 2 and 3 and compared to the initial photograph (month 0). The photographs had to be taken at the same time of day, at the same site in similar containers with the same camera to ensure continuous and accurate photographs for evaluation. The photographs were inspected and any abnormalities or changes noted in colour, texture or odour documented. Tables D.10 – D.12 illustrate the changes in visual appearance of each product over the 3-month period.

D.3.3.1 Face serum

Freshly prepared (**FS**) appeared smooth, runny and slightly yellow in colour with a very slight, but not unpleasant, clean smell. Visual inspection revealed no visible change in the colour or texture (relative to the initial photograph) in any samples stored at different conditions over the 3-month period. Although mass loss was confirmed in Section D.3.2.1, possibly due to the loss of moisture, the surface shine remained the same throughout the study, indicating the loss of moisture did not affect the visual appearance of the serum. However, from the photograph it can be observed that the sample stored at 40 °C/75% RH displayed phase separation at month 3. According to Stephane (2015:7), the loss of moisture could lead to phase separation, therefore affecting the visual appearance of the sample. No phase separation was observed in any other sample. The slight odour intensified over time to an egg-like, rather unpleasant smell. The sample stored at 40 °C/75% RH displayed the most unpleasant smell compared to those stored at 25 °C/60% RH and 30 °C/65% RH. No visible change in texture was observed in any sample after storage for 3 months.

Table D.10: Visual appearance of the **(FS)** at different storage conditions for each time interval

Initial	Month 1	Month 2	Month 3
25 °C/60% RH			
			
30 °C/65% RH			
			
40 °C/75% RH			
			

D.3.3.2 Eye cream

Freshly prepared **(EC)** appeared smooth, runny and an oatmeal colour with a slight, but not unpleasant, clean smell. Visual inspection revealed significant changes in the colour and texture (relative to the initial photograph) in all the samples stored at different climatic conditions over the 3 months. Samples stored at 25 °C/60% RH and 30 °C/65% RH displayed similar changes, where the overall colour changed from an oatmeal colour to a dark sand colour. The most intensive colour change occurred in the sample stored at 40 °C/75% RH, where the sample changed to a mocha colour after the 3-month period. The decrease in surface shine in all the samples indicated a loss of moisture, as confirmed in Section D.3.2.2. The influence of moisture loss on the visual appearance of a product is exhibited by the sample stored at 40 °C/75% RH, where phase separation is visible at month 2. According to Iwata & Shimada

(2012:95), it can occur because the low-melting-point oil component used to formulate the product becomes highly fluid, allowing lightweight particles to float. Phase separation was not observed in the other samples. Textural changes were also observed during visual examination. The texture of the samples stored at all three storage conditions changed from a runny, smooth texture to a thick, mousse-like consistency after 3 months. The presence of air bubbles can be observed in the samples, especially in the samples stored at 40 °C/75% RH after 3 months. This demonstrates the influence of higher temperature and humidity on product stability, with possible product shrinkage and hardening (Lin *et al.*, 1970:336). During storage, the slight odour intensified to an unpleasant, egg-like smell. The sample stored at 40 °C/75% RH revealed the most unpleasant smell compared to the samples stored at 25 °C/60% RH and 30 °C/65% RH.

Table D.11: Visual appearance of the (EC) at different storage condition for each time interval

Initial	Month 1	Month 2	Month 3
25 °C/60% RH			
			
30 °C/65% RH			
			
40 °C/75% RH			
			

D.3.3.3 Male cream

Table D.12: Visual appearance of **(MC)** at different storage conditions for each time interval

Initial	Month 1	Month 2	Month 3
25 °C/60% RH			
			
30 °C/65% RH			
			
40 °C/75% RH			
			

Freshly prepared **(MC)** appeared smooth, thick and a light cream colour with a very mild, but not unpleasant, clean smell. Visual inspection revealed no changes (relative to the initial photograph) in the colour and texture of the samples stored at 25 °C/60% RH and 30 °C/65% RH during storage. Although mass loss has been confirmed in Section D.3.2.3 in the samples stored at both climatic conditions, the surface shine remained the same during storage, indicating the possible loss of moisture did not influence the visible appearance of the cream. The sample stored at 40 °C/75% RH displayed significant changes in colour and texture. The colour of the sample changed from light cream to slightly yellow after 3 months. Phase separation was not observed in any sample at any storage condition. Furthermore, textural changes were observed in the sample stored at 40 °C/75% RH, following visual examination. The texture of the sample changed from a thick, smooth texture to a mousse-like,

almost 'baked soufflé' consistency after 3 months. Air bubbles could be observed in the samples, especially after 3 months in storage, which revealed the impact of higher humidity and temperature on product stability, causing possible shrinkage and hardening of the product (Lin *et al.*, 1970:336). The mild odour intensified over time to an unpleasant, egg-like smell. The sample stored at 40 °C/75% RH had the most unpleasant smell compared to the samples stored at 25 °C/60% RH and 30 °C/65 RH.

D.3.4 pH

The measurement of pH is a key indicator of topical formulation stability, especially regarding creams (Papaioanou *et al.*, 2018:30). Chemically, a change in the pH of a product can be indicative of possible degradation or ionisation of one or more of the compounds used in the formula. Moreover, the chemical transformation of compounds reflects their degradation or incompatibility, which can consequently cause chemically harmful effects in consumers (Ali *et al.*, 2013:1091). According to the Hach Company (2010:60), the concentration of hydrogen [H^+] and hydroxide ions [OH^-] remain constant in a stable formulation; in other words, the pH should remain constant throughout the stability testing to represent a stable product. The normal pH range of skin is between 4.6 – 5.8 (Leonardi *et al.*, 2002:565; Moraes *et al.*, 2017:31), therefore products intended for topical application should have a pH close to this range (Akhtar *et al.*, 2011:133). Barry (2002:512) proposed that a pH value within 3 – 9 is safe for topical use. A Mettler Toledo® pH meter (Mettler Toledo, United States of America) fitted with a Mettler Toledo® InLab® 410 electrode (Mettler Toledo, United States of America) (Figure B.5) was used to measure the pH of the respective Idexis products. The electrode was calibrated with Mettler Toledo® pH buffer solutions at various pH values (4, 7 and 10) prior to each interval measurement (months 0, 1, 2 and 3). The pH of each product at each storage condition was measured in triplicate after each time interval. All the products were considered stable, as no significant variations in pH were displayed based on the ICH Guidelines (2003:9). The samples stored at 40 °C/75% RH displayed the biggest decrease in pH for **(FS)**, **(EC)** and **(MC)**, which demonstrated the impact of higher temperature and humidity on formulation stability (Moraes *et al.*, 2017:31). The sudden decrease of the pH observed at month 1 in each product could be due to the formation of acidic degradation products or moisture loss (discussed in Section D.3.2). Due to the classified nature of this study, the excipients used to formulate each product is unknown to the student, therefore it is difficult to determine the cause of the decrease, which was then followed by a progressive increase seen from month 2.

D.3.4.1 Face serum

No significant changes, in agreement with the ICH Guidelines (ICH, 2003:9), were observed for **(FS)** following the determination of the percentage changes (relative to the initial pH) for all the

samples stored at different conditions. The pH value of all the samples ranged from 4.636 – 5.508, which is within the required range for topical use and without any risk of irritation (Barry, 2002:512; Bouranen, 2017:40). Samples stored at 25 °C/60% RH and 30 °C/65% RH exhibited a decrease in pH of around 2% after 3 months. The biggest decrease was observed in the sample stored at 30 °C/65% RH, which had a decrease of about 5%. The pH of **(FS)** remained relatively stable throughout the entire storage period, with a maximum decrease of approximately 5%. This product was considered stable, as it displayed a decrease of 0.118, 0.104 and 0.267 pH units over the 3-month period for the 25 °C/60% RH, 30 °C/65% RH and 40 °C/75% RH storage conditions, respectively.

Table D.13: pH of **(FS)** at different storage conditions for each time interval

	Initial	Month 1	Month 2	Month 3
25 °C/60% RH				
1	5.161	4.805	4.977	5.304
2	5.394	4.951	4.983	5.205
3	5.508	4.916	5.001	5.199
Median	5.394	4.916	4.983	5.205
30 °C/65% RH				
1	5.161	4.803	4.989	5.284
2	5.394	4.913	5.053	5.303
3	5.508	4.636	4.977	5.163
Median	5.394	4.803	4.977	5.284
40 °C/75% RH				
1	5.161	4.901	5.025	5.001
2	5.394	4.826	5.031	5.168
3	5.508	4.853	4.948	5.092
Median	5.394	4.853	5.025	5.092

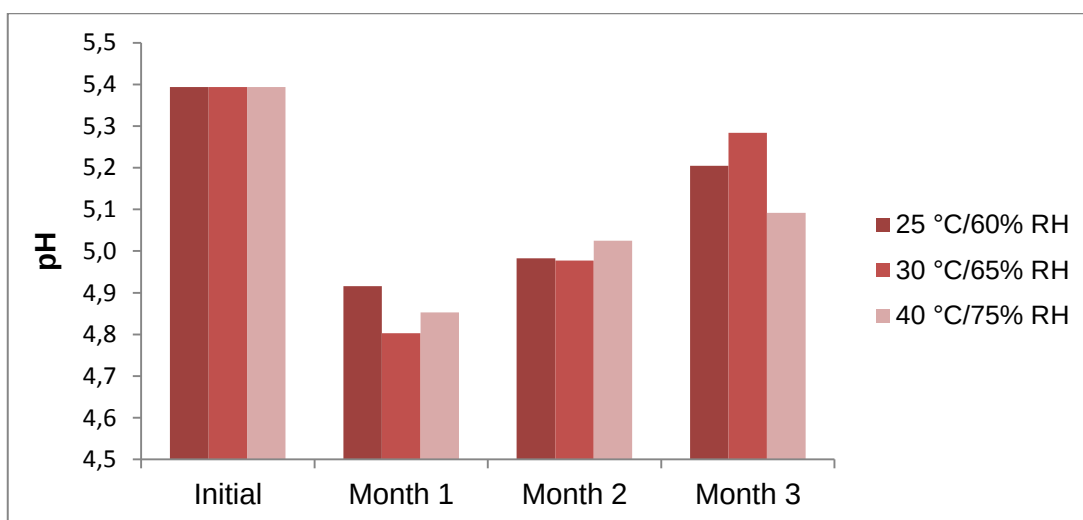


Figure D.16: Median pH for **(FS)** at different storage conditions for each time interval

D.3.4.2 Eye cream

Table D.14: pH of **(EC)** at different storage conditions for each time interval

	Initial	Month 1	Month 2	Month 3
25 °C/60% RH				
1	5.745	5.217	5.591	5.786
2	5.795	5.248	5.547	5.772
3	5.803	4.942	5.533	5.720
Median	5.795	5.217	5.547	5.772
30 °C/65% RH				
1	5.745	4.930	5.443	5.815
2	5.795	4.697	5.449	5.717
3	5.803	4.982	5.461	5.736
Median	5.795	4.930	5.449	5.736
40 °C/75% RH				
1	5.745	4.638	5.469	5.383
2	5.795	4.996	5.439	5.537
3	5.803	5.128	5.421	5.359
Median	5.795	4.996	5.439	5.383

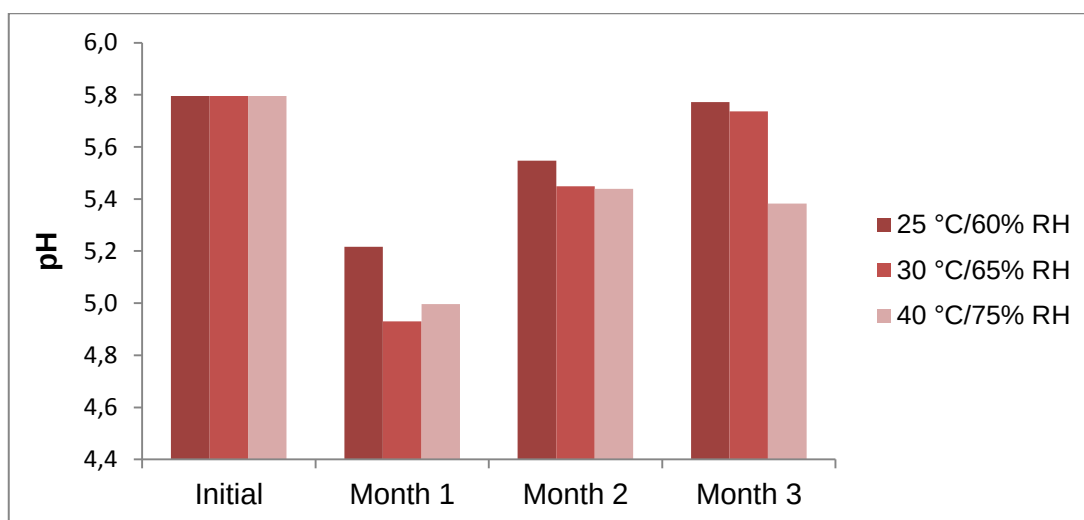


Figure D.17: Median pH for **(EC)** at different storage conditions for each time interval

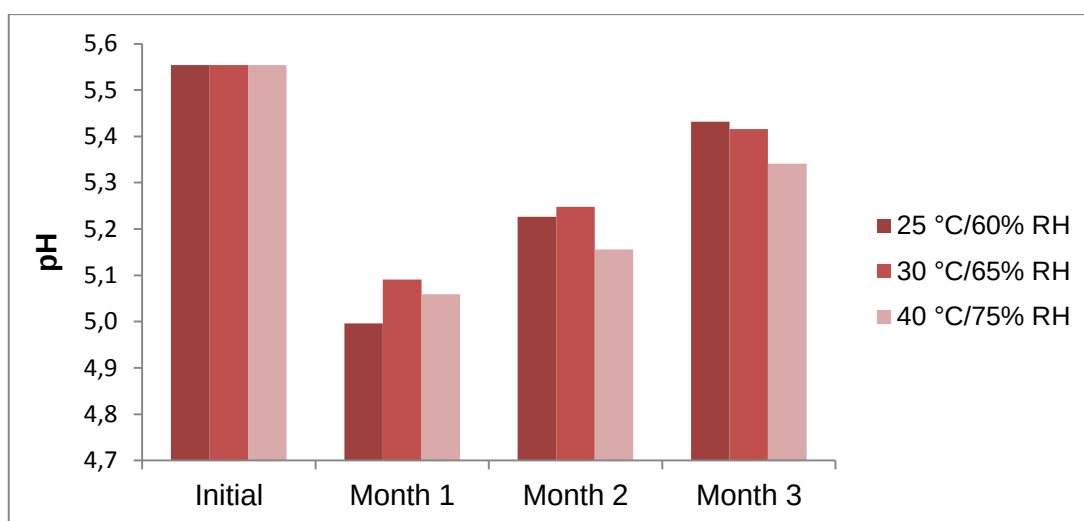
Based on the ICH Guidelines (ICH, 2003:9), no significant variations in the pH (relative to the initial pH) were observed for all the **(EC)** samples stored at different climatic conditions. The pH values of all the samples ranged between 4.638 and 5.815, which is within the required range for safe topical application (3 – 9) (Barry, 2002:512). Samples stored at 25 °C/60% RH and 30 °C/65% RH showed an insignificant decrease in pH after 3 months. The sample stored at 40 °C/75% RH displayed the largest decrease of roughly 3%. The pH of **(EC)** did remain relatively constant during storage, therefore the product was considered to be stable due to the minor decrease of 0.022, 0.025 and 0.355 pH units over the 3-month period for the 25 °C/60% RH, 30 °C/65% RH and 40 °C/75% RH storage conditions, respectively.

D.3.4.3 Male cream

According to the ICH Guidelines (ICH, 2003:9), no significant alterations were seen in the pH of the **(MC)** samples stored at different conditions. The pH range of all the samples remained between 4.930 and 5.612, which was considered suitable for topical application as it did not exceed the physiological pH range of the skin (4.6 – 5.8) (Leonardi *et al.*, 2002:565; Moraes *et al.*, 2017:31). Samples stored at 25 °C/60% RH and 30 °C/65% RH displayed a decrease in pH of approximately 3%, whereas the sample stored at 40 °C/75% RH exhibited the largest decrease of approximately 4% over the period of 3 months. The pH of **(MC)** remained stable over the 3-month period; therefore, it was considered stable as it displayed an insignificant decrease in pH of 0.159, 0.133 and 0.210 units for the 25 °C/60% RH, 30 °C/65% RH and 40 °C/75% RH storage conditions, respectively.

Table D.15: pH of (MC) at different storage conditions for each time interval

	Initial	Month 1	Month 2	Month 3
25 °C/60% RH				
1	5.548	4.930	5.273	5.434
2	5.554	5.015	5.194	5.370
3	5.612	4.996	5.226	5.432
Median	5.554	4.996	5.226	5.432
30 °C/65% RH				
1	5.548	5.085	5.226	5.515
2	5.554	5.137	5.248	5.384
3	5.612	5.091	5.292	5.416
Median	5.548	5.091	5.248	5.416
40 °C/75% RH				
1	5.548	5.007	5.155	5.413
2	5.554	5.088	5.204	5.341
3	5.612	5.059	5.156	5.329
Median	5.554	5.059	5.156	5.341

**Figure D.18:** Median pH for (MC) at different storage conditions for each time interval

D.3.5 Zeta-potential

Zeta-potential describes the electrokinetic potential in colloidal systems (Gumustas *et al.*, 2017:93). The stability of a product is greatly related to the zeta-potential (Gqebe *et al.*, 2016:580). Higher zeta-potential values (positive or negative) indicate higher product stability, while lower values tend to flocculate or coagulate, leading to poor product stability (Gumustas *et al.*, 2017:93; Lu & Gao, 2010:66). Zeta-potential values lower than - 30 mV and higher than + 30 mV are considered to have adequate repulsive forces between particles to attain physical

stability (Joseph & Singhvi, 2019:99). Optimal flocculation occurs when the zeta-potential is closer to 0 mV (Mehta, 1999:29; Narong & James, 2006:130). For this study, a Malvern Zetasizer Nano ZS (Malvern Instruments, United Kingdom) (Figure B.6) was used to determine the zeta-potential of each product. The samples were prepared as described in Section B.5.2. The zeta-potential was measured in triplicate for each product at each storage condition. Figures D.19 – D.21 illustrate the change in zeta-potential (mV) for each product over the 3-month period. Note that due to the negative values of the zeta-potentials, an increase in zeta-potential described a more positive value, while a decrease in zeta-potential described a more negative value.

D.3.5.1 Face serum

The zeta-potential of the **(FS)** remained within the range of less than - 30 mV or more than + 30 mV for all three storage conditions over the 3 months. As demonstrated in Figure D.19, the zeta-potential values remained highly negative over the testing period. The sample stored at 25 °C/60% RH displayed an increase of about 9 mV, whereas the sample stored at 30 °C/65% RH exhibited a smaller increase of roughly 4 mV. As expected, the sample stored at 40 °C/75% RH displayed the largest increase of approximately 10 mV over 3 months. Although the zeta-potential increased for all the samples at the different storage conditions, the values stayed within the range for product stability over the 3-month period.

Table D.16: Zeta-potential (mV) of **(FS)** at different storage conditions for each time interval

	Initial	Month 1	Month 2	Month 3
25 °C/60% RH				
1	-65.2	-61.9	-59.6	-53.6
2	-64.5	-57.8	-60.1	-56.1
3	-62.7	-61.2	-61.5	-55.2
Median	-64.5	-61.2	-60.1	-55.2
30 °C/65% RH				
1	-65.2	-65.8	-62.7	-60.6
2	-64.5	-68.6	-65.3	-60.1
3	-62.7	-66.1	-60.7	-61.0
Median	-64.5	-66.1	-62.7	-60.6
40 °C/75% RH				
1	-65.2	-62.7	-62.8	-53.0
2	-64.5	-60.8	-63.1	-53.6
3	-62.7	-64.4	-64.5	-55.3
Median	-64.5	-62.7	-63.1	-53.6

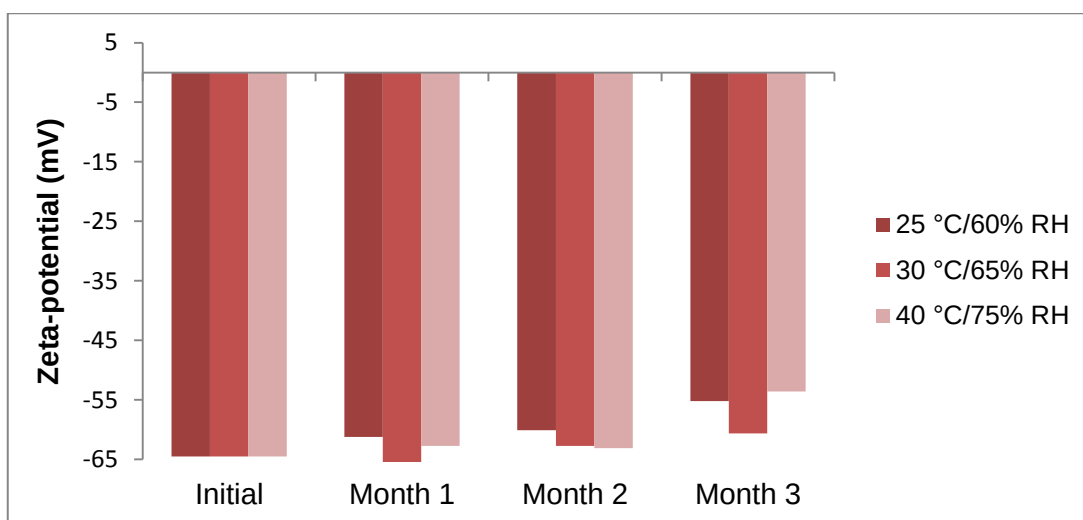


Figure D.19: Median zeta-potential (mV) for **(FS)** at different storage conditions for each time interval

D.3.5.2 Eye cream

Table D.17: Zeta-potential (mV) of **(EC)** at different storage conditions for each time interval

	Initial	Month 1	Month 2	Month 3
25 °C/60% RH				
1	-59.3	-61.5	-58.2	-60.8
2	-58.8	-61.7	-60.1	-61.4
3	-58.8	-64.1	-58.3	-62.8
Median	-58.8	-61.7	-58.3	-61.4
30 °C/65% RH				
1	-59.3	-60.7	-51.9	-61.7
2	-58.8	-62.2	-51.2	-59.6
3	-58.8	-58.1	-52.3	-62.2
Median	-58.8	-60.7	-51.9	-61.7
40 °C/75% RH				
1	-59.3	-64.9	-76.0	-51.4
2	-58.8	-63.6	-74.5	-53.7
3	-58.8	-66.5	-75.8	-53.8
Median	-58.8	-64.9	-75.8	-53.7

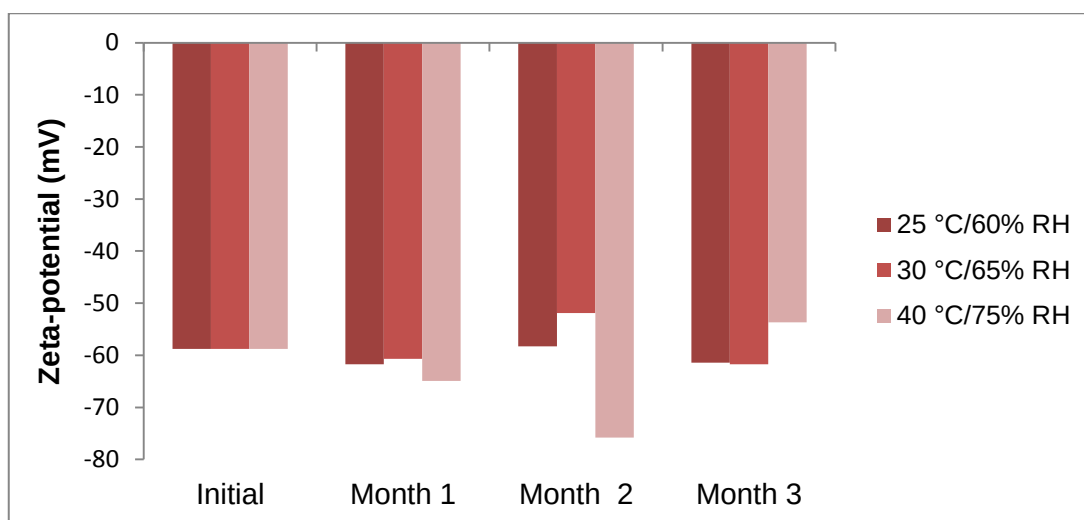


Figure D.20: Median zeta-potential (mV) for **(EC)** at different storage conditions for each time interval

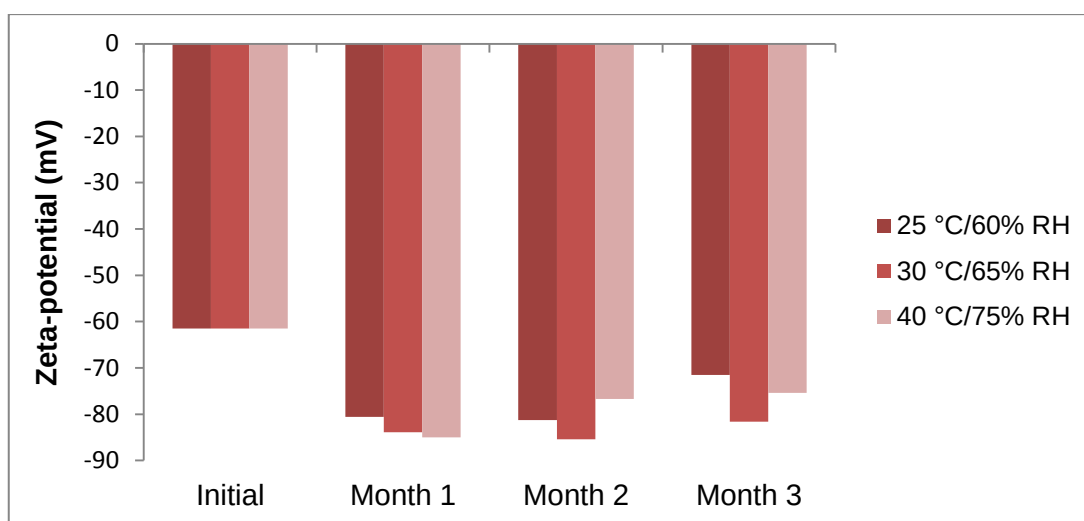
The zeta-potential of the **(EC)** remained within the range of less than - 30 mV or more than + 30 mV for all three storage conditions over the 3 months. The samples stored at 25 °C/60% RH and 30 °C/65% RH displayed an insignificant decrease of 2.7 mV and 2.2 mV, respectively. These samples demonstrated higher negative zeta-potentials relative to the initial measurement, which indicated an increase in product stability. The sample stored at 40 °C/75% RH exhibited an increase of approximately 6.0 mV over the 3-month period. Although the zeta-potential increased in the sample stored at 40 °C/75% RH, the values remained highly negative, therefore proposing stability.

D.3.5.3 Male cream

The zeta-potential of the **(MC)** remained within the range of less than - 30 mV or more than + 30 mV for all three storage conditions over the 3 months. The sample stored at 25 °C/60% RH exhibited a decrease of approximately 10 mV, whereas the sample stored at 40 °C/75% RH displayed a decrease of approximately 14 mV. The largest decrease in zeta-potential was exhibited in the sample stored at 30 °C/65% RH, with a decrease of roughly 20 mV. All the samples stored at different conditions displayed higher negative zeta-potentials relative to the initial measurement, indicating an increase in stability. As seen in Table D.18, the sample stored at 30 °C/65% RH was considered the most stable sample, with the largest decrease in zeta-potential compared to the other samples.

Table D.18: Zeta-potential (mV) of **(MC)** at different storage conditions for each time interval

	Initial	Month 1	Month 2	Month 3
25 °C/60% RH				
1	-59.8	-80.6	-81.3	-70.9
2	-61.5	-80.4	-80.3	-71.5
3	-62.0	-80.7	-82.5	-72.0
Median	-61.5	-80.6	-81.3	-71.5
30 °C/65% RH				
1	-59.8	-83.9	-86.7	-78.7
2	-61.5	-84.1	-84.8	-81.9
3	-62.0	-82.4	-85.4	-81.6
Median	-61.5	-83.9	-85.4	-81.6
40 °C/75% RH				
1	-59.8	-86.5	-76.7	-73.9
2	-61.5	-79.0	-77.4	-76.9
3	-62.0	-85.0	-75.9	-75.4
Median	-61.5	-85.0	-76.7	-75.4

**Figure D.21:** Median zeta-potential (mV) for **(MC)** at different storage conditions for each time interval

D.3.6 Particle size and distribution

Particle size has a direct effect on various physicochemical characteristics of a formulation, including the stability, appearance and chemical reactivity (ATA Scientific Instruments, 2019; Malvern Instruments Limited, 2015a). Changes in particle size can also influence the viscosity of a preparation. Decreasing the particle size increases the number of particles in a stable volume fraction, which consequently increases the particle-particle interactions, leading to an

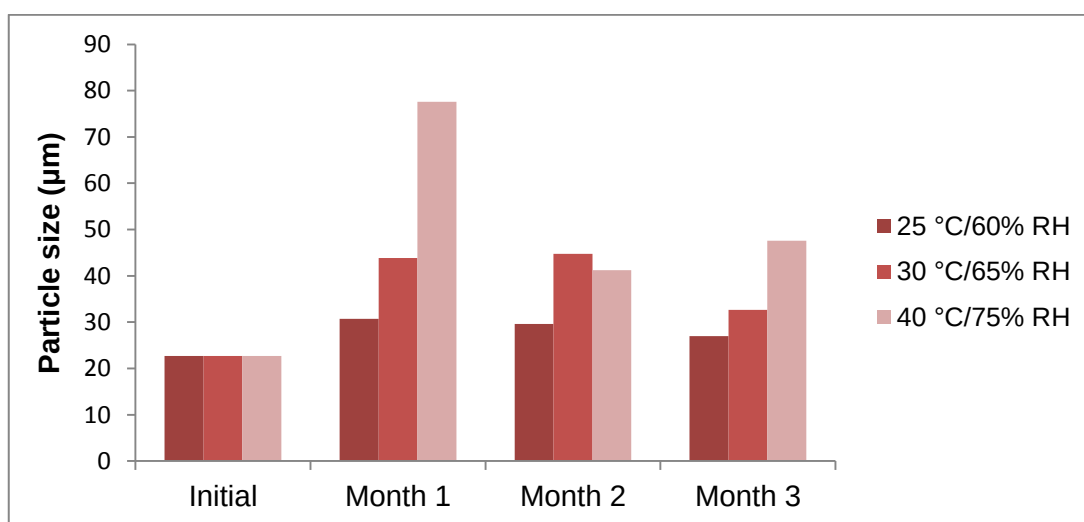
increase in viscosity (Malvern Instruments Limited, 2015b). In general, formulations containing smaller particles with identical electrical charge exhibit higher resistance to flocculation and sedimentation compared to systems with larger or uncharged particles, because Brownian motion becomes greater than the force of gravity (Buchmann, 2001:166; Ribeiro *et al.*, 2015:2496). Therefore, determination of particle size is an important indicator of product stability over time against various climatic conditions. A Malvern Mastersizer 2000 Particle Size Analyser (Malvern Instruments, United Kingdom) fitted with a Hydro 2000SM Dispersion Unit (Malvern Instruments, United Kingdom) (Figure B.10) was used to measure the particle size of each Idexis product. The samples were prepared as mentioned in Section B.5.3 and analysed at a stirring speed of 1 200 rpm. To conclude, the statistical distribution was calculated from the results using the derived diameters $D[m, n]$, which is an internationally approved method for specifying the mean and other moments of particle size. For this study, the $D(0.5)$ reading, which represented the size (μm) at which 50% of the sample was smaller and 50% larger, was used to determine the particle size of each preparation. Measurements were done in triplicate to determine the particle size for each product at each storage condition at the predetermined time intervals. Figures D.22 – D.24 illustrate a summary of the changes in particle size (μm) over time for each product.

D.3.6.1 Face serum

Significant changes, in accordance with the ICH Guidelines (ICH, 2003:9), were observed in the particle size (μm) of **(FS)** at all three storage conditions over the 3-month period. The sample stored at 25 °C/60% RH exhibited an increase of approximately 4 μm , while the sample stored at 30 °C/65% RH displayed a slightly larger increase of approximately 10 μm . Over the 3-month period, the largest increase, of roughly 25 μm , was demonstrated by the sample stored at 40 °C/75% RH. Overall, all three sample displayed a significant increase in particle size outside the acceptance range for stability testing. Due to Brownian motion, the particles constantly collided with each other, potentially becoming aggregated due to Van der Waals attraction forces. This could lead to flocculation, which is the formation of larger secondary particles (flocs) (Larsson *et al.*, 2012:209). It is evident from the results that the particles in the samples underwent flocculation as their size increased at all three storage conditions over time. The larger particles could possibly settle under the influence of gravity. The decrease in zeta-potential displayed in Table D.16 further confirmed the decrease in stability of **(FS)** over the 3-month period, especially at 40 °C/75% RH.

Table D.19: Particle size (μm) of **(FS)** at different storage conditions for each time interval

	Initial	Month 1	Month 2	Month 3
25 °C/60% RH				
1	22.839	30.639	29.571	27.189
2	22.536	30.753	29.727	26.714
3	22.702	30.791	29.598	26.985
Median	22.702	30.753	29.598	26.985
30 °C/65% RH				
1	22.839	42.221	42.255	32.679
2	22.536	43.851	44.758	32.142
3	22.702	43.928	45.921	33.174
Median	22.702	43.851	44.758	32.679
40 °C/75% RH				
1	22.839	77.560	41.226	47.578
2	22.536	76.993	41.195	47.063
3	22.702	77.663	41.868	48.548
Median	22.702	77.560	41.226	47.578

**Figure D.22:** Median particle size (μm) for **(FS)** at different storage conditions for each time interval

D.3.6.2 Eye cream

During storage, no significant changes were observed in the particle size (μm) of the **(EC)** over the 3-month testing period (ICH, 2003:9). Samples stored at 25 °C/60% RH and 30 °C/65% RH demonstrated a decrease in size of approximately 0.3 μm and 0.1 μm , respectively. The sample stored at 40 °C/75% RH displayed a small increase of approximately 0.3 μm over time. The particle size of all the stored samples remained stable over the 3-month period. From

Table D.20, it can be observed that the sample stored at 40 °C/75% RH experienced some degree of flocculation due to the small increase in particle size. The stability of the **(EC)** samples stored at 25 °C/60% RH and 30 °C/65% RH was also established by the highly negative zeta-potential values measured after 3 months, as shown in Table D.17.

Table D.20: Particle size (µm) of **(EC)** at different storage conditions for each time interval

	Initial	Month 1	Month 2	Month 3
25 °C/60% RH				
1	10.063	9.699	9.610	9.622
2	9.869	9.592	9.525	9.576
3	9.732	9.472	9.499	9.573
Median	9.869	9.592	9.525	9.576
30 °C/65% RH				
1	10.063	9.876	9.913	9.810
2	9.869	9.736	10.108	9.795
3	9.732	9.655	10.305	9.750
Median	9.869	9.736	10.108	9.795
40 °C/75% RH				
1	10.063	10.520	10.401	10.212
2	9.869	10.365	10.297	10.136
3	9.732	10.297	10.447	10.094
Median	9.869	10.365	10.401	10.136

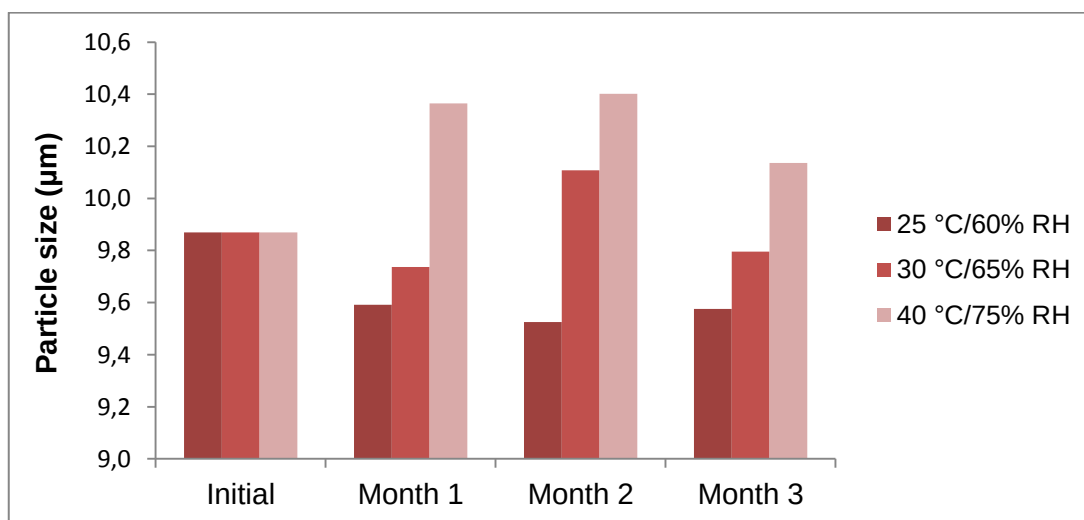


Figure D.23: Median particle size (µm) for **(EC)** at different storage conditions for each time interval

D.3.6.3 Male cream

Based on the ICH Guidelines (ICH, 2003:9), no significant variations were observed in the particle size (μm) of the **(MC)** samples stored at 30 °C/65% RH, which displayed an increase of approximately 1 μm . The sample stored at 25 °C/60% RH exhibited a decrease of about 2 μm , while the largest increase was displayed by the sample stored at 40 °C/75% RH, which increased from an initial particle size of 25.098 μm to a final particle size of 32.861 μm . It is evident from Table D.21 that the sample stored at 40 °C/75% RH underwent flocculation due to the large increase in particle size. Although the particle size of the sample stored at 40 °C/75% RH did not remain stable over the 3-month period, all three samples stored at the different conditions presented with high negative zeta-potential values, as demonstrated in Table D.18, which was indicative of increased product stability (Sun *et al.*, 2016:8525).

Table D.21: Particle size (μm) of **(MC)** at different storage conditions for each time interval

	Initial	Month 1	Month 2	Month 3
25 °C/60% RH				
1	24.729	30.431	19.655	19.966
2	25.295	30.320	22.406	23.724
3	25.270	30.135	23.490	25.385
Median	25.270	30.320	22.406	23.724
30 °C/65% RH				
1	24.729	25.565	22.584	24.360
2	25.295	28.041	25.917	26.087
3	25.270	28.374	27.037	26.766
Median	25.270	28.041	25.917	26.087
40 °C/75% RH				
1	24.729	26.467	28.499	30.017
2	25.295	26.702	29.926	33.400
3	25.270	26.834	30.652	35.165
Median	25.270	26.702	29.926	33.400

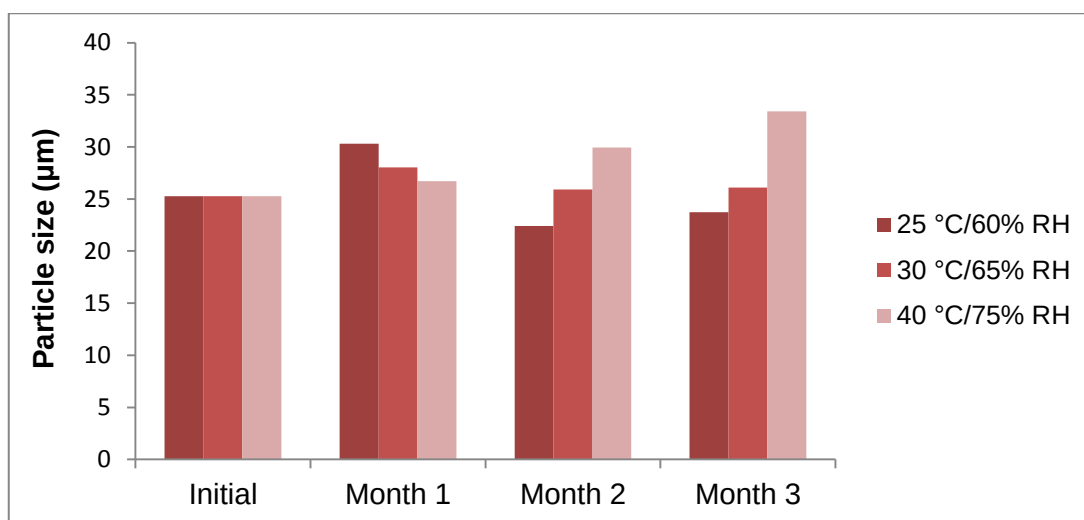


Figure D.24: Median particle size (µm) for **(MC)** at different storage conditions for each time interval

D.3.7 Viscosity

Viscosity characterises a fluid's resistance to flow, which is caused by internal friction (Anusavice *et al.*, 2012:32). The viscosity of a product can be perceived by the consumer when applying pressure to the product's surface (formulation 'pick-up') or spreading the product onto the skin (Postles, 2018:105). Thickening agents are generally used to adjust the viscosity of skin care products to improve application, rheological properties (flow and feel) and the appearance (Bouranen, 2017:3; Karsheva *et al.*, 2007:187; Riemer & Russo, 2017:43). According to Nastiti *et al.* (2017:44), viscosity greatly influences the stability of a formulation. As viscosity increases, the rate of sedimentation or creaming decreases (Postles, 2018:22). Therefore, the evaluation of viscosity is essential to determine the stability of the product in terms of consistency and indicate the product's behaviour at different climatic conditions over a 3-month period (ANVISA, 2004:34). A Brookfield Viscometer DV2T LV Ultra (AMETEK Brookfield, United States of America) (Figure B.14), connected to a thermostatic water bath pre-heated to a controlled temperature of ± 25 °C, was used to measure the viscosity of the samples from the different storage conditions at each time interval. The samples were placed in the pre-heated water bath to acclimatise 60 min prior to measurements. Appropriate Helipath spindles (AMETEK Brookfield, United States of America) were placed in each sample and set to a specified rate (rpm). For the purpose of this study, a T-E spindle was used to determine the viscosity of the Idexis products. The **(EC)** and **(MC)** were measured at a spindle rotation speed of 30 rpm, while the **(FS)** was measured at a spindle rotation speed of 200 rpm. The viscosity was measured every 10 s for 2 min. Rheocalc T software version 1.2 recorded approximately 32 readings, which were used to determine the viscosity of each sample. All three products displayed an increase in viscosity over the 3-month period. Viscosity generally increases with a decrease in temperature or an increase in intermolecular forces (Davarcioğlu, 2011:445; Fowles

& Boyes, 2010:32). The largest increase was observed in the samples stored at 25 °C/60% RH; therefore, it can be stated that the mechanism causing the increase was dependent on temperature. Due to the limited knowledge regarding the excipients used during formulation of each product, the exact mechanism responsible for the increase in viscosity was uncertain.

D.3.7.1 Face serum

The viscosity of **(FS)** did not remain stable during storage. The sample stored at 25 °C/60% RH demonstrated the largest increase of approximately 324 cP, whereas the sample stored at 30 °C/65% RH exhibited an increase of approximately 292 cP. The lowest increase was displayed by the sample stored at 40 °C/75% RH, with an increase of roughly 87 cP. This sample demonstrated an initial decrease in viscosity, which could be due to broken intermolecular forces (Van der Waals forces) (Davarcioğlu, 2011:445; Gerber, 2012:129). A polymer had been incorporated into all the products to decrease the skin permeability and systemic absorption of the female hormones. Xin *et al.* (2018:858) stated that the degradation of polymers could cause a decrease in viscosity. The total increase in viscosity over the 3-month period could be due to the loss of moisture, which was confirmed in Section D.3.2.1 (Gerber, 2012:128). However, it was uncertain as to exactly which mechanisms were responsible for the increase in viscosity of the **(FS)**. The selected dispensing container for the serum together with the increased viscosity could hinder the product's ability to be dispensed, leading to a decrease in the perceived quality of the product (Postles, 2018:110).

Table D.22: Viscosity (cP) of **(FS)** at different storage conditions for each time interval

Initial	Month 1	Month 2	Month 3
25 °C/60% RH			
551.950	599.808	747.658	876.135
30 °C/65% RH			
551.950	584.767	711.125	844.150
40 °C/75% RH			
551.950	442.767	577.600	639.042

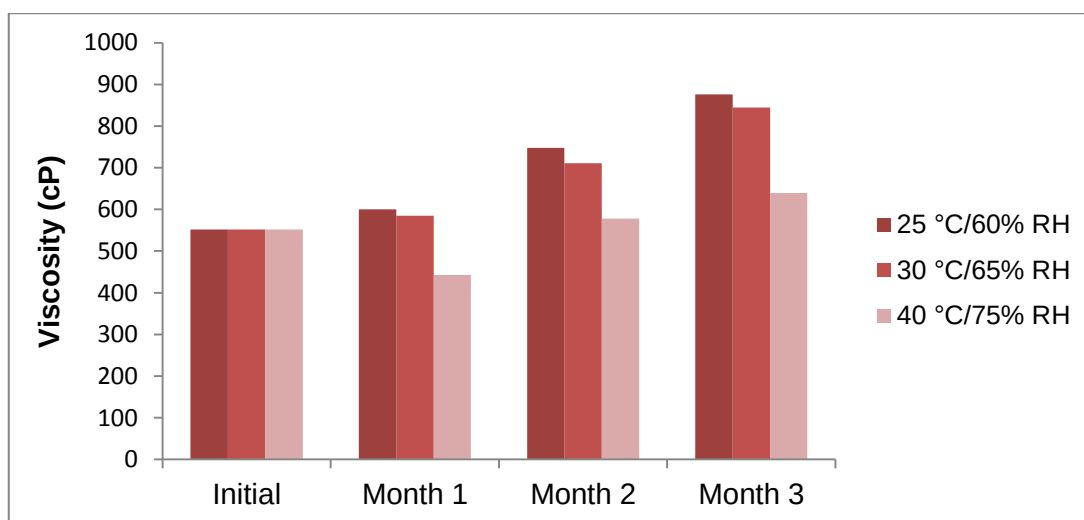


Figure D.25: Viscosity (cP) for **(FS)** at different storage conditions for each time interval

D.3.7.2 Eye cream

The viscosity of the **(EC)** samples did not remain stable during storage. The sample stored at 25 °C/60% RH exhibited an increase of approximately 7 580 cP. Samples stored at 30 °C/65% RH and 40 °C/75% RH displayed a large increase that was not within the FSR of the selected spindle/speed combination. The overall increase in viscosity could be due to moisture loss, which was confirmed in Section D.3.2.2. Due to the limited knowledge about the excipient used to formulate the **(EC)** and the error readings obtained for both samples stored at 30 °C/65% RH and 40 °C/75% RH, the exact mechanisms responsible for the increase in viscosity of the **(EC)** were not known.

Table D.23: Viscosity (cP) of **(EC)** at different storage conditions for each time interval

Initial	Month 1	Month 2	Month 3
25 °C/60% RH			
4 023.500	6 371.000	8 801.000	11 604.170
30 °C/65% RH			
4 023.500	7 562.833	9 815.500	EEEE*
40 °C/75% RH			
4 023.500	5 767.000	13 367.500	EEEE*

* Reading is over-range

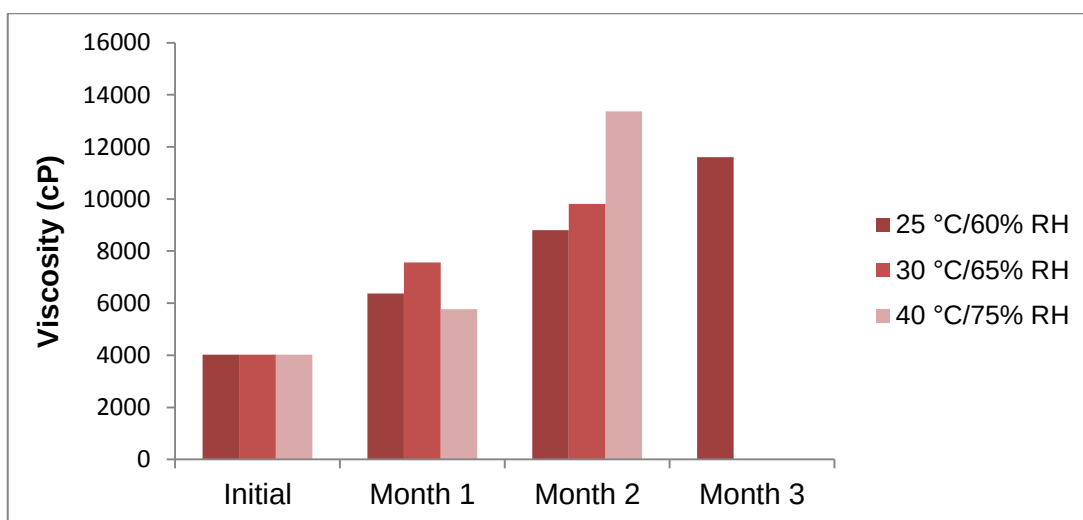


Figure D.26: Viscosity (cP) for **(EC)** at different storage conditions for each time interval

D.3.7.3 Male cream

Table D.24: Viscosity (cP) of **(MC)** at different storage conditions for each time interval

Initial	Month 1	Month 2	Month 3
25 °C/60% RH			
9 154.917	12 579.170	11 772.500	13 289.170
30 °C/65% RH			
9 154.917	12 211.670	11 261.670	13 206.670
40 °C/75% RH			
9 154.917	12 179.170	8 489.333	12 985.000

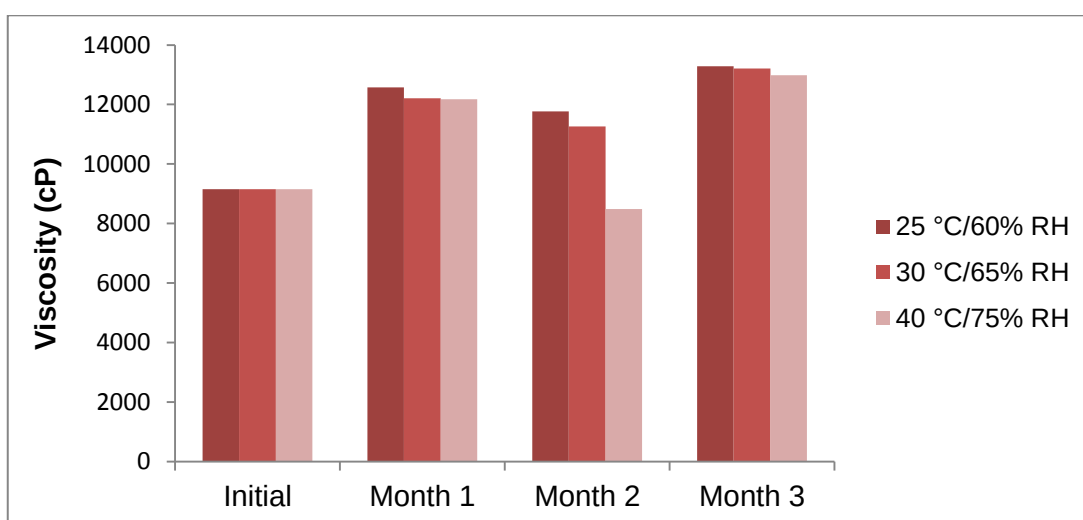


Figure D.27: Viscosity (cP) for **(MC)** at different storage conditions for each time interval

The viscosity of the **(MC)** samples did not remain stable during storage. The sample stored at 25 °C/60% RH demonstrated an increase of roughly 4 052 cP, whereas the sample stored at 40 °C/75% RH displayed an increase of about 3 830 cP. The largest increase was exhibited by the sample stored at 30 °C/65% RH, with a total increase of approximately 4 143 cP over the 3-month period. The total increase in viscosity can be due to the loss of moisture, which was confirmed in Section D.3.2.3. Moreover, certain polymers possess the ability to form hydrogen bonds (Ren *et al.*, 2008:16238). An increase in hydrogen bonds has been shown to increase viscosity (Deshkar *et al.*, 2016:394). In general, hydrogen bonds weaken with increasing temperature, which explains why the sample stored at 25 °C/60% RH displayed higher viscosity compared to the sample stored at 40 °C/75% RH (Dračinský, 2017:20). The exact mechanisms responsible for the increase in viscosity of the **(MC)** during storage were not known.

D.4 Conclusion

For stability testing to be meaningful, it is important to establish parameters that will be evaluated, define the acceptance criteria, as well as the methods that will be used to measure the characteristic variations over the predetermined period (Vieira *et al.*, 2009:516). For this study, the stability of the provided Idexis products was evaluated in conformity with the ICH Guidelines (2003:9). According to O'Donnell & Bokser (2005:1027), knowledge of the stability of a pharmaceutical or cosmetic product is required for three primary reasons. Firstly, the product must appear fresh, appealing and professional for the entire period it remains on the shelf; any change in the physical appearance or odour can cause consumers to lose confidence in the product and the company. Secondly, since the product is dispensed in multi-dose containers, dosage uniformity during usage must be ensured; phase separation can result in non-uniform dosage patterns. Thirdly, the active ingredient must be available to the consumer during the estimated shelf life; degradation can cause non-availability or 'dose dumping' of the active to the consumer.

The assay concentrations of oestriol, oestradiol and progesterone in the **(FS)**, **(EC)** and **(MC)** displayed significant changes and did not remain within the acceptable 5% variation limit (relative to the initial value) during the storage period (ICH, 2003:9). This indicated product instability against increased temperature and humidity due to possible moisture loss, which was confirmed during mass loss studies shown in Tables D.7 – D.9. Moreover, drug-excipient interaction could have occurred, resulting in the formation of impurities and hazardous compounds, which could compromise product quality, safety and performance (Hotha *et al.*, 2016:107). During storage, increased concentrations were displayed by all three female hormones, at all three storage conditions for all three products over the 3-month period. Possible reasons for the depredation could be attributed chemical interactions, as well as insufficient protection by the selected antioxidants incorporated into each product. Another

possible factor that should not be excluded is inconsistent mixing of the bulk formulation before packaging, which could have resulted in uneven distribution of the hormone concentrations among the containers from which samples were taken during HPLC analysis. The assay results obtained during this study gave a clear indication that the products were not stable at 25 °C/60% RH, 30 °C/65% RH or 40 °C/75% RH. Due to the limited information regarding the formulations, no possible reason could be recognised for the instability displayed by the products other than moisture loss.

Moisture loss due to elevated storage conditions can be monitored through mass loss studies. During storage, the mass of the **(FS)**, **(EC)** and **(MC)** did not remain stable. Overall, the samples stored at all three storage conditions displayed a decrease in mass that was not within the acceptable 5% variation limit (relative to the initial values), as described by the ICH Guidelines (2003:9), which could be attributed to the porosity of the container or the container lid not sealing sufficiently, allowing moisture to escape through the closure gaps or the container walls to the outer environment. The evaporation of moisture from the product could lead to product instability as a result of phase separation (Stephane, 2015:7). The lowest decrease in mass was displayed by the **(MC)** samples stored at 40 °C/75% RH, with a decrease within the acceptable range. According to Waterman *et al.* (2016:1572), moisture loss is relative to the difference in the RH between the inner and outer atmospheres. The small change in mass observed in this sample could be due to the moisture equilibrium being reached much quicker between the moisture content within the container headspace and the surrounding atmosphere. The results obtained during viscosity testing and visual examination corresponded with the loss of moisture observed over the 3-month period. All three products stored at all three storage conditions demonstrated an increase in viscosity, while visual examination revealed textural changes, especially in the **(EC)** and **(MC)**; both formulations became thicker with a mousse-like consistency.

The visual appearance of the **(EC)** and **(MC)** showed significant changes over the 3-month period. The samples stored at 40 °C/75% RH displayed the biggest change in colour and texture. The **(EC)** samples changed from an oatmeal colour to a mocha colour, whereas the **(MC)** samples changed from a light cream colour to a slightly yellow colour over the 3-month period. In general, elevated temperatures promote degradation, which could affect the colour of a formulation (Aulton, 2013:844; Naveed & Sajid, 2016:81). Similar textural changes were observed in both products with the formation of air bubbles, which could have been the result of product shrinkage, and possibly contributed to the thick, mousse-like consistency. Overall, the **(FS)** samples stored at different conditions displayed no significant change in colour; however, the sample stored at 40 °C/75% RH displayed phase separation after 3 months, possibly due to the loss of moisture. Regarding the odour, the slight, but not unpleasant clean smell emitted by all three products intensified to an unpleasant, egg-like smell over the 3-month period.

The pH of the **(FS)**, **(EC)** and **(MC)** stored at different climatic conditions remained stable over the 3-month period. Human skin is highly sensitive to extreme pH variations, while the stratum corneum can remain unaffected, tolerating a pH range from 3 – 9 (Barry, 2002:512). Therefore, all three products were considered safe for topical use without any risk of irritation. The **(FS)** and **(MC)** samples proved to be more stable stored at 30 °C/65% RH, while the **(EC)** samples displayed the best stability stored at 25 °C/60% RH. Possible antioxidant activity could explain the stability regarding the pH observed in all three products by preventing accelerated oxidative degradation.

Zeta-potential can be influenced by various factors, the most important one being the pH. As the pH decreases the surface charge of a particle (i.e. zeta-potential) becomes more positive (Layek & Singh, 2017:228; Kurama & Karaguzel, 2013:580). This statement corresponds with the result obtained during stability studies, as the zeta-potential values became more positive as the pH of the samples decreased over time. Zeta-potential values lower than - 30 mV or higher than ± 30 mV represent product stability due to adequate repulsive forces between the particles (Joseph & Singhvi, 2019:99). The **(FS)**, **(EC)** and **(MC)** displayed highly negative zeta-potentials over the 3-month period, proposing stability against elevated temperature and humidity, with lower probability to coagulate or flocculate. Although the zeta-potential value increased during storage, indicating a decrease in stability, the values remained within the acceptable range over the 3 months. The **(MC)** proved to be the most stable formulation, displaying a decrease in zeta-potential at all three storage conditions over the 3-month period.

The **(FS)** displayed an increase in the particle size at all three storage conditions over the 3-month period. Both the **(EC)** and **(MC)** exhibited an increase in particle size in the samples stored at 40 °C/75% RH. These results corresponded with the increased zeta-potential values, as well as the phase separation revealed during visual examination. This could be a result of stronger Van der Waals forces between the particles, which led to flocculation and sedimentation. The **(EC)** and **(MC)** samples stored at 25 °C/60% RH and 30 °C/65% RH were considered to be more stable, considering both samples displayed highly negative zeta-potential values, as well as decreased particle sizes over time.

The viscosity of the **(FS)**, **(EC)** and **(MC)** displayed an overall increase over the 3-month period. The **(EC)** samples stored at 30 °C/65% RH and 40 °C/75% RH failed to provide useful readings, as the centipoise (cP) value of both samples were not within the FSR of the spindle/speed combination used (15 600 cP). From visual inspection, it can be stated that the viscosity increased at both conditions. Possible reasons for the increase in viscosity can be ascribed to moisture loss, which was confirmed during mass loss testing. However, the exact mechanism responsible for the increase in viscosity is not known.

The changes observed in the physical and chemical stability of the **(FS)**, **(EC)** and **(MC)** during storage possibly altered the efficacy, safety and quality of each product. No product completely met the ICH criteria for stability.

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APPENDIX E:

FRANZ CELL DIFFUSION STUDIES OF TOPICAL FORMULATIONS CONTAINING OESTRIOL, OESTRADIOL AND PROGESTERONE

E.1 Introduction

In postmenopausal women, skin ageing results from the synergistic effects of oestrogen deficiency (hormonal ageing), age and genetic factors (intrinsic ageing), as well as UVR (photoageing). HRT has been found to be effective in not only reducing menopausal symptoms (i.e. hot flushes and sleep disturbances), but also preventing or reversing skin ageing (Gupta *et al.*, 2016:95; Pritchard, 2001:353; Vaillant & Callens, 1996:67). However, the widespread use of HRT came to a sudden halt in the early 2000s after the initial results were released of the WHI trial and the study was ended. Interim analysis led to concerns about hormone-related adverse effects, primarily an increased risk of breast cancer with the use of combination therapy (oestrogen and progesterone) (Palacios *et al.*, 2019:1). Due to the climacteric impact on skin and its evident improvement with the use of HRT (Phillips *et al.*, 2001:665; Sator *et al.*, 2001:43; Thornton, 2013:268), attempting the topical delivery of female hormones could prove advantageous, as it will improve skin texture, elasticity and dryness, as well as reduce the appearance of wrinkles while limiting systemic absorption and the risk of hormone-induced breast cancer (Farrell, 2016:e93; Rzepecki *et al.*, 2019:89).

The skin offers a convenient and accessible site for the administration of actives (Alkilani *et al.*, 2015:438). Topical drug delivery results in the accumulation of APIs in the upper skin layers, an effect that is generally desired for cosmetic products, whereas systemic absorption (through the skin layers) is required for transdermal drug delivery (Chuang *et al.*, 2017:1309; Garg *et al.*, 2013:969; Kanfer, 2017:53). Human skin is classically considered a target for numerous hormones, of which the induced effects have been characterised over years of research (Zouboulis, 2000:230). Topical administration of female hormones can be a practical alternative to oral delivery, since it allows direct application to the targeted area, avoids first-pass metabolism and has a decreased risk of systemic adverse effects (Hagen & Baker, 2017:1623). The skin itself, however, constitutes a formidable barrier and presents difficulties for topical drug delivery (Uchechi *et al.*, 2014:194). The skin is a multi-layered structure, consisting of three distinct layers (Lu *et al.*, 2016:499). The outermost layer, known as the stratum corneum, primarily provides protection and functions as the main barrier, preventing water loss and the penetration of potentially damaging chemicals and pathogens from the surrounding environment into the body (Marks, 2004:2017S; Van Smeden *et al.*, 2004:295). Keratin-filled corneocytes are surrounded by a lipophilic matrix composed of cholesterol, long chain ceramides and free fatty acids with the characteristic absence of phospholipids (Bouwstra & Ponc, 2006:2080;

Foldvari, 2000:418; Naik *et al.*, 2000:318). The lipid composition plays an integral part in regulating the permeation and absorption of actives through the skin (Baroni *et al.*, 2012:259; Hendriks *et al.*, 2006:259). As a result, the stratum corneum serves as an API flux regulator and rate-limiting barrier as it influences the amount of active that can move across the skin layers (Baroni *et al.*, 2012:259; Hendriks *et al.*, 2006:259; Williams, 2003:28). It is therefore essential that the API intended for topical delivery possesses the ideal physiochemical properties to promote skin permeation and absorption (Ruela *et al.*, 2016:529). Moreover, the selection of an appropriate formulation is important, since the interaction of the vehicle with the skin can alter the ability of the penetrant (Nalamothu, 2015:1).

The assessment of transcutaneous permeation of drug molecules is one of the fundamental steps in the preliminary design and evaluation of transdermal and topical delivery systems (Godin & Touitou, 2007:1152), as it provides insight into understanding the efficacy and safety of the drug before initiating clinical studies (Hermsmeier *et al.*, 2018:5416). A range of methods have been developed to measure skin permeation, including *in vitro* (using animal or human skin samples), *in vivo* (i.e. on human participants) and *in silico* (computational model) (Rezaee & Abdollahi, 2017:237). The most reliable data is obtained from *in vivo* human studies, which involve the application of an API (incorporated within a vehicle) to specific body sites of research participants, followed by the collection of data using non-invasive optical methods, tape stripping or blood samples (Kattou, 2017:13). However, *in vivo* studies are often infeasible and unethical as the adverse effects of a given compound are not entirely predictable (Rezaee & Abdollahi, 2017:238). A significant challenge for research regarding skin permeation of APIs is the development of robust methods that can consistently correlate *in vivo* and *ex vivo* data, so as to shorten and economise the processes residing under the scope of dermal delivery (Godin & Touitou, 2007:1153; Kattou, 2017:13).

Mathematical models can be utilised to better understand skin permeation. The fundamentals of mathematical modelling of topical and transdermal delivery were set in the 1940s to 1970s. During that period, it was recognised that solubility and partitioning were significant factors that influenced skin permeation (Mitragotri *et al.*, 2011:115). Stephen Rothman first identified the importance of the physicochemical characteristics of an API and the major influence of the selected vehicle on permeation (Rothman, 1943). Higuchi (1960) recognised that Fick's first law could be used to characterise the transport process in skin. The resultant equations defined fundamental relationships between drug concentrations, release rates and transport properties, which laid the foundation of the modern principles of skin permeation (Mitragotri *et al.*, 2011:115). In the years that followed, the scientific activity, with regard to the mathematical characterisation of numerous skin processes, has increased dramatically (Kattou, 2017:19; Mitragotri *et al.*, 2011:116).

The permeation of compounds across the skin barrier is considered to be primarily driven by passive diffusion (Prausnitz & Langer, 2008:1262; Vitorino *et al.*, 2015:2702), as there has been limited evidence to indicate there are any active transport processes involved (Hadgraft, 2001:2). Therefore, following Higuchi's observation, Fick's first law has been utilised to describe steady-state and passive diffusion of uncharged compounds across homogeneous barriers (Schaefer & Redelmeier, 2011:216; Williams, 2013:681). The law states that the rate of diffusion of a compound across a unit area is directly proportional to the concentration gradient (Mondal *et al.*, 2012:60). Moreover, this mathematical model (Equation E.1) can be used to evaluate the transport and penetration of APIs across skin during *in vitro* studies (Dancik *et al.*, 2008:182; Hadgraft, 2001:2).

$$J = DK\Delta C / h \quad \text{Equation E.1}$$

Where:

- J = Diffusive flux
- D = API diffusion coefficient
- K = Skin-vehicle partition coefficient
- ΔC = Concentration difference
- h = Diffusional path length (stratum corneum thickness)

Generally, the applied concentration is greater than the concentration present in the skin; hence, Equation E.1 can be simplified to Equation E.2 (Hadgraft, 2001:2).

$$J = k_p C_{app} \quad \text{Equation E.2}$$

Where:

- k_p = Permeability coefficient (= KD / h)
- C_{app} = Applied concentration

This mathematical model can provide further information in terms of diffusivity, as it also relates the drug flux. According to the Fickian relationship, an increase in API diffusivity results in a corresponding increase in percutaneous drug flux (Naik *et al.*, 2000:320). Moreover, the diffusion and delivery of APIs can be influenced by the physicochemical properties and characteristics of both the API and the skin (Naik *et al.*, 2000:320; Williams, 2013:677).

For this study, Idexis aimed to formulate topical products that deliver therapeutically effective concentrations of oestriol, oestradiol and progesterone to the skin. The formulator utilised polymers to inhibit skin penetration and systemic absorption, which consequently reduces the

risk of breast cancer associated with elevated hormone concentrations. Due to the classified nature of this study, limited information was received regarding the formulation of the products. Therefore, three **(NOE)** (as discussed in Appendix C) were formulated with the aim of comparing the *in vitro* diffusion study results obtained for the simple formulations with that of the complex Idexis formulations to evaluate the efficacy of the vehicle and polymer used to inhibit skin penetration and systemic absorption of the respective hormones. The delivery of topical and transdermal systems is evaluated by use of *in vitro* diffusion studies, therefore, *in vitro* membrane release and skin diffusion studies were performed by utilising the vertical Franz cell method (Salamanca *et al.*, 2018:3; Williams, 2013:683). Release studies were performed prior to diffusion studies to determine if the female hormones were released from the **(NOE)** (**(CoOE)**, **(CaOE)** and **(EOE)**) and the Idexis products (**(FS)**, **(EC)** and **(MC)**), respectively. Thereafter, skin diffusion studies together with tape stripping, were performed to determine whether transdermal delivery and/or topical delivery was achieved for both the **(NOE)** and the Idexis products.

E.1.1 Physiochemical properties of the selected female hormones

E.1.1.1 Aqueous solubility

Solubility describes the ability of a liquid, solid or gaseous chemical substance (i.e. solute) to dissolve in a solvent (liquid, solid or gaseous) to form a homogenous solution. The solubility of an API is highly dependent on the solvent used, along with pH, temperature and pressure (Lynch *et al.*, 2001:1549) and is measured as the saturation concentration, where any additional solute added to the solution does not increase its concentration (Savjani *et al.*, 2012:1). This value indicates the amount of API that will dissolve in the specific solvent, and as a result, the amount available for absorption through the skin (Khadka *et al.*, 2014:304; Steele & Austin, 2009:24). Hence, solubility is an important contributing factor to consider when formulating topical or transdermal products (Singh *et al.*, 2017:674). Naik *et al.* (2000:319) proposed that an API with an aqueous solubility higher than 1 mg/ml is considered ideal for topical or transdermal drug delivery. According to the Encyclopaedia Britannica (2016), the permeation of a compound is directly proportional to its solubility; consequently, low aqueous solubility often results in poor skin permeation (Kulkarni & Nagarsenker, 2008:466; Orme, 1984:60; Savjani *et al.*, 2012:1). Moreover, the solubility and permeability of an API is related to its lipophilic character (Stocks, 2013:96). In this study, oestradiol was considered to be the most lipophilic hormone (log P of 4.0) followed by progesterone (log P of 3.9) and oestriol (log P of 2.4), which can be correlated when comparing the aqueous solubility of the respective hormones summarised in Table E.1.

Table E.1: Aqueous solubility (mg/ml) of the respective female hormones obtained from literature

Hormone	Aqueous solubility (mg/ml)
Oestriol	0.1190 ^a
Oestradiol	0.0015 ^b
Progesterone	0.0168 ^c

a) Drugbank, 2007; b) Shareef *et al.*, 2006:880; c) Haskins, 1949:229

From Table E.1, it can be observed that oestriol, oestradiol and progesterone are practically insoluble in an aqueous medium, and so it can be stated that the solubility of the hormones is not ideal for permeation across the skin. However, a saturated formulation of an API will effectively provide the maximum flux (maximum flow rate across a unit area), regardless of the selected vehicle and solubility of the API therein (Kuswahyuning *et al.*, 2015:113; Naik *et al.*, 2000:320; Singh, 2016:239). Formulators are familiar with the statement of Higuchi (1960), which specifies that under ideal circumstances, all saturated formulations of a particular API will yield the same steady-state flux through the skin. Simply put, an API having a solubility of 1 mg/ml and 10 mg/ml in vehicles 1 and 2, respectively, will penetrate the skin at an equal rate when delivered from saturated formulations in both vehicles, provided that the vehicles do not interact with or alter the membrane in any way (Naik *et al.*, 2000:320). However, during this study saturated formulations were not formulated, although, for future reference, this technique could be utilised to improve the permeation flux of hormones (poor aqueous soluble APIs) across the skin if required. Note that in normal skin, percutaneous absorption is not greater than the maximum flux (Singh, 2016:239).

E.1.1.2 Lipophilicity

Lipophilicity is the affinity of a chemical compound to a lipophilic environment (Stoner *et al.*, 2008:135; Waterhouse, 2003:376). The log P describes the tendency of a neutral compound to dissolve in an immiscible two-phase system of lipid (organic solvents, oils, fats) and water (Bannan *et al.*, 2016:4015). Therefore, it is a useful quantitative parameter for demonstrating: 1) the lipophilic nature of an API, which also reflects 2) the thermodynamic tendency of an API to preferentially partition in different mediums (Domine *et al.*, 1996:47). The skin is a complex multi-layered membrane that changes from a lipophilic outer layer (stratum corneum) to a more aqueous internal structure (lower epidermis and dermis) (Abd, 2016:10).

APIs with high log P values are considered more lipophilic, and will therefore partition into the lipophilic stratum corneum more effortlessly; however, partitioning into the hydrophilic epidermis is more challenging, which often results in decreased permeation rates, and consequently, the temporary retention of the APIs within the skin. This process is referred to as the reservoir

effect (Holmgaard & Nielsen, 2009:20). The reservoir function of the skin is a key determining factor of the duration of action of topically applied APIs (Roberts *et al.*, 2004:3). It is generally considered that the higher the log P value, the greater the probability of the API being retained within the skin (Akomeah *et al.*, 2004:343; Hagedorn-Leweke & Lippold, 1988:220). Steroids have been reported to demonstrate a reservoir effect in the stratum corneum, where continued permeation is observed for a significant time period, even after the removal of the donor phase from the skin's surface. This effect has been attributed to the increased level of partitioning into the intercellular lipids and the moderately slow release rate from this compartment in the stratum corneum (Cross *et al.*, 2003:760).

Table E.2: Log P of each respective female hormone

Hormone	Log P
Oestriol	2.4 ^a
Oestradiol	4.0 ^b
Progesterone	3.9 ^c

a) Moffat *et al.*, 2004:987; b) Moffat *et al.*, 2004:986; c) Moffat *et al.*, 2004:1486

According to Naik *et al.* (2000:319), an API with a log P value between 1 and 3 is considered ideal for topical delivery; however, Williams (2013:680) proposed that a log P value between 1 and 4 could also be deemed acceptable. From Table E.2, it can be stated that oestriol, oestradiol and progesterone are considered ideal for topical delivery as their log P values are within the acceptable range (Williams, 2013:680).

E.2 Methods

E.2.1 HPLC analysis of oestriol, oestradiol and progesterone samples

To determine and quantify each respective female hormone (oestriol, oestradiol and progesterone) present in the samples collected throughout *in vitro* diffusion studies, a validated HPLC analytical method was developed. As discussed in Appendix A, this analytical method was successfully validated and proved to be sensitive, responsive and reliable for the quantification of each female hormone concentration present in the **(FS)**, **(EC)** and **(MC)**, as well as the **(CoOE)**, **(CaOE)** and **(EOE)**.

The samples intended for HPLC analysis were extracted from the Franz cell receptor chamber during *in vitro* diffusion studies and analysed with an Agilent® 1200 Series HPLC system fitted with an Agilent® 1200 gradient pump, UV detector and autosampler injection mechanism (Agilent Technologies, Unites States of America). The system was operated on ChemStation Rev. A.10.03 data acquisition and analysis software. A reverse phase Venusil® XBP C₁₈ (2) column (150 x 4.6 mm) with high purity silica particles (5 µm) was used (Agela® Technologies,

Unites States of America). The mobile phases consisted of Milli-Q[®] water (in-lab) with 0.1% H₃PO₄ and acetonitrile (HPLC grade). Specific chromatographic conditions (listed in Table E.3), as well as a constant temperature of 25 °C, were maintained throughout analysis in the laboratory. A standard solution was prepared prior to each analysis by dissolving ± 10 mg oestradiol in 50 ml methanol, thereafter 10 ml of the solution was transferred into a 200 ml volumetric flask to which ± 10 mg oestriol and ± 20 mg progesterone were added and made up to volume with methanol. The standard solution was injected as follows to create a standard curve for each analysis:

- 1 µl
- 5 µl
- 10 µl
- 15 µl
- 20 µl

Table E.3: Chromatographic conditions used during the analysis of samples extracted from the receptor chamber to determine the concentration of each female hormone

Injection volume	20 µl
Run time	15 min
Detection wavelength	210 nm
Flow rate	1.0 ml/min
Retention time	Oestriol: ± 3.8 min Oestradiol: ± 5.1 min Progesterone: ± 7.6 min

During HPLC analysis of the samples extracted from the receptor phase of every Franz diffusion cell, following the completion of membrane release and skin diffusion studies, a second peak eluted at 5.036 min, which interfered with oestradiol, forming a split peak (as displayed in Figure E.2.c). Not only does this complicate the accurate integration of oestradiol for quantification, but it may potentially be an impure peak. Therefore, peak purity was determined by comparing the UV spectra of 1) oestradiol in the standard solution with 2) the split peak in the receptor phase sample. Figure E.1 illustrates an integrated chromatogram following the HPLC analysis of a standard solution injected at the default volume (20 µl), from which the UV spectrum of oestradiol (5.120 min) was used as a reference. The oestradiol peak was pure as indicated by the green band (displayed in Figure E.3.b). From the UV spectrum it can be observed that oestradiol was absorbed at 280 nm (as shown in Figure E.3.a), which corresponds with the spectrum provided by literature (Moffat *et al.*, 2004:1351, 1964). From the UV spectrum of the split peak (as displayed in Figure E.4.a), it can be observed that the

compound was absorbed at 240 nm. Hence, it can be stated that the peak that eluted at 5.036 min in the chromatogram of the receptor phase samples was not oestradiol. It is further established by the impurity of the peak, which is indicated by the red band (as seen in Figure E.4.b). Hence, oestradiol together with progesterone was not quantified during this study as its peak (7.349 min) was too small to integrate accurately (as demonstrated in Figure E.2.d).

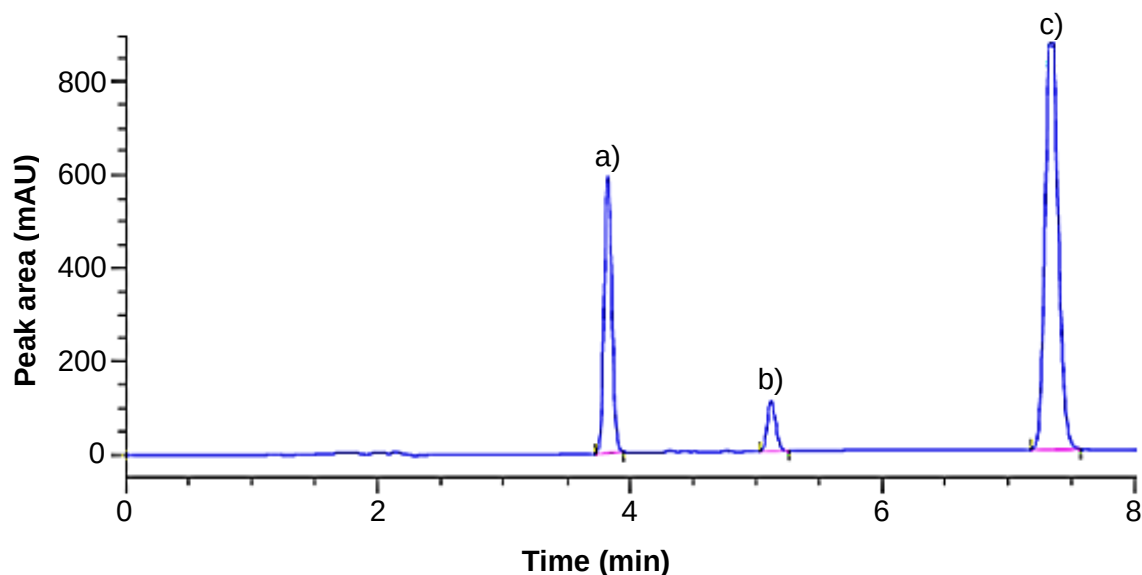


Figure E.1: Integrated chromatogram of: a) oestriol (± 3.8 min), b) oestradiol (± 5.1 min) and c) progesterone (± 7.3 min) following the HPLC analysis of a standard solution

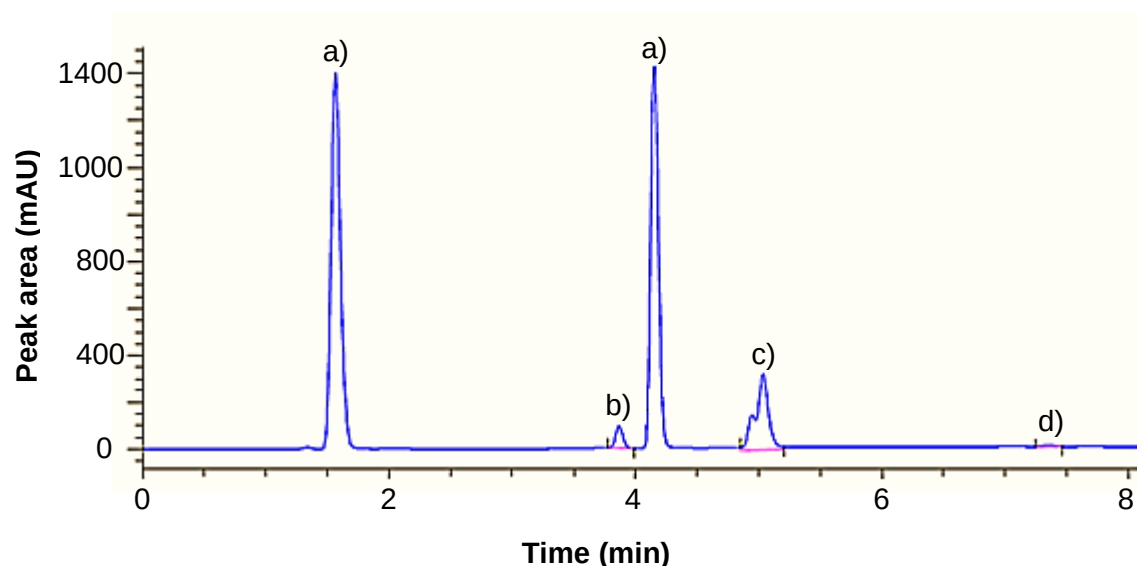


Figure E.2: Integrated chromatogram of: a) impurity/formulation compounds (± 1.6 min and ± 4.2 min), b) oestriol (± 3.8 min), c) oestradiol (± 5.0 min) and d) progesterone (± 7.4 min) following the HPLC analysis of a sample extracted from the Franz cell receptor chamber during an *in vitro* diffusion study

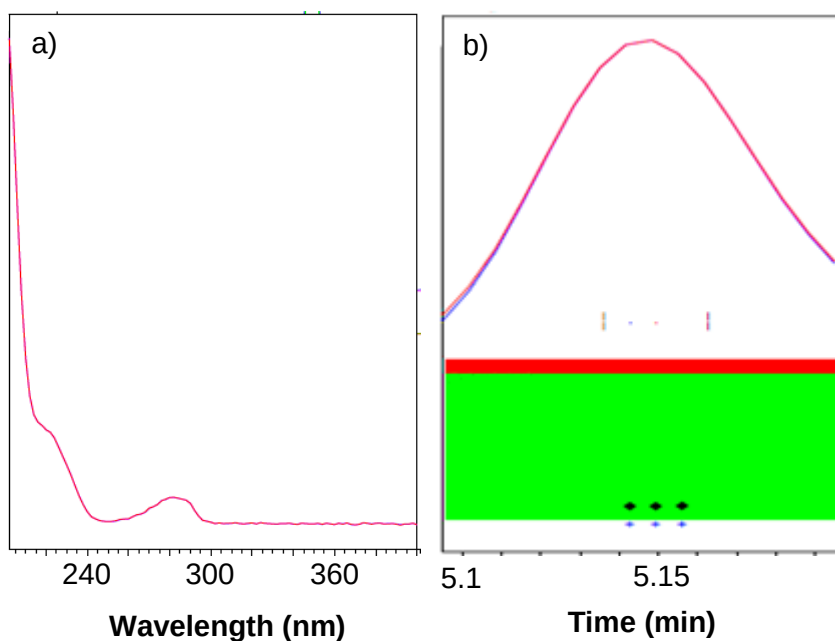


Figure E.3: a) UV Spectra and b) purity of oestradiol peak (5.148 min)

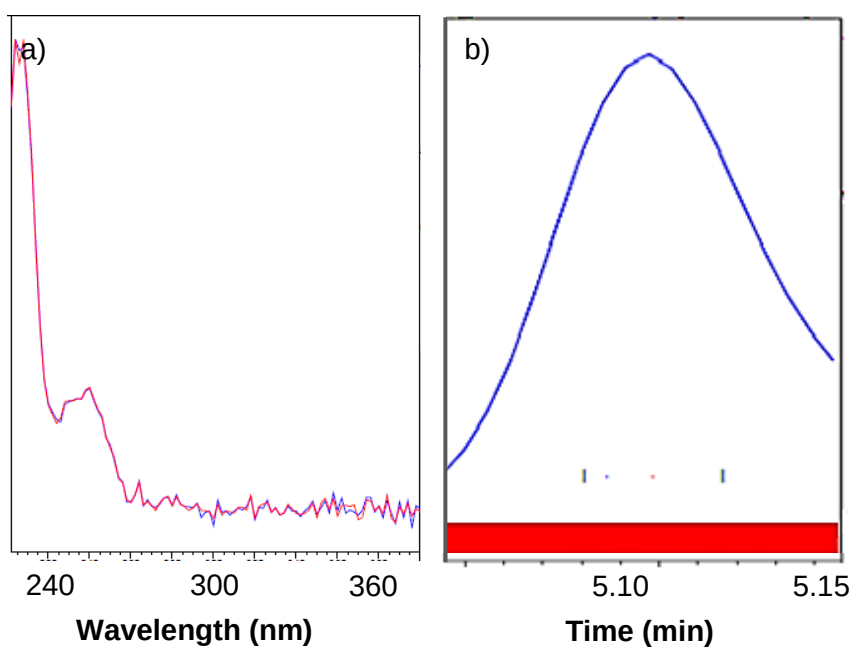


Figure E.4: a) UV Spectra and b) purity of split peak (5.036 min)

Figure E.5 illustrates a chromatogram of the split peak at a lower wavelength (254 nm). Although the peak is too small to determine the wavelength at which the compound was absorbed, the red band indicates the impurity of the peak that eluted at 5.036 min.

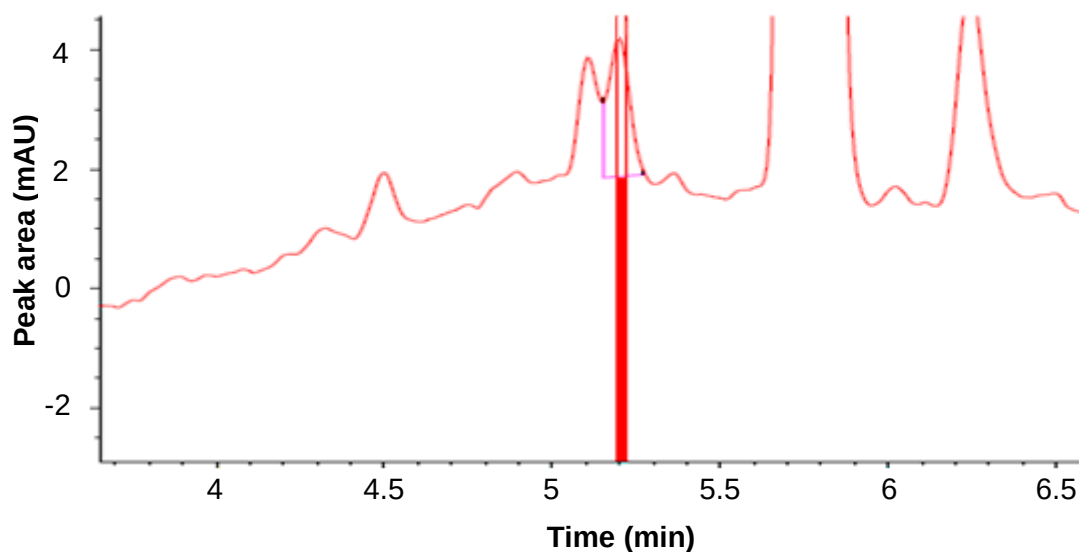


Figure E.5: Chromatogram of the receptor phase sample at a lower wavelength (254 nm)

E.2.2 *In vitro* diffusion studies: vertical Franz cell method

In vivo studies prove to be the most optimal method for drug development and risk analysis by determining the rate and extent of API absorption through the skin. However, *in vitro* studies are often favoured due to the ethical and economic considerations coupled with *in vivo* studies (Karadzovska & Riviere, 2013:569). To accurately predict *in vivo* percutaneous absorption of APIs, robust and validated *in vitro* methods are required (Bartosova & Bajgar, 2012:4671; Ruela *et al.*, 2016:529). Vertical Franz diffusion cells are the most widely used *in vitro* method to assess drug delivery into and across the skin (Kim *et al.*, 2019:418). Moreover, this method presents with numerous advantages, which include (Carrer *et al.*, 2018:73; Salamanca *et al.*, 2018:148; Sartorelli *et al.*, 2000:134):

- minimum handling of biological tissue;
- experimental conditions are easily controlled;
- low cost;
- no constant sample collecting;
- small amount of drug needed for analysis;
- time-efficiency, and
- good reproducibility.

Franz cells consist of two chambers, donor and receptor, which are separated by either a polyvinylidene fluoride (PVDF) synthetic membrane (during release studies) or dermatomed

human skin samples (during permeation studies) (Simon *et al.*, 2016:235). During *in vitro* studies, it is essential that diffusional sink conditions (i.e. initial API concentration in the receptor chamber is zero) are maintained, allowing adequate drug release over an extensive time period for accurate analysis (Naik *et al.*, 2016:18; Ng *et al.*, 2010b:1440). Although perfect sink conditions are practically impossible to achieve in a static diffusion cell, for experimental purposes, sink conditions occur when the API concentration in the receptor chamber is not greater than 10% of its saturation solubility concentration (Ng *et al.*, 2010b:1440). However, according to the European Medicines Agency (EMA, 1999:12), the concentration of the API present in the receptor chamber, should not exceed 30% of its saturation concentration.

E.2.2.1 Experimental parameters

According to Modi & Shah (2015:1) controlling *in vitro* conditions to resemble the *in vivo* environment as closely as possible allows reliable extrapolation of data. A study conducted by Klein (2013:565) revealed the influence that different experimental parameters have on the outcome of *in vitro* permeation studies carried out in Franz cell systems. These experimental parameters include temperature, stirring conditions and membrane type (Ng *et al.*, 2010b:1432).

E.2.2.1.1 Temperature

Temperature represents a key parameter, as it directly affects API diffusivity and solubility (Ng *et al.*, 2010b:1440). As a rule, percutaneous flux displays a two-fold increase with every 10 °C rise in temperature. However, a study conducted by Akomead *et al.* (2004:340) reported an approximate two-fold increase in the percutaneous flux of methyl paraben, butyl paraben and caffeine, with an increase of 7 – 8 °C in the receptor temperature. Ogiso *et al.* (1998:71) and Clarys *et al.* (1998:282) attributed this enhancement to an increase in fluidity of stratum corneum lipids, which could lead to a corresponding expansion in intercellular space, and therefore an increase in skin permeation. Hence, temperature should be monitored closely as fluctuations could significantly influence the barrier structure and API diffusion rate, and consequently the results obtained during *in vitro* studies (Ohara *et al.*, 1995:283; Shahzad *et al.*, 2015:2). For this study, two water baths were preheated to:

1. 37 °C (normal *in vivo* environment) to submerge the receptor phase of the Franz cell, and
2. 32 °C (normal external skin temperature) to preheat the Idexis products and the **(NOE)** (Williams, 2013:685).

Additionally, it was determined that temperature fluctuations can occur during sampling, as it can cause the formation of air bubbles just below the membrane, leading to a momentary (< 1 min) drop in temperature. Air bubbles can be removed via the sampling port by tilting the

Franz cell; this action should be performed as quickly as possible so as to minimise the extent of cooling (Ng *et al.*, 2010b:1440).

E.2.2.1.2 Stirring conditions

A disadvantage of vertical Franz cell configuration is that the receptor fluid may be poorly stirred, resulting in inaccurate low permeation for lipophilic APIs that experience major diffusional resistance in aqueous layers (Miller & Kasting, 2011:705). Hence, sufficient stirring of the receptor fluid is essential for (Friend, 1992:239; Ng *et al.*, 2010b:1440):

- maintaining both uniform drug distribution and thermal equilibrium, and
- minimising the unstirred boundary layer and diffusional resistance.

Inefficient stirring can occur when there is: 1) fast stirring at the base of the receptor chamber, but minor stirring in the upper portion, or 2) efficient mixing throughout the majority of the chamber, but not within the vicinity of the sampling port (Ng *et al.*, 2010b:1440). These effects might cause deviation from ideal sink conditions, as the API is not distributed uniformly throughout the receptor cell (Friend, 1992:239). The unstirred boundary layer adjacent to the membrane acts as an additional diffusion-limiting barrier (Vrana *et al.*, 2005:849). It has been shown that as the stirring rate increases, the thickness of the unstirred boundary layer decreases, and therefore the permeation rate of the API increases (Naik *et al.*, 2016:17; Friend, 1992:239). However, excessive stirring could cause vortex formation, which is unwanted as it can cause disruption of the unstirred boundary layer (Sesto Cabral *et al.*, 2014:623). Variation in the thickness of this layer between respective Franz cells could produce wide-ranging permeation results (Brodin *et al.*, 2010:141). The ideal stirrer bar-speed combination produces the highest possible stirring rate without creating a vortex (Ng *et al.*, 2010b:1440). For this study, a PTFE magnetic stirring bar (7.5 x 2.0 mm) operating at 750 rpm was used.

E.2.2.1.3 Membrane type

The FDA has recommended the use of porous synthetic membranes to assess the performance of topical formulations as they act as a support without establishing a rate-limiting barrier (FDA, 1997). Unlike human skin samples, these membranes are inert and do not present biological variability subjected to the anatomical location, race and age of the skin donor, skin sample preparation and storage method (Carrer *et al.*, 2018:74). Shah *et al.* (1989) conducted a study using different microporous synthetic membranes with similar pore size and thickness to evaluate the permeation of hydrocortisone from two commercially marketed creams. They found that the drug flux was consistent, independent of the type of membrane (Shah *et al.*, 1989:58). Conversely, Wu *et al.* (1992) investigated 10 types of synthetic membranes with different pore size and thickness to evaluate drug release from commercial ointments. They

found that membranes with different characteristics display different release rates (Wu *et al.*, 1992:1154), therefore careful attention must be given when selecting a membrane for *in vitro* permeation studies (Ng *et al.*, 2010a:209). Moreover, differences in the release rate observed with each membrane might be explained in terms of the API's affinity for the membrane (Jung *et al.*, 2012:275). For this study, PVDF synthetic membrane filters (Pall® Life Sciences, United States of America) with a pore size of 0.45 µm and 25 mm diameter were used, as they have high hydrophobicity, chemical and thermal stability, as well as mechanical strength (Zhang *et al.*, 2014:82).

E.2.2.2 Vertical Franz cell components

For many years, vertical Franz cell diffusion systems have been recognised as a useful *in vitro* method for determining the permeation of drug molecules across the skin (Klein, 2013:565). Therefore, this method was used to determine the release of the selected hormones from both the **(NOE)** and Idexis products, respectively. Furthermore, this method was used to establish whether the respective hormones were delivered transdermally.

E.2.2.2.1 Preparation of receptor phase

The receptor medium used during *in vitro* studies should serve the same function as blood does *in vivo*; consequently, phosphate buffer solution (PBS) at pH 7.4 was used as the receptor phase for both release and skin diffusion studies (OECD, 2011:29). Furthermore, PBS was used as it would not affect the integrity of the human skin samples, leading to inaccurate permeation results (Williams, 2013:685). The PBS (1 000 ml) was prepared as follows:

- Weigh approximately 1.5 g of NaOH in a clean glass beaker and dissolve in 400 ml Milli-Q® water using a magnetic stirring rod (Solution 1).
- Weigh approximately 6.5 g of potassium dihydrogen orthophosphate (KH₂PO₄) in a clean glass beaker and dissolve in 250 ml Milli-Q® water using a magnetic stirring rod (Solution 2).
- Add Solution 1 to Solution 2 while continuously stirring, and fill to volume with Milli-Q® water (1 000 ml).

The pH of the solution was measured using a Mettler Toledo® pH meter (Mettler Toledo, United States of America), fitted with a Mettler Toledo® In Lab® 410 electrode (Mettler Toledo, United States of America). The pH was set and adjusted to 7.4 by adding either:

- H₃PO₄ to lower the pH, or
- NaOH to increase the pH.

E.2.2.2.2 Test formulations and the preparation of the donor phase

During *in vitro* diffusion studies, three Idexis products and three **(NOE)** (as discussed in Appendix C) were investigated. In addition, a full placebo study (12 Franz cells) was run using only dermatomed skin and water in the donor phase to determine the concentration levels of naturally occurring hormones in the skin, which was then subtracted from the diffused hormone concentrations to calculate the true average concentration diffused ($\mu\text{g/ml}$). Figures E.6 and E.7 illustrate the formulations, respectively, which were tested during the membrane release and skin diffusion studies.

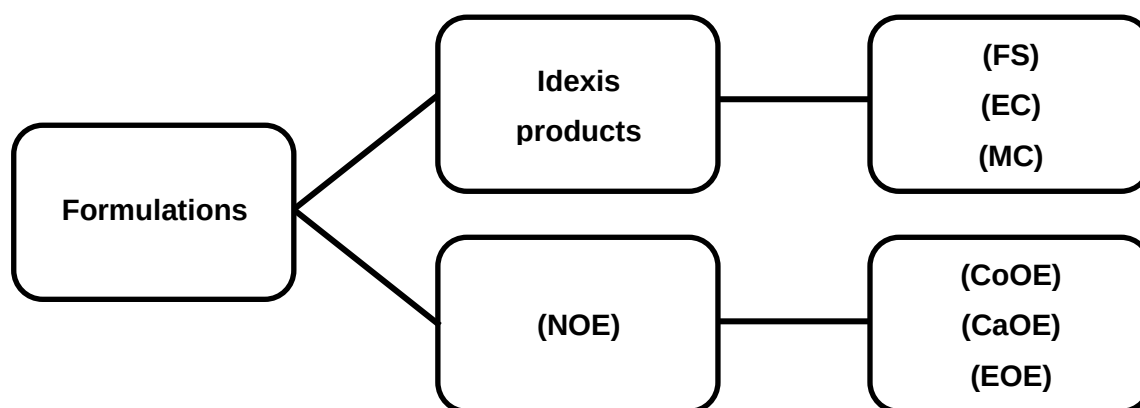


Figure E.6: Diagrammatic representation of the formulations tested during both membranes release and skin diffusion studies

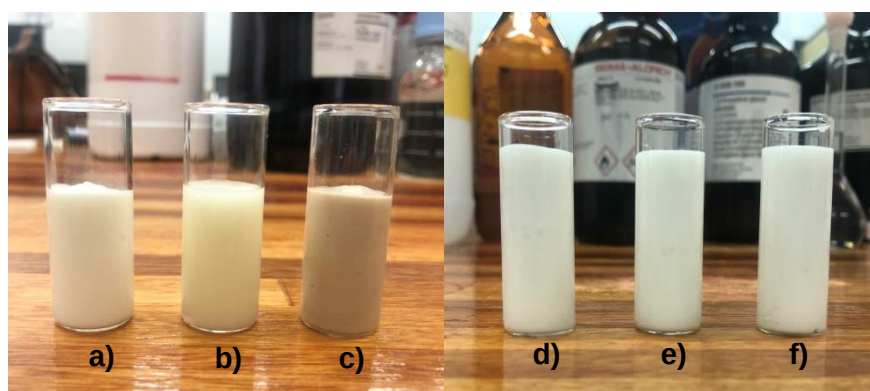


Figure E.7: Idexis products: a) **(MC)**, b) **(FS)** and c) **(EC)** together with **(NOE)**: d) **(CoOE)**, e) **(CaOE)** and c) **(EOE)** tested

E.2.2.3 Membrane release studies

Twelve vertical Franz diffusion cells, each composed of a donor (top) and receptor (bottom) chamber were used to conduct the membrane release studies. During each study, two Franz cells served as a control (as their donor phase contained only water), while the other 10 Franz cells contained the respective test formulations. Prior to performing the release studies, the

formulations were pre-heated to 32 °C in a controlled water bath. A second water bath was used to pre-heat the receptor phase (PBS (pH 7.4)) to 37 °C.

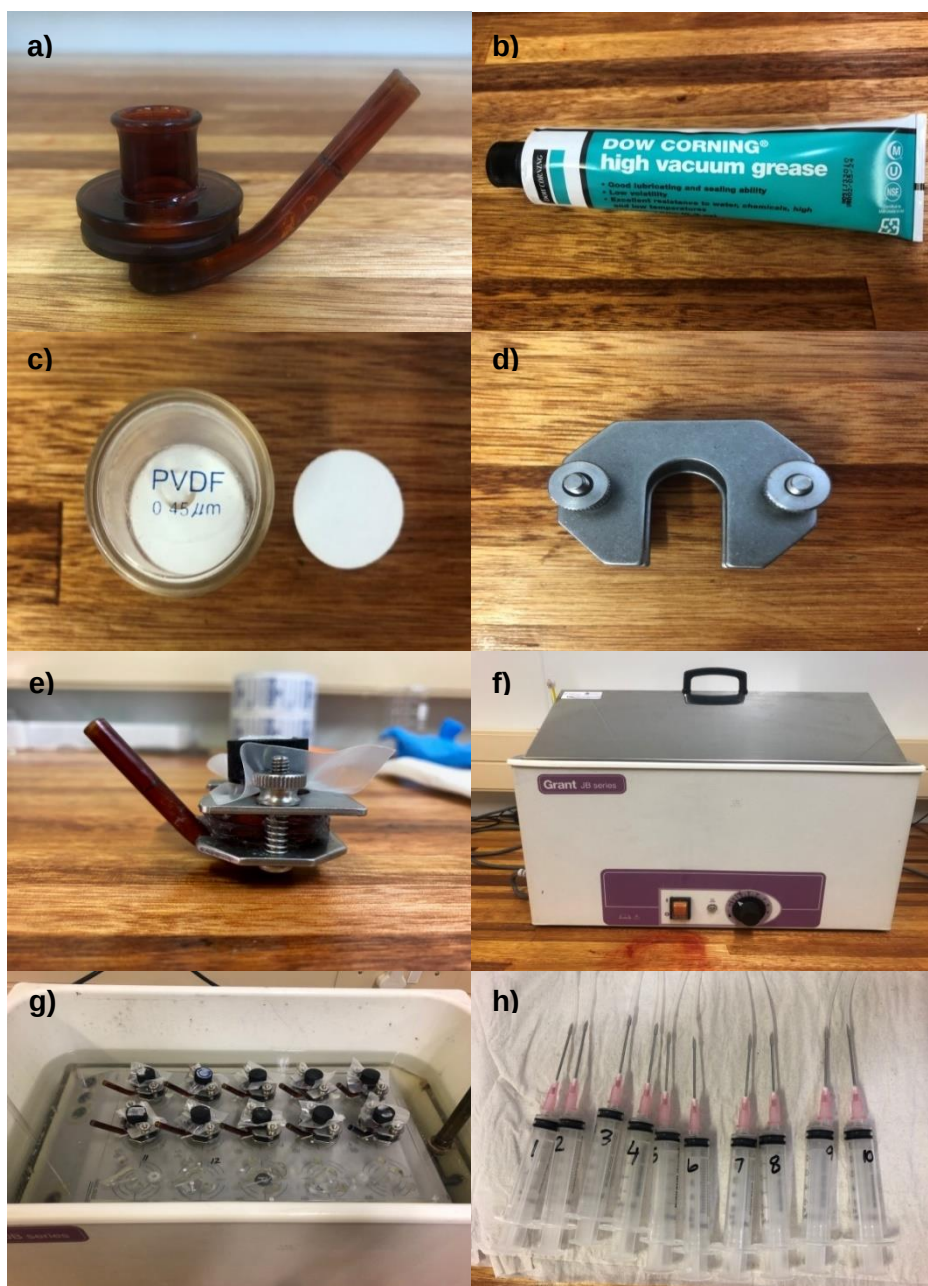


Figure E.8: Apparatus and materials used during membrane release studies in order of use: a) Franz cell with donor (top) and receptor chamber (bottom), b) Dow Corning® high vacuum grease, c) PVDF synthetic membrane, d) metal horseshoe clamp to secure Franz cell chambers, e) Franz cell after filling the chambers, f) Grant® water bath, g) assembled Franz cells in diffusion cell stand, placed on a Variomag® magnetic stirring plate within the preheated water bath and g) marked syringes used for hourly extractions for 6 h

Dow Corning® high vacuum grease was applied to the connecting side of both chambers. A magnetic stirrer (7.5 x 2.0 mm) was added to the receptor chamber prior to placing the PVDF synthetic membrane (Pall® Life Sciences, United States of America) onto the greased side of the receptor chamber. The donor chamber was then placed on top of the receptor chamber, after which vacuum grease was generously applied to seal the sides of the two connected chambers to avoid any possibility of leakage. Lastly, a metal horseshoe clamp was used to secure the two connecting chambers (donor chamber on top of receptor chamber) together to ensure they stayed intact throughout the entire study (this was repeated for all 12 Franz cells). The receptor chamber, with a diffusion surface of 1.075 cm², has a filling capacity of approximately 2 ml; therefore, 2 ml of the preheated PBS (pH 7.4) was used to fill each receptor chamber through the sampling port. Each receptor chamber was visually examined after being filled to confirm that no air bubbles were trapped within the chamber. The donor chambers were then filled with 1 ml of the selected formulation and sealed with a piece of Parafilm® and plastic cap to avoid evaporation of any actives during the study. The assembled Franz cells were placed in a diffusion cell stand within the preheated Grant® water bath (Grant Instruments, United Kingdom) at 37 °C (± 0.5 °C) on a Variomag® magnetic stirring plate (Variomag, United States of America). The lid of the water bath was kept closed throughout the study, except during sample extraction. Hourly, for 6 h, the entire content in the receptor chamber was extracted through the sampling port using the assigned syringe and replaced with new PBS (pH 7.4). The extracted samples were transferred to vials for HPLC analysis (as discussed in Section E.2.1) to determine the concentration and release rate of the actives from the various formulations.

E.2.2.4 *In vitro* skin diffusion

In vitro Franz cell skin diffusion studies together with tape stripping are widely used methods for investigating the transport kinetics and penetration depth of APIs (Escobar-Chávez *et al.*, 2008:104; Salamanca *et al.*, 2018:148). By performing both methods, it can be determined whether the hormones were retained within the stratum corneum-epidermis (SCE) or the epidermis-dermis (ED), representing topical delivery or if the hormones reached the receptor phase, which is indicative of transdermal delivery. The quantification of the APIs within the skin is important for drug delivery research (Escobar-Chávez *et al.*, 2008:104), and therefore data was obtained following the analysis of the respective samples using HPLC.

E.2.2.4.1 Skin ethics and collection

Informed consent can be defined as an individual's right to be presented with all the pertinent information regarding participation in the study to make a decision on whether or not to partake (Madden *et al.*, 2007:61). Due to the ethical implications involved with the use of human skin (biological waste) to study transdermal and topical drug delivery, signed informed consent had

to be obtained from the research participant, the medical practitioner, as well as a witness prior to surgery. The medical practitioner explained the informed consent to the patient and that participation was voluntary, and that the patient could choose to withdraw from the study at any moment. To protect the identity of the patient, any personal information was removed from the excised skin and replaced with an allocated reference number. The name of the donor as well as the respective reference number was recorded in the donor logbook to ensure that if the patient chose to withdraw, the correct skin could be discarded. This information was only available to authorised personnel and postgraduate students. While no formal recruitment occurred for this study, the participants had to voluntarily donate skin (with informed consent) and meet the predetermine criteria.

Female Caucasian skin was used to perform the diffusion studies. The skin samples were obtained from various clinics after the completion of cosmetic abdominal surgery. All the donors were over the age of 21 years in accordance to the National Health Act 61 of 2003, Chapter 8 (South Africa, 2012:31). Skin obtained from men was discarded due to the fact that men have thicker skin compared to women (Lee & Hwang, 2002:188). Despite the similarity in skin thickness (Freeman *et al.*, 1962:298), there are structural differences in the stratum corneum of Caucasian and African skin. The stratum corneum in African skin consists of more cell layers and requires more tape stripping compared to Caucasian skin (Weigand *et al.*, 1974:567). African skin has an increased level of lipid content (Reinertson & Wheatley, 1959:49), lower ceramide content (Sugino *et al.*, 1993:597) and lower TEWL (Warrier *et al.*, 1996:235). Studies have shown a decrease in transcutaneous penetration (Berardesca & Maibach, 1996:668) and diverse vasoactive reactions to substances in African skin compared to Caucasian skin (Berardesca & Maibach, 1989:787). Furthermore, there are significant variations in skin absorption across different regions of the body due to differences in stratum corneum hydration, thickness and lipid composition (Abd *et al.*, 2016:165). Hence, the continuous use of only female Caucasian abdominal skin should reduce the variation in the results obtained. Moreover, abdominal skin is convenient to use due to the large area available and easier to obtain, as abdominoplasty is a common procedure performed on females.

Ethics approval was obtained from the North-West University Health Research Ethics Committee (NWU-HREC) under the larger study, "*The use of human skin (biological waste) to study transdermal and topical delivery*" (Ethics no: NWU-00111-17-A1). Individual ethics approval was also granted by the NWU-HREC (Ethics no: NWU-00111-17-A1-06) for this sub-study. In accordance with SOP-22-003 (collection of skin), the skin was collected and transported in an appropriate container that stated the content was bio-waste, after which the excised skin was stored for a maximum of six months at - 20 °C directly after arriving at the laboratory. Note that this storage condition is appropriate as non-viable (but intact) skin can be used to investigate percutaneous permeation (OECD, 2011:28).

E.2.2.4.2 Preparation of dermatomed skin

It is well-documented that the penetration of lipophilic compounds ($\log P > 3$) through human *in vitro* skin samples is greatly reduced when full-thickness skin (i.e. dermis included in the skin samples; 500 – 1 000 μm thick) is used (SCCP, 2006:7; Wilkinson *et al.*, 2006:410). The dermis is a highly aqueous structure that is considerably less well-perfused *in vitro* when compared to *in vivo*; hence this layer may bring about a permeability barrier for lipophilic compounds (Wilkinson *et al.*, 2006:410). The dermis may retain permeates that, *in vivo*, would have been removed via systemic uptake. Consequently, either the dermis must be removed before *in vitro* studies, or potential *in vitro* retention in the dermis and epidermis (without the stratum corneum) must be taken into consideration when interpreting the results (SCCP, 2006:4). Conversely, for hydrophilic compounds, the stratum corneum provides a rate-limiting barrier, and therefore the use of full thickness skin (with dermis) in permeation studies will not give widely varying results from dermatomed skin (without dermis) (Moser *et al.*, 2001:108). For this study, dermatomed skin samples were used to perform *in vitro* skin diffusion studies.

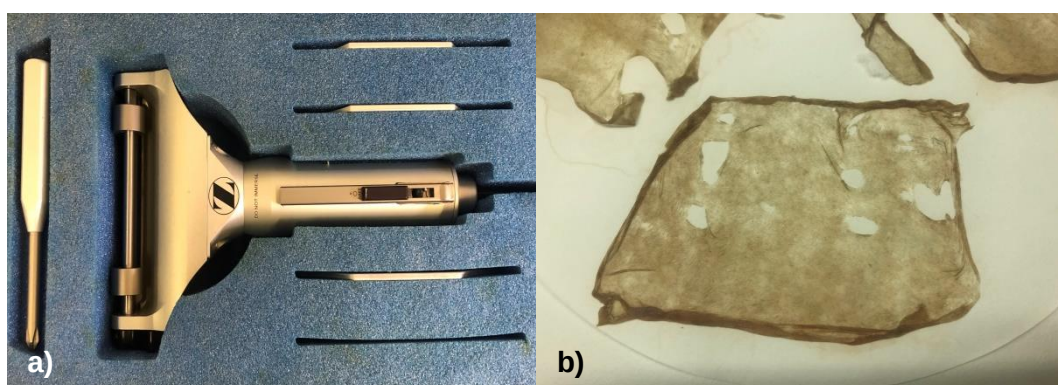


Figure E.9: a) Zimmer™ Electric Dermatome (Zimmer TDS, United Kingdom) and b) dermatomed ($\pm 400 \mu\text{m}$) skin samples on Whatman® filter paper

Prior to the preparation of the dermatomed skin samples, the donated skin was thawed at room temperature (25°C) and closely inspected to ensure there was no damage, abnormalities or stretch marks that could influence the results of this study. Only skin that complied with the standards was used. A Zimmer™ electric dermatome (Zimmer TDS, United Kingdom) (as seen in Figure E.9.a) was utilised to cut skin samples with a continuous thickness of approximately $400 \mu\text{m}$, by pressing on the skin with the apparatus at a $30 - 45^\circ$ angle. The skin samples, with the stratum corneum facing upwards, were placed on Whatman® filter paper (as displayed in Figure E.9.b) to dry and covered with aluminium foil before being stored for a maximum period of 6 months in the freezer (-20°C) for future use. Prior to each diffusion studies, the skin samples were cut into circular pieces (15 mm diameter), so that each circle could fit between the two chambers of a vertical Franz cell.

E.2.2.4.3 Skin diffusion studies

The use of *in vitro* diffusion cells to evaluate skin permeability has advanced into a major research methodology, providing valuable information regarding the relationship between the skin, active ingredient and the formulation (Ng *et al.*, 2010a:210). By making use of *in vitro* methods, percutaneous absorption of APIs can be determined through passive diffusion across the non-viable stratum corneum (Venter *et al.*, 2001:169). The aim of the skin diffusion studies was to determine the API flux from: 1) the simple **(NOE)** (as discussed in Appendix C) and 2) the complex Idexis products in order to evaluate the efficacy of the vehicle and polymers used to facilitate the targeted intradermal delivery of the respective hormones. The same cell preparation method used during membrane release studies (as described in Section E.2.3.3) was utilised; however, for this study, the circular dermatomed skin samples with the stratum corneum facing upwards were used, replacing the PVDF synthetic membranes between the donor and receptor chambers. It was predetermined that a single 12 h extraction would take place after the study had commenced; after 12 h the total volume of the receptor chamber was extracted and analysed by means of HPLC.

E.2.2.4.4 Tape stripping

Tape stripping is an effective method for the evaluation of the efficacy and quality of topical formulations by determining the localisation and distribution of an API within the stratum corneum (Escobar-Chávez *et al.*, 2008:104; Lademann *et al.*, 2009:317). This method is performed by removing the cell layers of the stratum corneum through the repeated application of adhesive tapes (Escobar-Chávez *et al.*, 2008:104). For this study, the aim of tape stripping was to indicate whether topical delivery of the hormones occurred within either the SCE and/or the ED.

Upon completion of the skin diffusion study, the Franz cell chambers were disassembled and the skin was visually inspected for any damage or abnormalities. The skin samples were then removed and pinned to a solid surface on a piece of Parafilm® and patted dry with a paper towel to remove any remaining product. 3M Scotch® Magic™ Tape was cut into 16 pieces that could adequately cover the diffusion area (1.075 cm²), which was outlined by the imprint made on the sample by the Franz cell. To prevent any possible contamination, the first strip was discarded after use. The following 15 strips, containing the API, stratum corneum and sections of the epidermis were placed into a polytop containing 5 ml methanol, which served as the extraction solution. The remaining skin was cut into smaller pieces and placed in an additional polytop containing 5 ml methanol. The polytops were stored overnight ($\pm$ 8 h) at $\pm$ 4 °C; thereafter, a volume was extracted from each using a syringe and filtered through a 0.45 µm PTFE membrane filter into an amber autosampler vial for HPLC analysis (Pellett *et al.*, 1997:91). This process was repeated for each Franz cell of the respective study. By analysing the samples

extracted from the polytops containing: 1) the tape strips and 2) the remaining skin, it was possible to establish the concentration of the respective hormones present in the SCE and ED, respectively.

E.2.2.5 Data analysis

For each study (membrane release, skin diffusion and tape stripping), the samples were analysed using HPLC. A linear line was then utilised to estimate the concentration of each of the selected hormones (oestriol, oestradiol and progesterone) in the respective Franz cells (Ng *et al.*, 2010a:213). For membrane release studies, the average flux ($\mu\text{g}/\text{cm}^2\cdot\text{h}$) of each of the selected hormones, which had permeated through the PVDF synthetic membrane, was determined by means of the slope of the linear regression curve plotted against time (h) (obtained at hourly intervals for 6 h) (Mustapha *et al.*, 2011:159; Ng *et al.*, 2010a:213; Shakeel *et al.*, 2007:E3). Steady-state flux was observed in both **(NOE)** and Idexis products between 3 h and 6 h. For skin diffusion studies (transdermal delivery), the amount per area diffused ($\mu\text{g}/\text{cm}^2$) of each hormone which had permeated through the abdominal skin after 12 h was determined, in conjunction with the average concentration ($\mu\text{g}/\text{ml}$) of each respective hormone that diffused into the skin after 12 h (topical delivery).

Through the membrane release and skin diffusion studies, the release of the female hormones from the respective formulations could be established. Membrane release studies have shown to be beneficial during the testing of nano-sized delivery systems (i.e. liposomes, nano-suspensions and -emulsions) (Jug *et al.*, 2017:177). The use of intact human skin samples is considered the most favourable *in vitro* method to predict the *in vivo* behaviour of topically applied actives (Ruela *et al.*, 2016:531; Todo, 2017:33). Moreover, *in vitro* diffusion studies are essential in identifying whether the targeted-site was reached. For topical drug delivery, APIs penetrate the stratum corneum and retain within the skin, whereas transdermal drug delivery requires that the topically applied APIs penetrate through the stratum corneum and epidermis without accumulating in the dermal layers, and consequently producing a systemic effect (Alkilani *et al.*, 2015:439; Garg *et al.*, 2013:969; WHO, 2006:23).

E.2.2.6 Statistical analysis

Descriptive and inferential statistics were used to analyse data from this study. Descriptive statistics include the calculation of the median and the mean concentrations (including the SD) (Sheskin, 2000:1, 4), whereas inferential statistics include an analysis of variance (ANOVA) and the non-parametric Kruskal-Wallis test. Any significant difference between the compared values was indicated by a p-value of < 0.05 . Box-plots were used to graphically present the data.

During the membrane release studies, skin diffusion studies, as well as tape stripping, the following three comparisons were investigated in terms of the respective formulations:

- **(FS), (EC) and (MC)**
- **(CoOE), (CaOE) and (EOE)**
- **(FS), (CoOE), (CaOE) and (EOE)**

Note that only the **(FS)** was compared to the **(CoOE)**, **(CaOE)** and **(EOE)**, as all four formulations contain 2% oestriol. To investigate whether the flux values varied significantly between the respective formulations, a one-way ANOVA was performed followed by a normal probability plot to visually test for normality. The Levene's test was applied to test for homogeneity of variances as the second assumption in order to apply the ANOVA. If these assumptions held, Tukey's Unequal N honest significance difference (HSD) post-hoc test was applied. The aforementioned statistical analyses were performed for both membrane release and skin diffusion studies. For tape stripping, the data was also compared in terms of the SCE and ED, together with the respective formulations. Therefore, a two-way ANOVA was performed by means of TIBCO Statistica™ (data analysis software system), version 13.3 (StatSoft, 2018) to determine whether knowledge of both the skin layers (i.e. SCE and ED) and the formulation was imperative to model the accumulation of oestriol in the tape strips. Since no statistically significant interaction between formulation and skin layer was found, a one-way ANOVA for each skin layer was performed. Normality did not hold, therefore the non-parametric Kruskal-Wallis test was performed with multiple comparisons for the ED. A t-test was performed to compare the two IDEXIS products in terms of the SCE, which was then confirmed with the non-parametric Mann-Whitney U test.

Log transformation is an established technique used to address data that is exponentially distributed or skewed (Feng *et al.*, 2014:105; James, 2016:50). Moreover, the log function is especially useful for data with a few large inputs. Generally, the differences between the values are maintained, even though lower values become more spread out while higher values become more condensed (James, 2016:51). Additionally, log transformation is used to rescale sample observations so that the assumptions of selected statistical analyses (i.e. normality and homogeneity of variances) are fulfilled (Curran-Everett, 2018:343). For this study, log transformation of data was applied to investigate the statistical significance of effects during skin diffusion studies and tape stripping.

Before performing statistical analysis of data, a null hypothesis should be proposed, denoting that there is no difference (relationship) between the groups with regards to the variables of interest (Ali & Bhaskar, 2016:665; McCluskey & Lalkhen, 2007b:209). From then on statistical analysis can be completed, followed by the interpretation of the results by relying on the appreciation and consideration of the null hypothesis, p-value, study power, concept of statistical significance, pitfalls of multiple comparisons, as well as one versus two-tailed tests, prior to conducting the study (McCluskey & Lalkhen, 2007b:208). The p-value, also known as

the probability value, is the probability of obtaining results that are equivalent or more extreme than what was truly observed, assuming the null hypothesis is correct (Ali & Bhaskar, 2016:665; Dahiru, 2008:22). Moreover, the p-value measures the likeliness that any differences observed between the groups were simply due to chance (Dahiru, 2008:22). P-values range between 0 (0%) and 1 (100%), where values closer to 1 indicate no difference between the groups, whereas values closer to 0 denote that the difference observed is not likely to be due to chance (Dahiru, 2008:22). While a p-value ≤ 0.05 is generally considered statistically significant (Concato & Hartigan, 2016:1166), numerous medical journals often report the p-value as highly significant or very significant, depending on the closeness of the value to 0 as opposed to simply reporting the data as significant or not significant (Dahiru, 2008:22).

E.3 Results and discussion

Central tendency is described as “the statistical measure that identifies a single value as representative of an entire distribution” (Manikandan, 2011:140). Different measures of central tendency can be utilised to determine what might be referred to as the normal, expected, typical or average values of a given dataset (McCluskey & Lalkhen, 2007a:127). It is of great importance to determine whether the mean or median will be used to describe the distribution data, as both are considered appropriate measures of central tendency. Mean is commonly used and considered a good representation of the data, since it uses every value in the data set; however, one significant disadvantage presented by mean is its sensitivity to extreme outliers. Therefore, mean is not an appropriate measure when the distribution is skewed (Manikandan, 2011:140). All data obtained from membrane release studies, skin diffusion studies and tape stripping will be described in terms of the median values to avoid the influence of extreme outliers; even so, the mean values will still be reported.

A placebo *in vitro* skin diffusion study was also performed to determine the amount of endogenous oestriol, oestradiol and progesterone present within the excised skin. Progesterone was the only hormone detected during HPLC analysis; however, since progesterone will not be investigated during this study (as previously discussed in Section E.2.1), the small concentrations quantified within the SCE and ED will have no influence on the outcome.

E.3.1 Membrane release studies

The results obtained on the Idexis products and **(NOE)** from the membrane release studies are outlined in Table E.4, which indicates the average percentage (%) oestriol released from each formulation over a period of 6 h, together with the respective average and median flux ($\mu\text{g}/\text{cm}^2\cdot\text{h}$). Comparative box-plots are presented in Figures E.22 to E.24 for the Idexis products and the **(NOE)**. Figures E.10 – E.21 graphically illustrate the results obtained during the membrane release studies for the respective formulations.

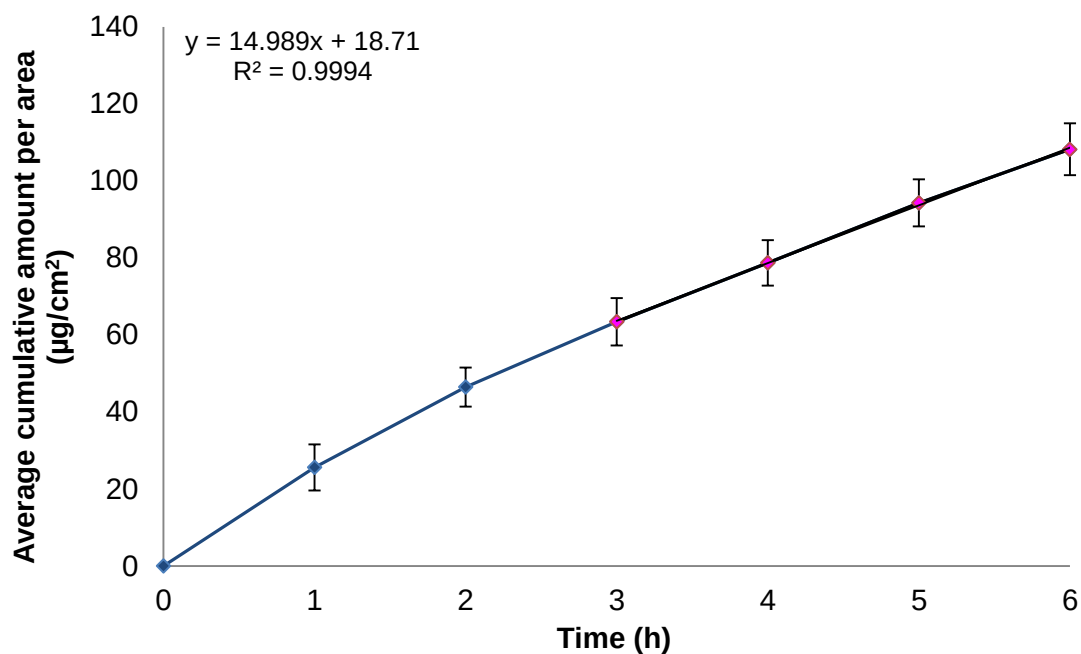


Figure E.10: Average cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of oestriol from the (FS) that was released through the membranes to indicate the average flux from 3 – 6 h (n = 10)

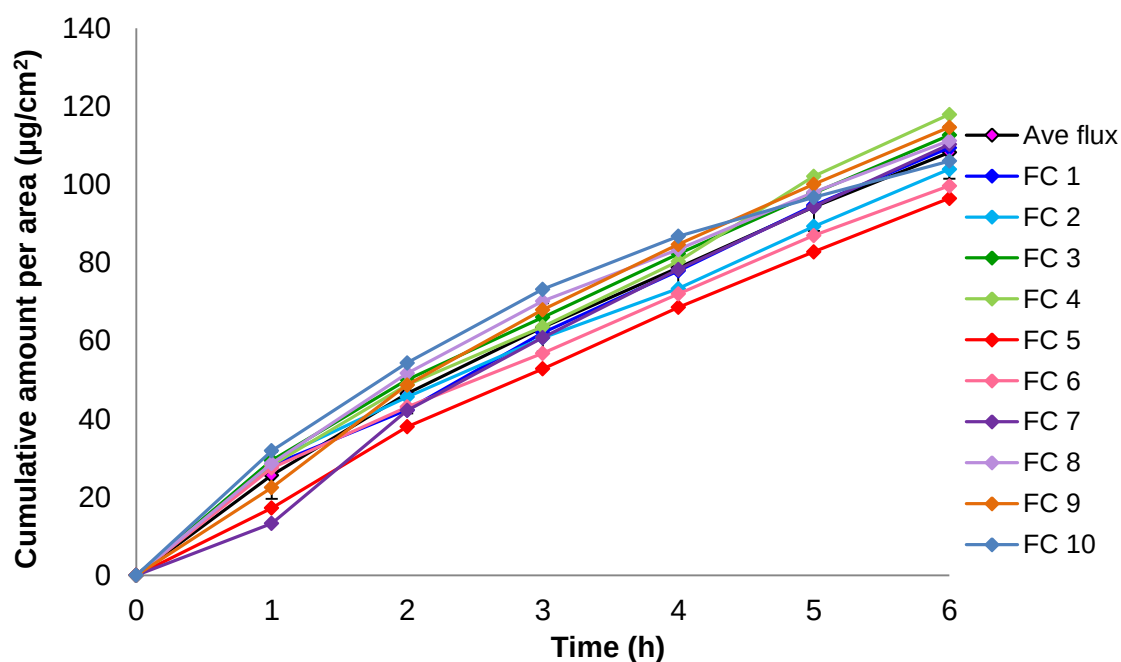


Figure E.11: Cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of oestriol from the (FS) that was released through the membranes of each individual Franz cell over 6 h (n = 10)

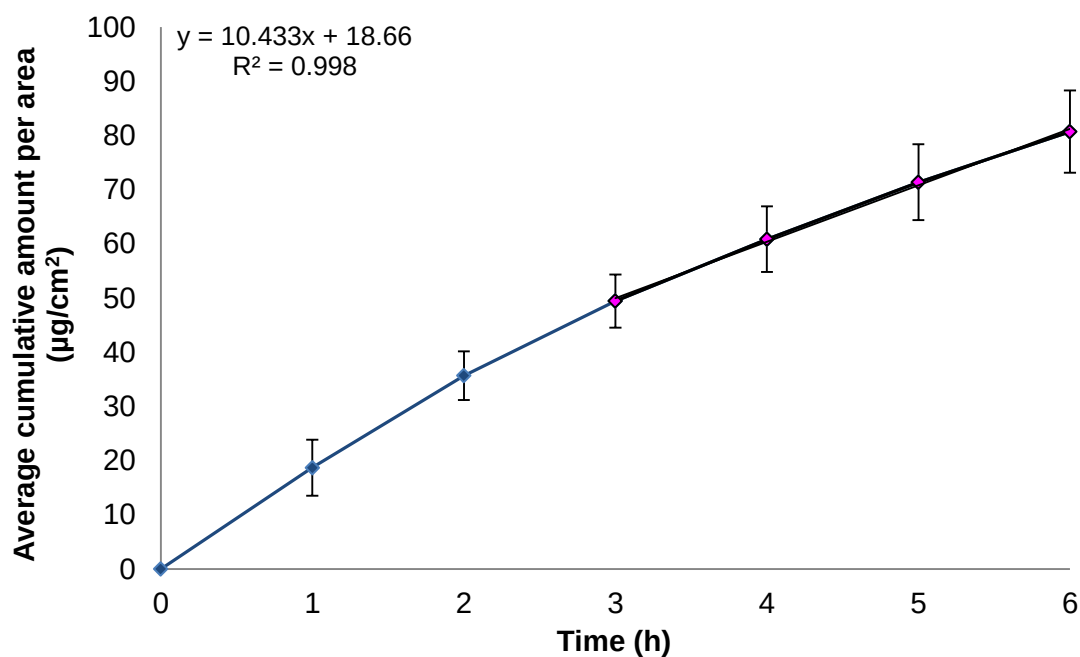


Figure E.12: Average cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of oestriol from the (EC) that was released through the membranes to indicate the average flux from 3 – 6 h ($n = 8$)

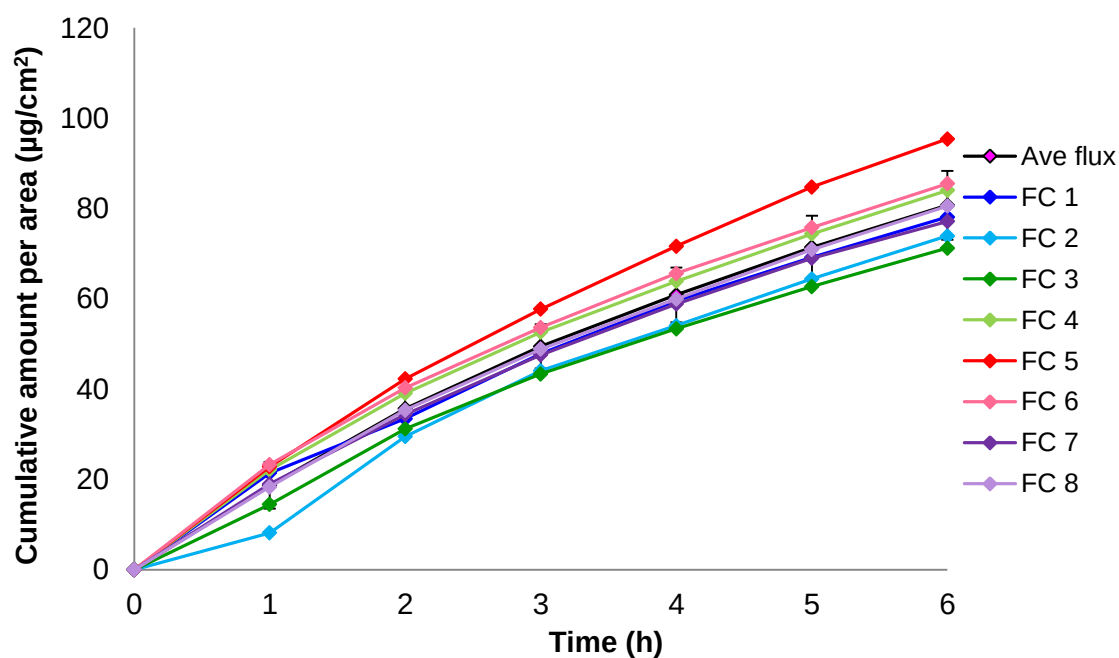


Figure E.13: Cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of oestriol from the (EC) that was released through the membranes of each individual Franz cell over 6 h ($n = 8$)

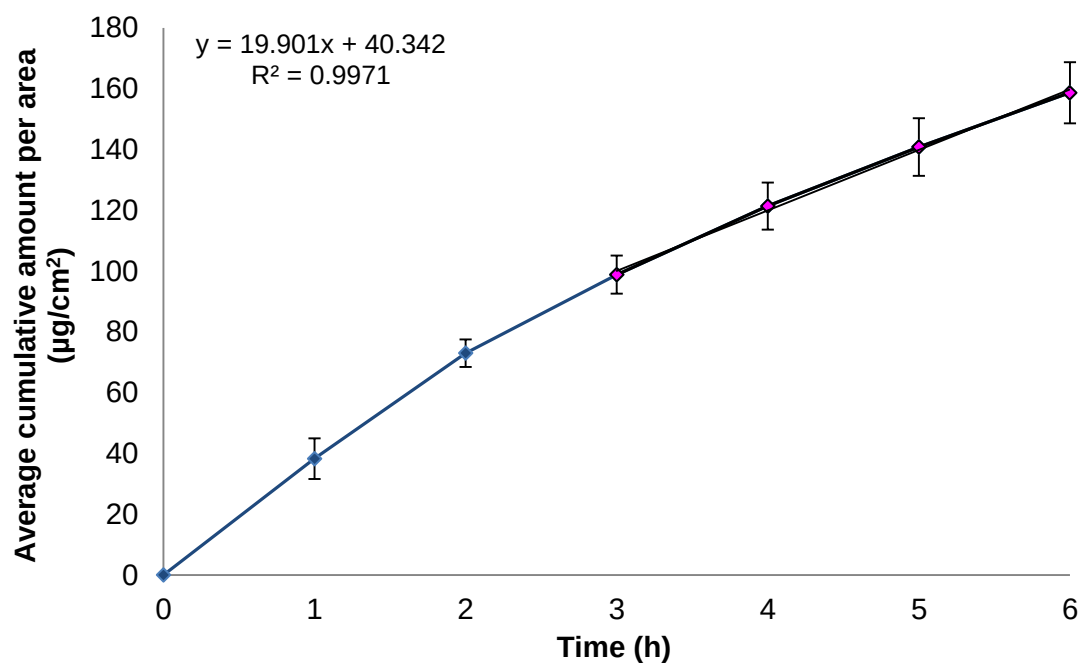


Figure E.14: Average cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of oestriol from the (MC) that was released through the membranes to indicate the average flux from 3 – 6 h ($n = 8$)

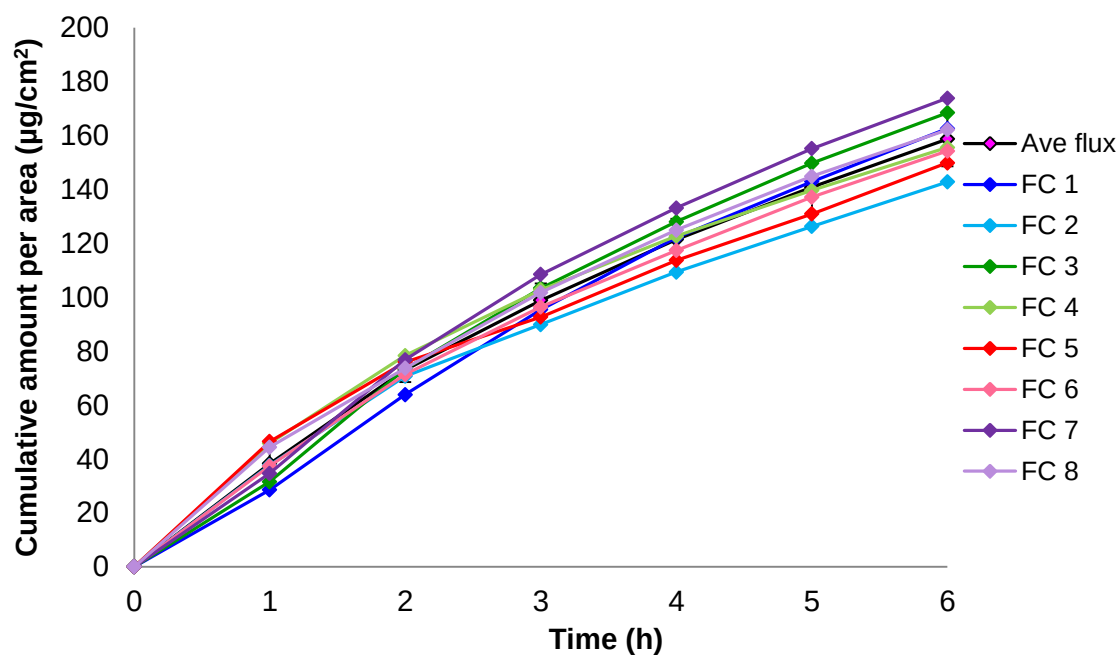


Figure E.15: Cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of oestriol from the (MC) that was released through the membranes of each individual Franz cell over 6 h ($n = 8$)

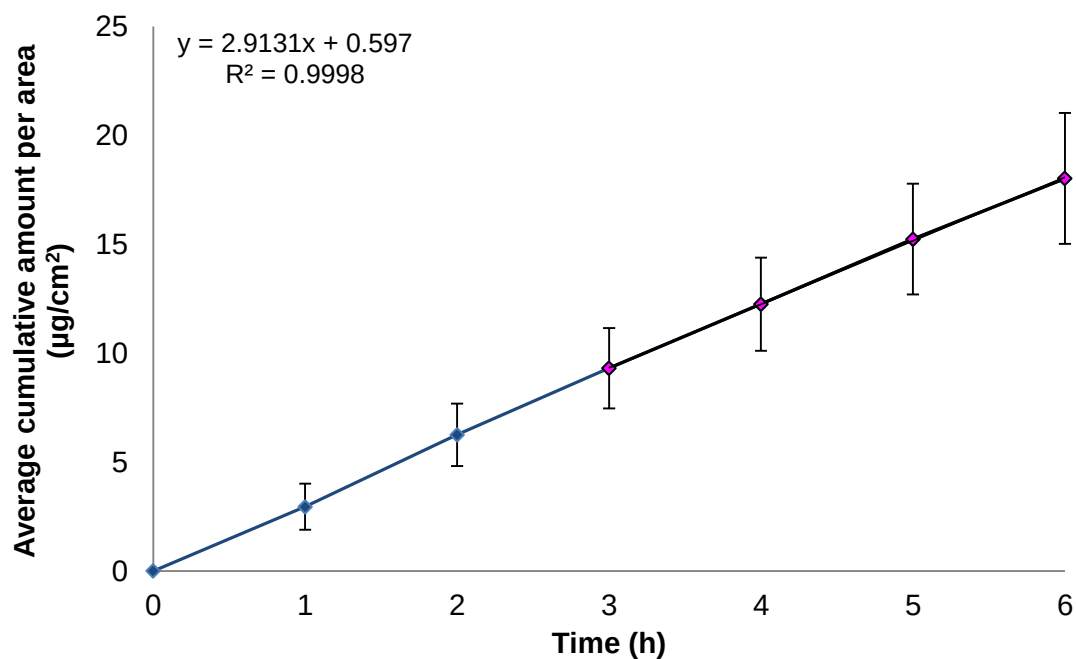


Figure E.16: Average cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of oestriol from the (CoOE) that was released through the membranes to indicate the average flux from 3 – 6 h (n = 10)

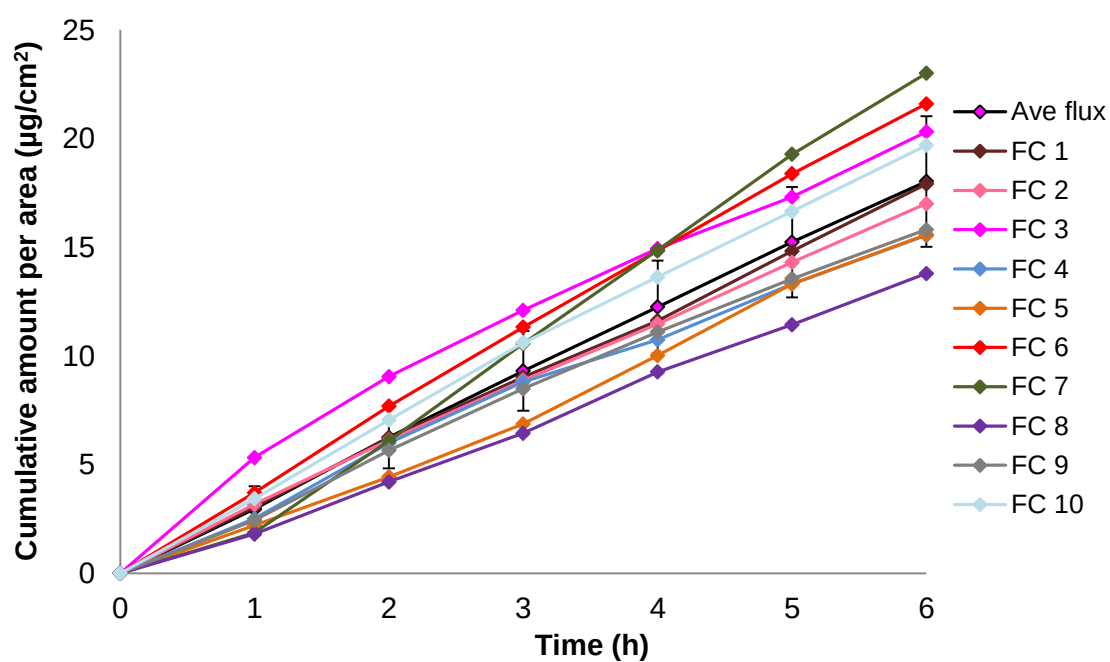


Figure E.17: Cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of oestriol from the (CoOE) that was released through the membranes of each individual Franz cell over 6 h (n = 10)

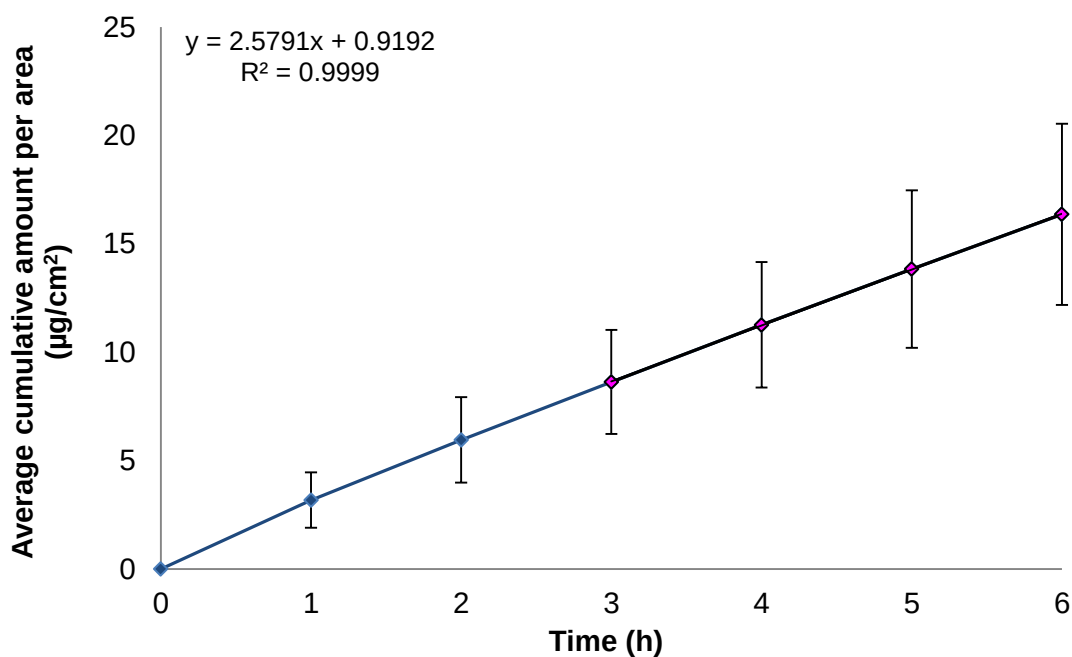


Figure E.18: Average cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of oestriol from the **(CaOE)** that was released through the membranes to indicate the average flux from 3 – 6 h (n = 10)

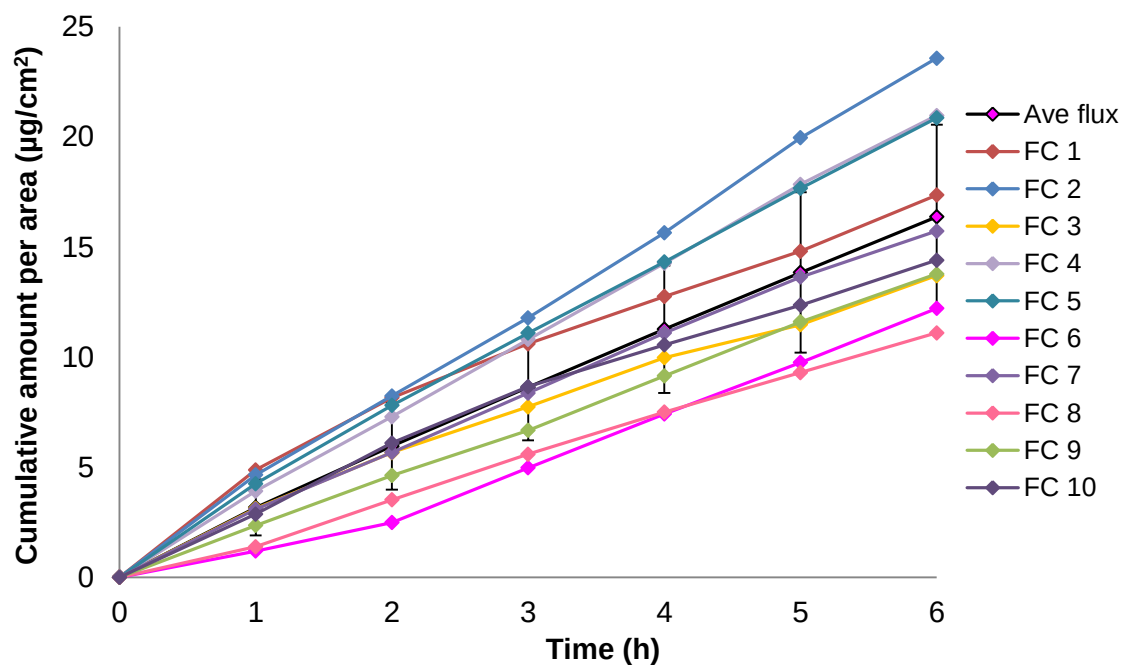


Figure E.19: Cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of oestriol from the **(CaOE)** that was released through the membranes of each individual Franz cell over 6 h (n = 10)

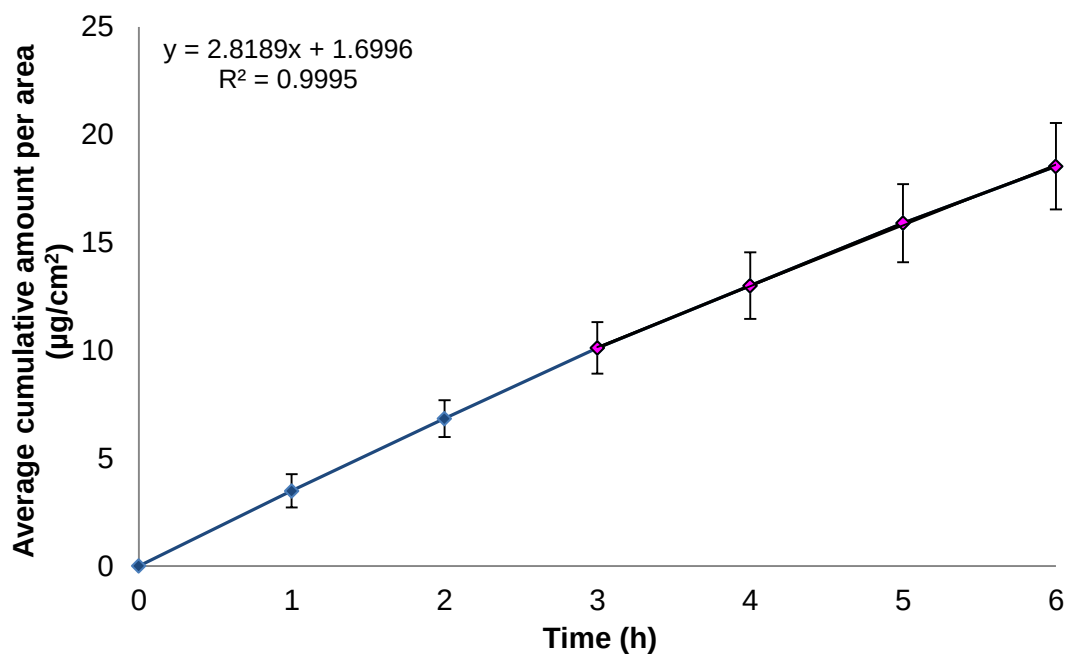


Figure E.20: Average cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of oestriol from the (EOE) that was released through the membranes to indicate the average flux from 3 – 6 h (n = 10)

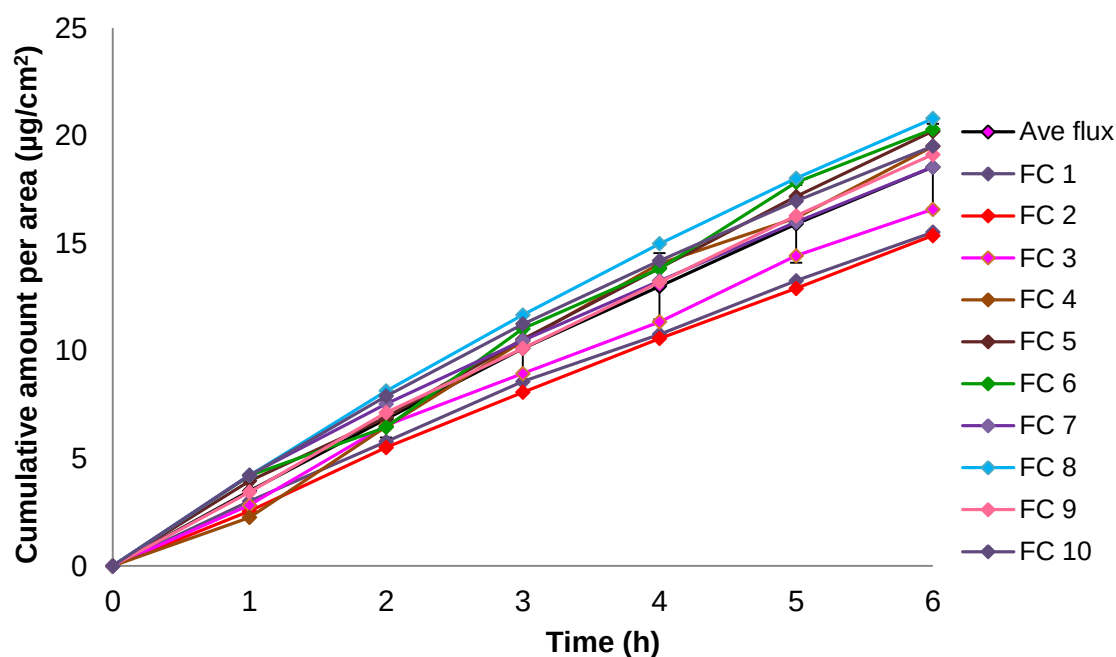


Figure E.21: Cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of oestriol from the (EOE) that was released through the membranes of each individual Franz cell over 6 h (n = 10)

Table E.4: The average percentage released (%), together with the average and median flux ($\mu\text{g}/\text{cm}^2\cdot\text{h}$) for oestriol from the Idexis products and the **(NOE)** after a 6 h membrane release study (n = number of Franz cells used)

Formulation	n	Average %released (%)	Average flux ($\mu\text{g}/\text{cm}^2\cdot\text{h}$)	Median flux ($\mu\text{g}/\text{cm}^2\cdot\text{h}$)
(FS)	10	0.291 ± 0.017	14.989 ± 1.446	16.297
(EC)	8	0.868 ± 0.077	10.433 ± 0.947	12.224
(MC)	8	0.853 ± 0.051	19.901 ± 2.768	23.261
(CoOE)	10	0.048 ± 0.008	2.913 ± 0.529	2.847
(CaOE)	10	0.043 ± 0.010	2.579 ± 0.613	2.357
(EOE)	10	0.041 ± 0.005	2.819 ± 0.327	3.081

From Table E.4, it can be observed that oestriol concentrations were detected in the receptor chambers of the Franz cells during the 6 h membrane release studies. Therefore, it can be stated that oestriol was released from the different vehicles, whereafter diffusion through the PVDF synthetic membranes occurred. The average flux ($\mu\text{g}/\text{cm}^2\cdot\text{h}$) of each of the selected hormones, which had permeated through the PVDF synthetic membrane, was determined by means of the slope of the linear regression curve plotted against time (h) (obtained at hourly intervals for 6 h) (Mustapha *et al.*, 2011:159; Ng *et al.*, 2010a:213; Shakeel *et al.*, 2007:E3). Steady-state flux was observed in both **(NOE)** and Idexis products between 3 h and 6 h.

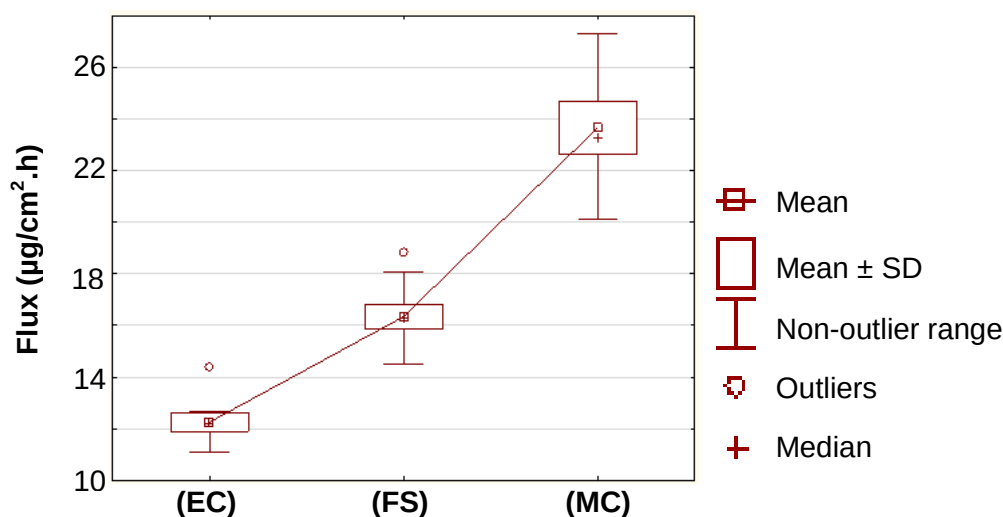


Figure E.22: Box-plot indicating the flux ($\mu\text{g}/\text{cm}^2\cdot\text{h}$) of oestriol from the Idexis products during membrane release studies over 6 h

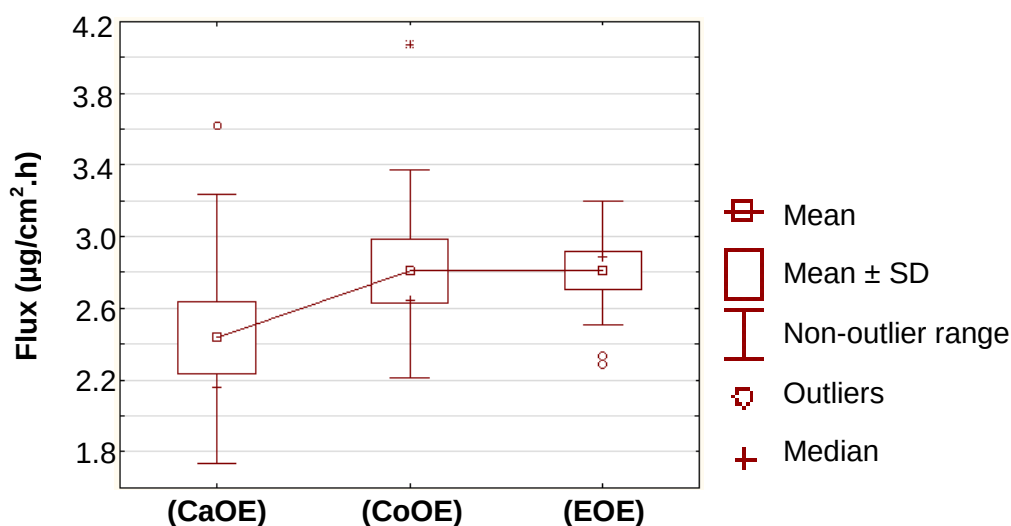


Figure E.23: Box-plot indicating the flux ($\mu\text{g}/\text{cm}^2\cdot\text{h}$) of oestriol from the **(NOE)** during membrane release studies over 6 h

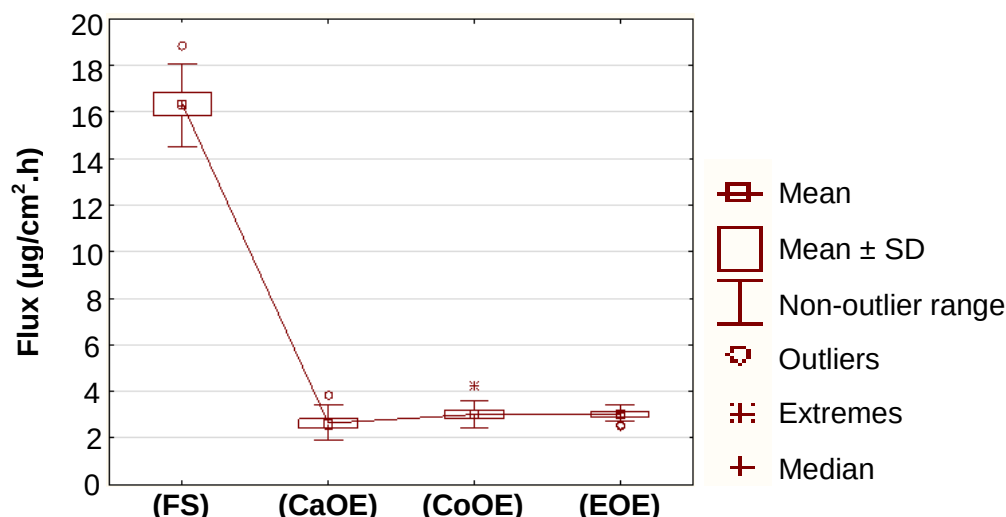


Figure E.24: Box-plot indicating the flux ($\mu\text{g}/\text{cm}^2\cdot\text{h}$) of oestriol from the **(FS)** and the **(NOE)** during membrane release studies over 6 h

Among multiple factors that could influence drug permeation across the skin from topical and transdermal formulations, diffusion of the API from the vehicle towards the surface of the skin and the resultant partitioning into the stratum corneum are of great importance (El Gendy *et al.*, 2002:824). Consequently, drug release is essential: 1) to ensure the effective topical treatment (for skin ageing), and 2) for the consumer's positive perception of the product and the company. A reasonably high viscosity is advantageous for the manual application of the formulation on smaller skin areas (Binder *et al.*, 2019:725; Surber & Kottner, 2016:32). Due to the intended application areas of the respective IDEXIS products (i.e. the face and under the eyes), a high viscosity is desired. High viscosity hinders the 'run-off' of the formulation, therefore it remains on the application area for a longer period of time, which in turn extends the time the API is

available for diffusion (Binder *et al.*, 2019:726). However, during the release studies, the respective formulations were placed within an enclosed donor chamber, so possible 'run-off' will not have had an influence on the results, hence, the diffusion of the API within the vehicle towards the skin surface (membrane) is the primary factor. Apart from skin adhesion, viscosity influences the rate of drug release (Tanwar & Jain, 2012:2). Chime *et al.* (2014:97) and Razavi *et al.* (2014:1321) proposed that the rate of drug release increases with a decrease in vehicle viscosity.

When comparing the IDEXIS products, the **(EC)** obtained the lowest median flux (12.224 $\mu\text{g}/\text{cm}^2\cdot\text{h}$), followed by the **(FS)** (16.297 $\mu\text{g}/\text{cm}^2\cdot\text{h}$), and lastly, the **(MC)** with the highest median flux (23.261 $\mu\text{g}/\text{cm}^2\cdot\text{h}$). The viscosity of the formulations decreased in the order of **(MC)** > **(EC)** > **(FS)** (see Section D.3.7), whereas the median flux increased in the order of **(EC)** < **(FS)** < **(MC)**. The apparent lack of correlation displayed between viscosity and the median flux suggested that the viscosity of the formulation was not the primary factor affecting the diffusion of the API, and that drug diffusion across the membrane mainly depends on the drug-vehicle interaction as opposed to viscosity (El Gendy *et al.*, 2002:826).

When comparing the **(NOE)**, the **(EOE)** obtained the highest median flux (3.081 $\mu\text{g}/\text{cm}^2\cdot\text{h}$), followed by **(CoOE)** (2.847 $\mu\text{g}/\text{cm}^2\cdot\text{h}$), and lastly, **(CaOE)** with the lowest median flux (2.357 $\mu\text{g}/\text{cm}^2\cdot\text{h}$). A study conducted by Viljoen *et al.* (2015:2048), investigating the penetration enhancing effects of selected natural oils, reported that formulations containing emu oil displayed higher median flux values compared to those containing coconut oil. The results correlate with the observations made by Viljoen *et al.* (2015:2048), as the **(EOE)** displayed the highest median flux, followed by the **(CoOE)**. Moreover, a study led by Larsen *et al.* (2001:403) found that the addition of castor oil resulted in a marked reduction in drug release from oil vehicles; a possible explanation for the low median flux displayed by the **(CaOE)**. The viscosity of the formulations decreased in the order of **(CoOE)** > **(EOE)** > **(CaOE)** (see Section C.6.4), whereas the median flux increased in the order of **(CaOE)** < **(CoOE)** < **(EOE)**. The apparent lack of correlation between viscosity and median flux suggests that the viscosity of the formulations did not attribute to the diffusion of the API across the membrane.

When comparing the complex **(FS)** with the simple **(NOE)**, the **(FS)** obtained the highest median flux (16.297 $\mu\text{g}/\text{cm}^2$), followed by the **(EOE)**, **(CoOE)**, and lastly the **(CaOE)** with the lowest median flux (2.357 $\mu\text{g}/\text{cm}^2$). The viscosity of the formulations decreased in order of **(CoOE)** > **(EOE)** > **(CaOE)** > **(FS)**, whereas the median flux increased in order of **(CaOE)** < **(CoOE)** < **(EOE)** < **(FS)**. The highest median flux displayed by the **(FS)** could be attributed to the low viscosity, which facilitates the diffusion of the API molecule from the vehicle towards the skin surface (El Gendy *et al.*, 2002:824).

It should be mentioned there was a difference in the order the formulations (Idexis and **(NOE)**) released oestriol when looking at the average %released and the median flux. This difference could be attributed to the fact that the average %release was investigated for the entire period of the membrane release study (6 h), while the median flux was selected between 3 and 6 h of the study.

E.3.2 Skin diffusion studies

All samples collected from the Franz cell receptor chambers during skin diffusion studies were analysed by means of HPLC and summarised in Table E.5 in terms of the average %diffused, average concentration diffused ($\mu\text{g}/\text{ml}$), average amount per area diffused ($\mu\text{g}/\text{cm}^2$) and the median amount per area diffused ($\mu\text{g}/\text{cm}^2$) after 12 h for the respective Idexis products and **(NOE)**. Franz cells that leaked (i.e. increase or decrease in the initial receptor chamber volume) were omitted from the dataset. From Table E.5, it can be observed that one Franz cell was removed from the dataset for the **(CoOE)**, while three Franz cells were removed from the respective datasets for the **(FS)** and **(CaOE)** due to leakage.

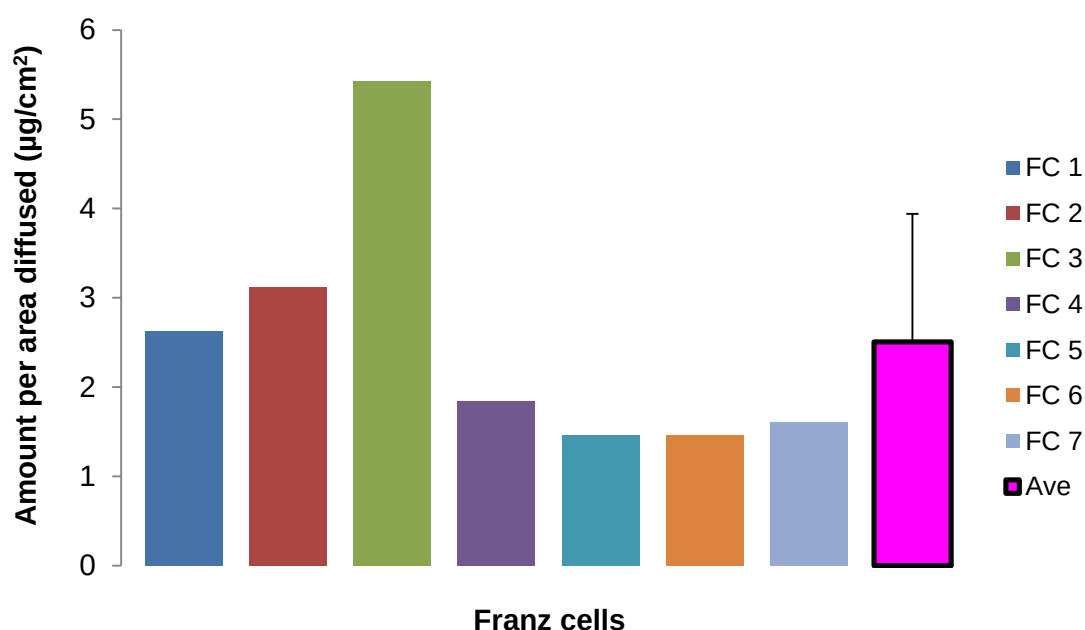


Figure E.25: The amount per area diffused ($\mu\text{g}/\text{cm}^2$) of oestriol from the **(FS)** after the 12 h diffusion study (n = 7)

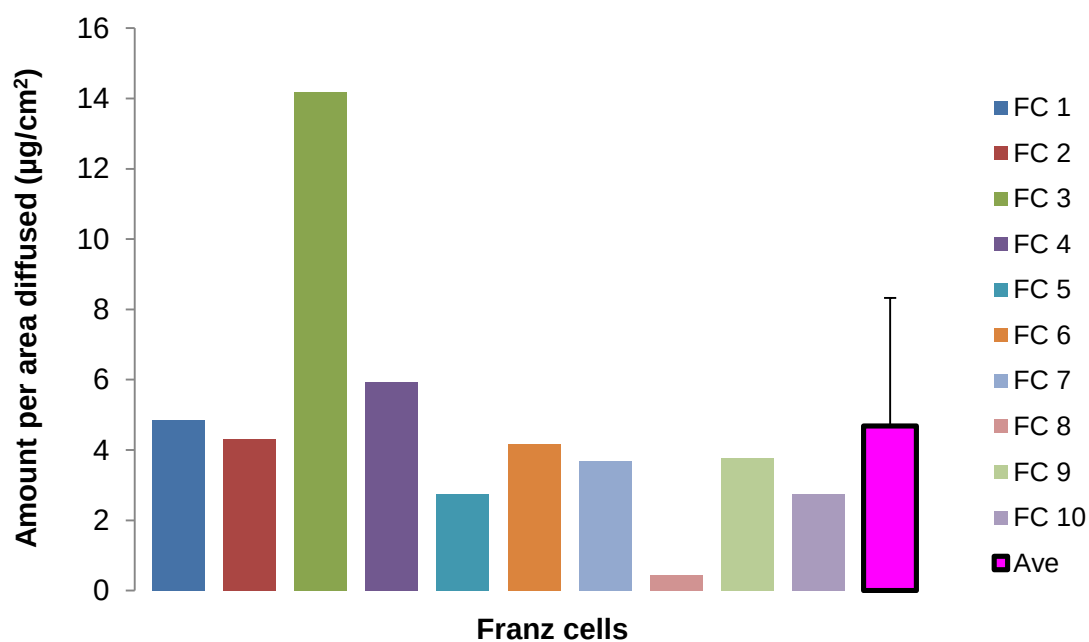


Figure E.26: The amount per area diffused ($\mu\text{g}/\text{cm}^2$) of oestriol from the (EC) after the 12 h diffusion study (n = 10)

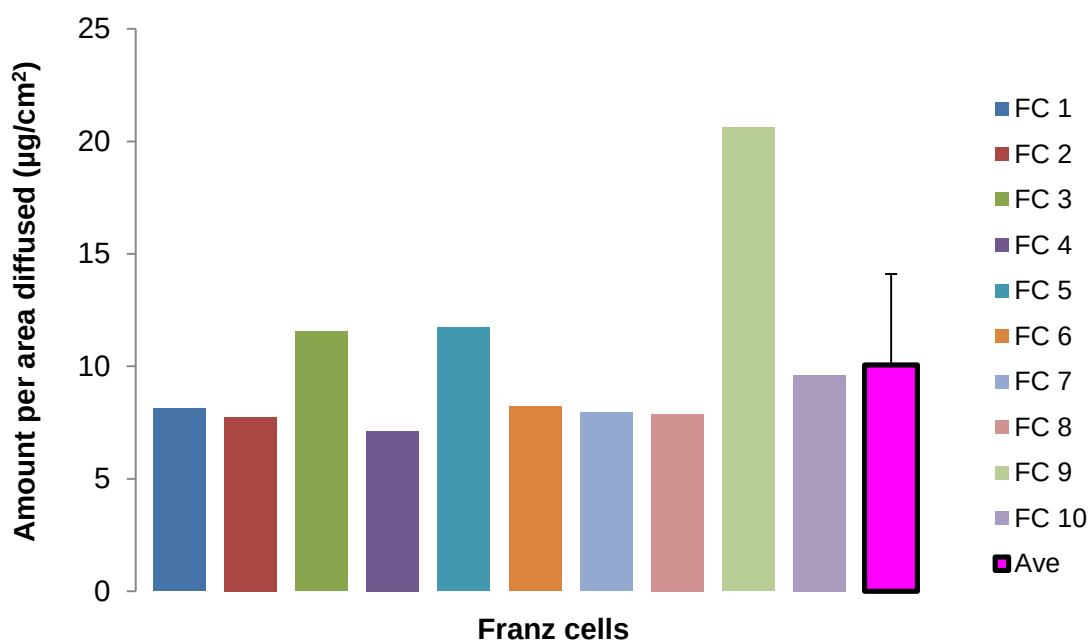


Figure E.27: The amount per area diffused ($\mu\text{g}/\text{cm}^2$) of oestriol from the (MC) after the 12 h diffusion study (n = 10)

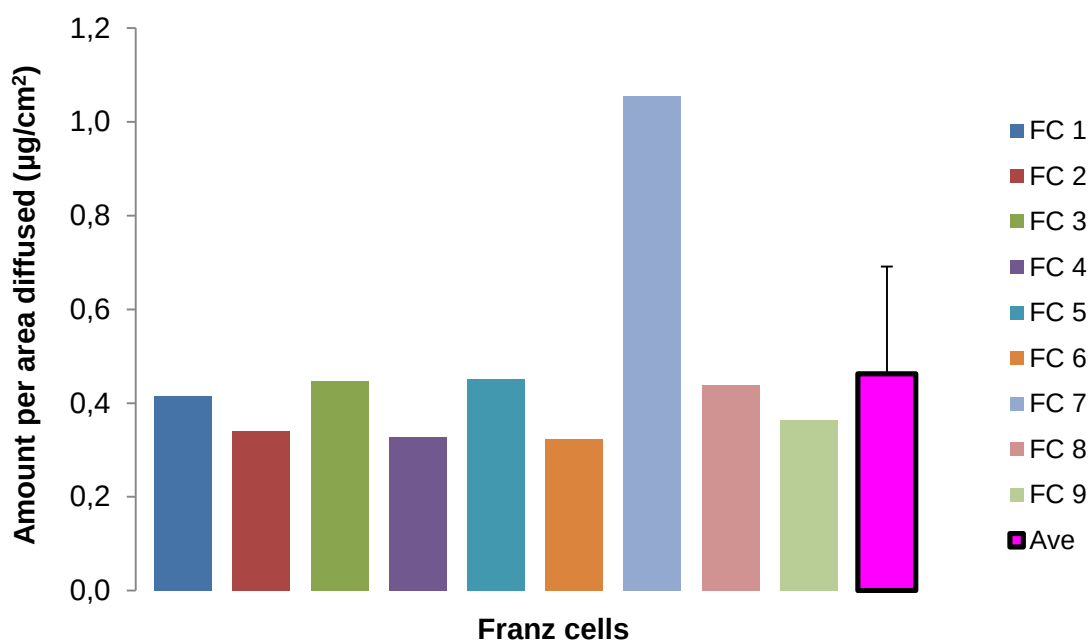


Figure E.28: The amount per area diffused ($\mu\text{g}/\text{cm}^2$) of oestriol from the **(CoOE)** after the 12 h diffusion study (n = 9)

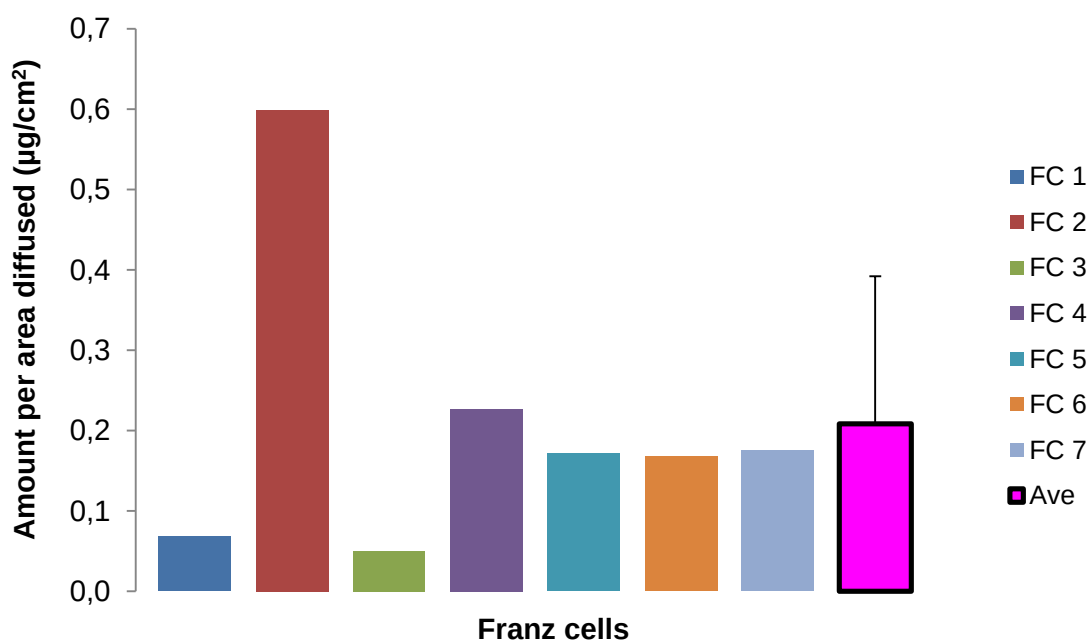


Figure E.29: The amount per area diffused ($\mu\text{g}/\text{cm}^2$) of oestriol from the **(CaOE)** after the 12 h diffusion study (n = 7)

Table E.5: The average percentage diffused (%), average concentration diffused ($\mu\text{g/ml}$), together with the average and median amount per area diffused ($\mu\text{g/cm}^2$) of oestriol from the Idexis products and the **(NOE)** (n = number of Franz cells used)

Formulation	n	Average %diffused (%)	Average concentration diffused ($\mu\text{g/ml}$)	Average amount per area diffused ($\mu\text{g/cm}^2$)	Median amount per area diffused ($\mu\text{g/cm}^2$)
(FS)	7	0.007 ± 0.004	1.348 ± 0.713	2.506 ± 1.326	1.845
(EC)	10	0.050 ± 0.037	2.517 ± 1.858	4.680 ± 3.455	3.971
(MC)	10	0.054 ± 0.021	5.409 ± 2.061	10.058 ± 3.833	8.168
(CoOE)	9	0.001 ± 0.001	0.249 ± 0.116	0.463 ± 0.215	0.414
(CaOE)	7	0.001 ± 0.000	0.112 ± 0.091	0.208 ± 0.170	0.171

After HPLC analysis, the concentration ($\mu\text{g/ml}$) of oestriol present in the receptor phase was quantified, which was above the LOD ($0.0022 \mu\text{g/ml}$) and LOQ ($0.0067 \mu\text{g/ml}$) values for oestriol (as determined in Section A.4.7) in each of the respective formulations, with the exception of the **(EOE)** as no oestriol concentration was quantified in the receptor phase. From Table E.5, it can be observed that the median amount per area diffused was very low in relation to the initial %oestriol present in the respective formulations (as displayed in Tables A.1 and C.9), thus only a small amount of oestriol was delivered transdermally (i.e. reached the systemic circulation) from each of the formulations. **(EOE)** did not deliver oestriol transdermally.

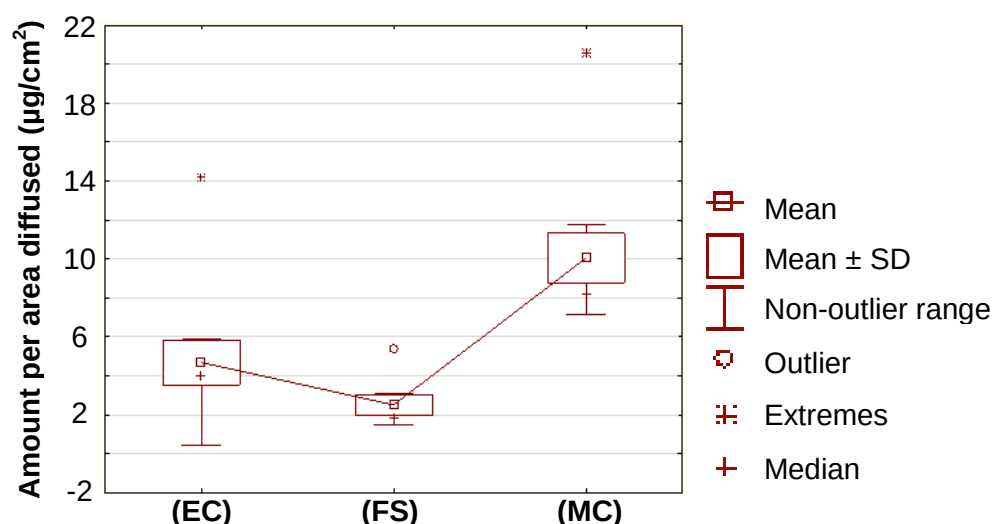


Figure E.30: Box-plot indicating the mean and median amount per area diffused ($\mu\text{g/cm}^2$) of oestriol from the Idexis products ((EC) and (MC) both had $n = 10$, (FS) had $n = 7$) after 12 h

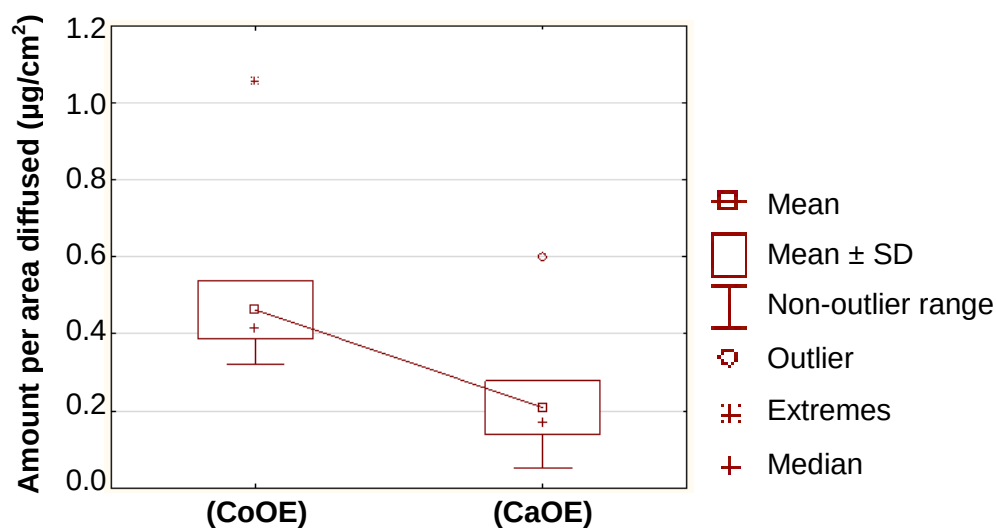


Figure E.31: Box-plot indicating the mean and median amount per area diffused ($\mu\text{g}/\text{cm}^2$) of oestriol from the **(NOE)** ((CoOE) had $n = 9$, (CaOE) had $n = 7$) after 12 h

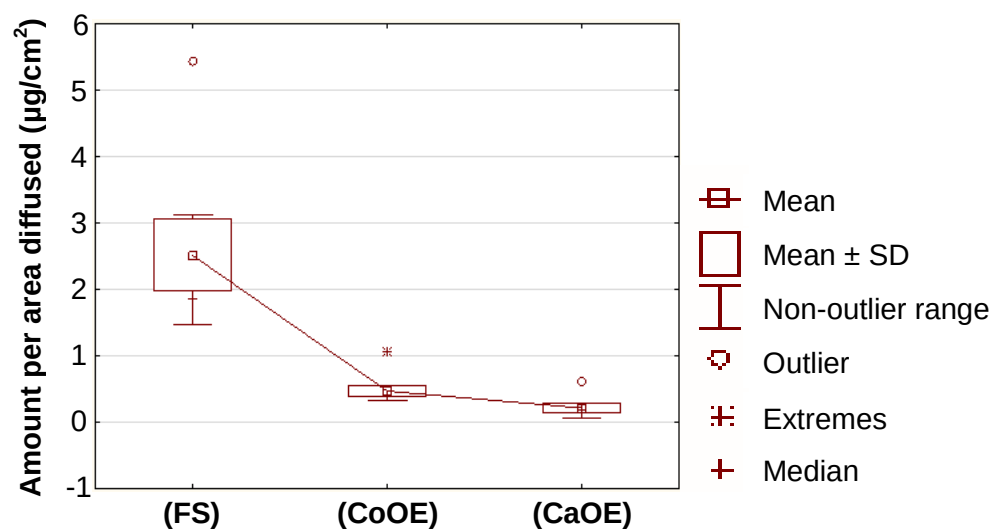


Figure E.32: Box-plot indicating the mean and median amount per area diffused ($\mu\text{g}/\text{cm}^2$) of oestriol from the **(FS)** and the **(NOE)** ((FS) and (CaOE) had $n = 7$, (CoOE) had $n = 9$) after 12 h

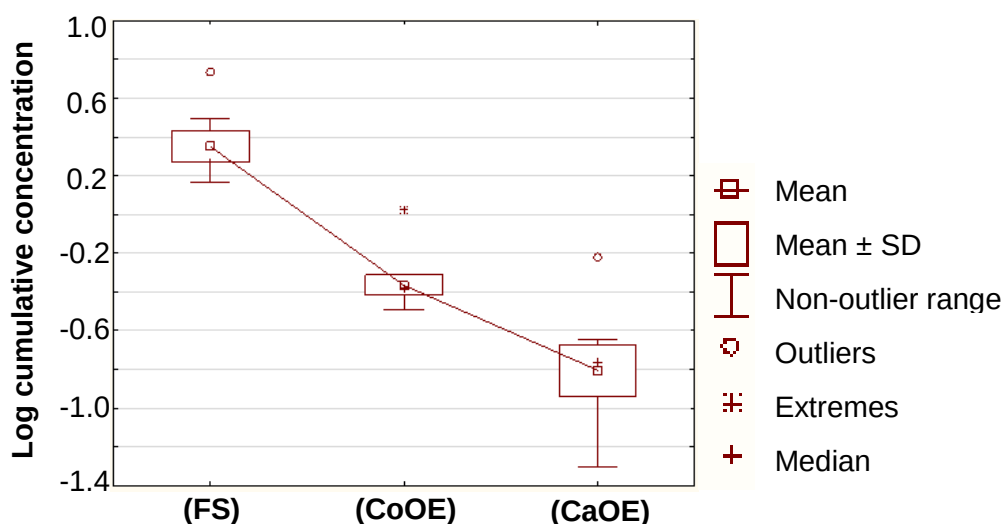


Figure E.33: Box-plot indicating the log cumulative concentration of oestriol from the **(FS)** and the **(NOE)** (**(FS)** and **(CaOE)** had $n = 7$, **(CoOE)** had $n = 9$) after 12 h

When comparing the Ilexis products, the **(MC)** obtained the highest median amount per area diffused ($8.168 \mu\text{g}/\text{cm}^2$), followed by the **(EC)** ($3.971 \mu\text{g}/\text{cm}^2$) and lastly, the **(FS)** with the lowest median amount per area diffused ($1.845 \mu\text{g}/\text{cm}^2$). Due to the classified nature of this study, limited information was made available with regard to the formulation of the respective Ilexis products. However, one clear difference among the respective products was the formulation vehicle in which the oestriol was applied onto the skin, which determines the rate of permeation given that thermodynamic activities vary from formulation to formulation (Stahl *et al.*, 2011:13). A general approach to increase skin permeation is to incorporate solvents that: 1) favour a high API concentration in the stratum corneum by increasing the thermodynamic activity of the API in the vehicle and by this means promote the interfacial API transport into the stratum corneum, or 2) to incorporate solvents that have the ability to penetrate the stratum corneum themselves and thereby alter the skin barrier function (Møllgaard & Hoelgaard, 1983:186).

The highest median amount per area diffused obtained for the **(MC)** could be due to the increased availability of oestriol from the vehicle compared to the **(EC)** and **(FS)** (see mean recovery of each formulation in Section D.3.1), which may have increased the concentration gradient and act as a driving force to enhance the diffusion of oestriol across the skin (Fu & Kao, 2010:430). Moreover, the vehicle could have potentially influenced the permeation of the API through the skin by means of vehicle-skin interactions (Benson *et al.*, 2005:213); the vehicle of the **(MC)** could have interacted with and altered the skin barrier (more than **(EC)** and **(FS)**), leading to an increase in the permeation of oestriol across the skin.

The viscosities of the Ilexis products decreased in order of **(MC)** > **(EC)** > **(FS)** (see Section D.3.7), whereas the median amount per area diffused increased in order of **(FS)** < **(EC)** < **(MC)**. Batheja *et al.* (2011) reported a positive relationship between increased viscosity and

skin permeation, which could be attributed to the increased contact time on the skin surface that is achieved by adding thickening or gelling agents to topical preparations. The results correlate with the observation made by Batheja *et al.* (2011), as the highly viscous **(MC)** displayed the highest median amount per area diffused. However, Welin-Berger *et al.* (2001:309) stated that as the viscosity of a formulation decreases, the permeability generally increases. The abovementioned factors affecting the permeation of oestriol across the skin from the respective Idexis products are merely speculation, as the exact mechanisms responsible for the varied skin diffusion results displayed by the respective Idexis products are not known.

When comparing the **(NOE)**, the **(CoOE)** obtained the highest median amount per area diffused ($0.414 \mu\text{g}/\text{cm}^2$), followed by the **(CaOE)** ($0.171 \mu\text{g}/\text{cm}^2$). Note that no concentration of oestriol was quantified in the receptor phase of the **(EOE)** diffusion study, thus oestriol did not diffuse through the skin into the systemic circulation (i.e. was not delivered transdermally) from this formulation. The same optimised formula and method (as discussed in Appendix C) was used to formulate the **(NOE)**, with the only variable being the natural oils used to serve as the oil phase and penetration enhancer. Therefore, the penetration enhancing effects of the selected natural oils will be discussed. Fatty acids are responsible for the penetration enhancing effect of natural oils (Čižinauskas *et al.*, 2017:1536). As summarised in Table C.7, the lipid composition of the respective natural oils vary significantly; therefore, varied skin diffusion results were expected.

The high median amount per area diffused ($\mu\text{g}/\text{cm}^2$) exhibited by the **(CoOE)** could possibly be attributed to the high concentration of lauric acid (C12:0) and myristic acid (C14:0) present in coconut oil. Viljoen *et al.* (2015:2051) proposed that oils high in SFA levels, particularly lauric and myristic acid, increase the API flux across skin. Furthermore, a study led by Kravchenko *et al.* (2003:579) demonstrated that the incorporation of lauric acid in a transdermal therapeutic system increased the anticonvulsive effect of phenazepam 3-fold compared to the control. The authors attributed the effect to an increase in the bioavailability of phenazepam from the applied matrix, which was evident in the significant increase in percutaneous absorption. The lower median amount per area diffused displayed by **(CaOE)** compared to the **(CoOE)** could be due to the presence of linoleic acid (C18:2) in the castor oil, which contains two double bonds within its carbon chain (Akinshina *et al.*, 2016:17448). The double bonds create conformational kinks within the chain (Croguennec, 2016:43), making it more difficult for the fatty acid to insert itself within the lipid bilayers of the stratum corneum, which consequently, increases the lag time. However, once the chains are packed within the skin, their kinked-structure disrupts the ordered orientation of the lipid region, resulting in an increase in the fluidity of the stratum corneum (Haque & Talukder, 2018:174), and in turn, higher flux of the API. Lastly, the **(EOE)** showed no apparent diffusion through the skin, as no oestriol concentration was quantified during HPLC analysis of the receptor phase. Emu oil is rich in palmitic acid (C16:0), which has a linear shape

and low solubility. Subsequently, this reduces its ability to disrupt the densely packed lipid region within the stratum corneum and to insert itself into the lipid bilayers (Cho & Shin, 2012:10), which ultimately results in little or no effect (Viljoen *et al.*, 2015:2050). In addition, due to the lipophilic nature of longer chain SFAs, they have a higher affinity to the lipids in the stratum corneum, and therefore, delay their own permeation and that of other permeates across the skin, as seen in this case with oestriol (Kanikkannan & Babu, 2045:1).

When comparing the **(FS)** and **(NOE)**, the **(FS)** obtained the highest median amount per area diffused ($1.845 \mu\text{g}/\text{cm}^2$), followed by the **(CoOE)** ($0.414 \mu\text{g}/\text{cm}^2$) and lastly, the **(CaOE)** with the lowest median amount per area diffused ($0.171 \mu\text{g}/\text{cm}^2$). The difference in the median amount per area diffused could be attributed to the viscosities, which decrease in order of **(CoOE) > (CaOE) > (FS)**. According to Baroli (2010:33), viscous formulations reduce the diffusion coefficient of API molecules in the vehicle, therefore impeding its skin partitioning and consequent absorption. Cross *et al.* (2001:149) demonstrated that viscous formulations hinder the skin penetration of benzophenone-3 under infinite-dose conditions (very thick application). However, when the viscous formulation was thinly applied, therefore under finite-dose conditions (in-use), skin penetration improved. However, the apparent lack of correlation between the viscosity of the formulation and the median amount per area diffused suggests that another mechanism is responsible for the API diffusion across the skin.

Lastly, it can be stated that the polymers used to reduce/impede the transdermal delivery of oestriol from the Idexis products were not effective, as the complex products all delivered an increased concentration of oestriol across the skin compared to the simple **(NOE)**.

E.3.3 Tape stripping

Topical formulations must have the ability to penetrate the stratum corneum and permeate to the target-site in quantities adequate to exert a therapeutic effect (Hagen & Baker, 2017:1623). For this study, the ED served as the target-site as the purpose of the complex Idexis products was for the incorporated female hormones to be retained within the skin after topical application. Numerous factors can influence this process, such as the physicochemical properties of the selected API, the formulation used, as well as the mode of application (Hagen & Baker, 2017:1623). Table E.6 displays the results obtained by utilising the tape stripping technique to determine the average and median concentration ($\mu\text{g}/\text{ml}$) of oestriol present in the SCE and ED after completing the skin diffusion studies for the respective Idexis products and **(NOE)**.

Table E.6: Average and median concentration ($\mu\text{g/ml}$) of oestriol in the SCE and ED (n = number of Franz cells used)

Formulation	n	Average concentration in SCE ($\mu\text{g/ml}$)	Median concentration in SCE ($\mu\text{g/ml}$)	Average concentration in ED ($\mu\text{g/ml}$)	Median concentration in ED ($\mu\text{g/ml}$)
(FS)	7	0.000 ± 0.000	0.000	1.025 ± 0.297	0.921
(EC)	10	0.322 ± 0.206	0.314	0.727 ± 0.494	0.700
(MC)	10	1.021 ± 0.554	0.811	2.348 ± 1.427	2.281
(CoOE)	9	2.377 ± 1.292	2.207	3.634 ± 3.049	2.143
(CaOE)	7	2.639 ± 1.289	2.641	5.347 ± 2.065	6.222
(EOE)	7	0.778 ± 0.323	0.725	1.058 ± 0.687	1.191

E.3.3.1 Stratum corneum-epidermis concentration

Figures E.34 – E.38 display the results of the average oestriol concentration obtained in the SCE from the respective Idexis products and the **(NOE)** after tape stripping was completed.

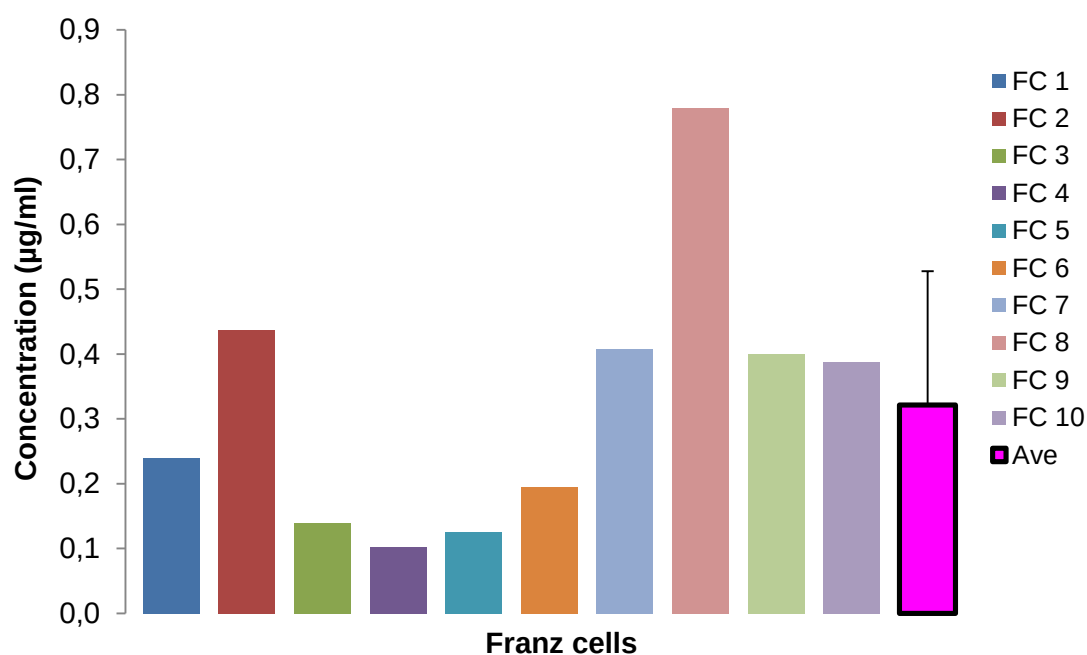


Figure E.34: Oestriol concentration ($\mu\text{g/ml}$) from the **(EC)** present in the SCE (n = 10)

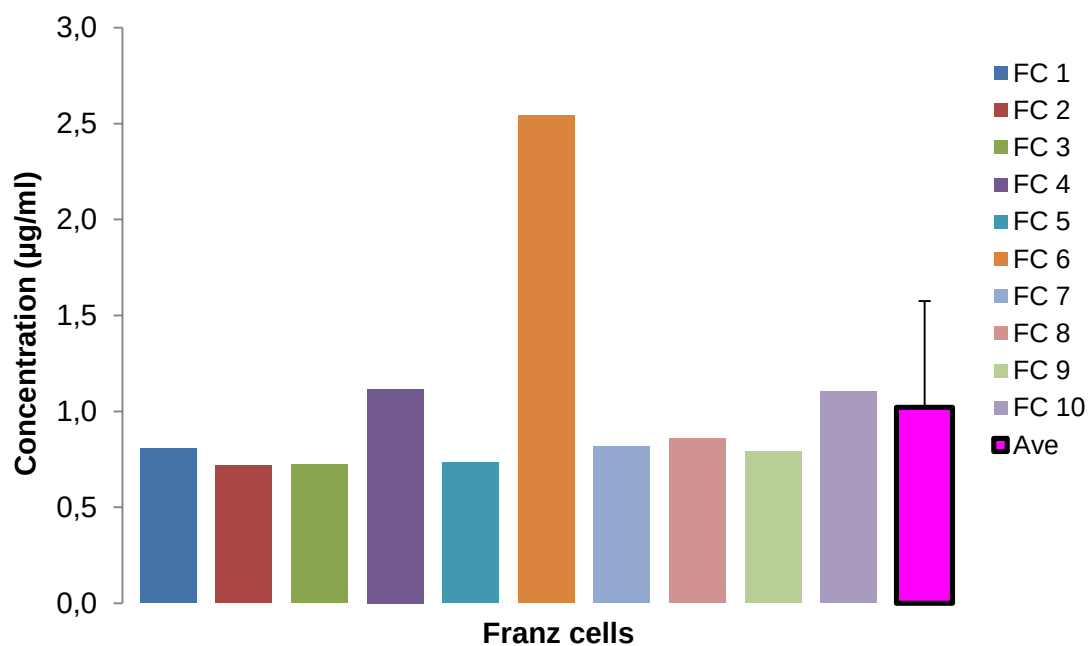


Figure E.35: Oestriol concentration (µg/ml) from the **(MC)** present in the SCE (n = 10)

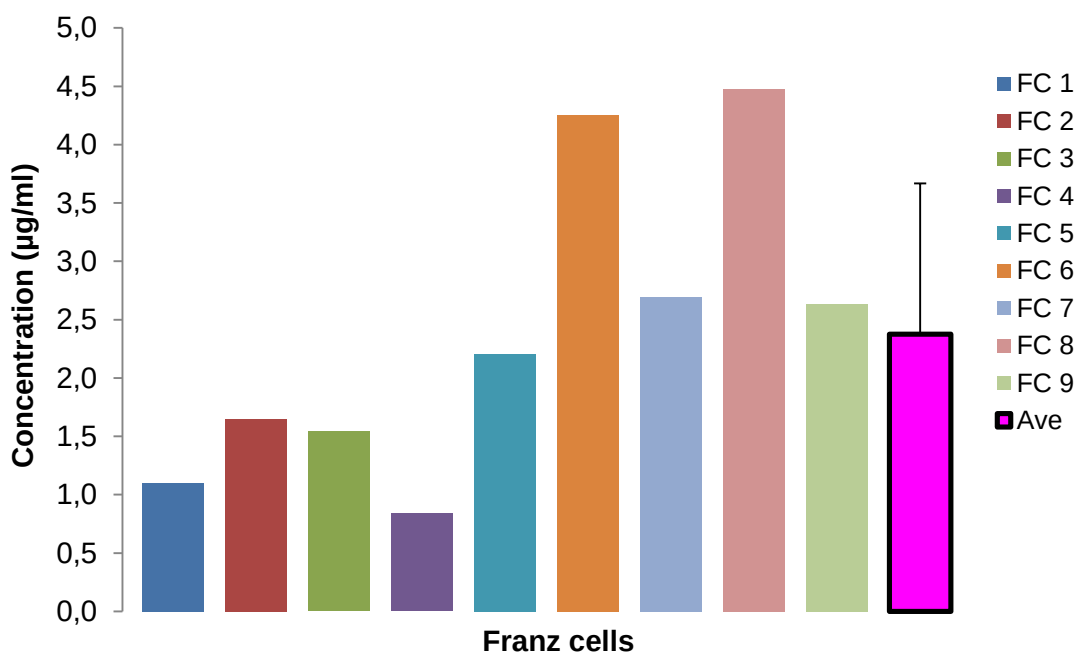


Figure E.36: Oestriol concentration (µg/ml) from the **(CoOE)** present in the SCE (n = 9)

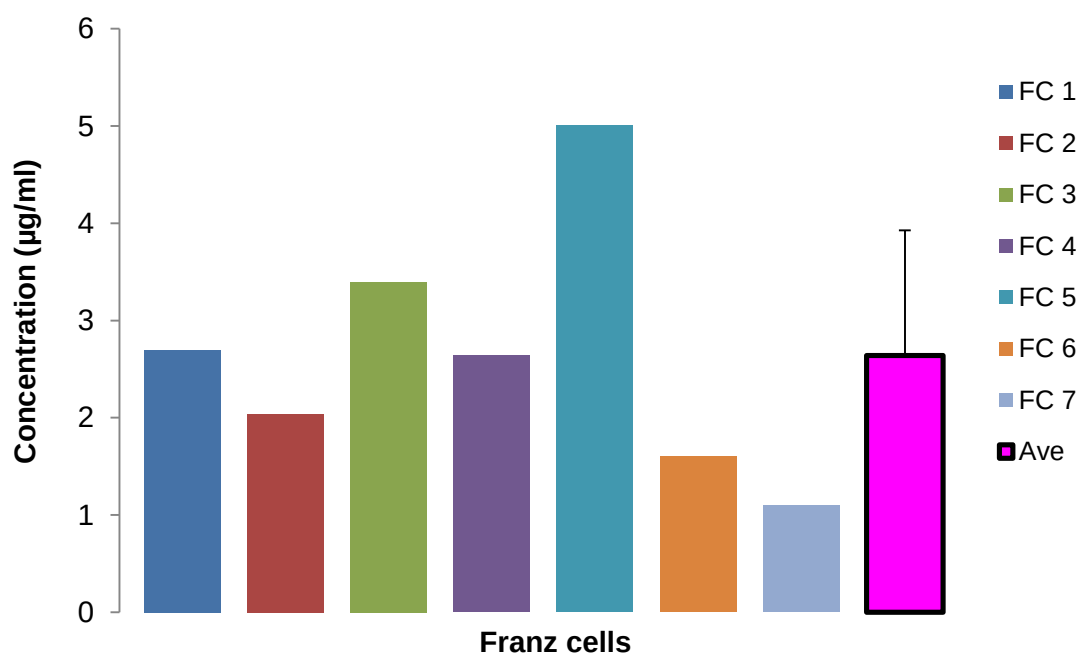


Figure E.37: Oestriol concentration (µg/ml) from the **(CaOE)** present in the SCE (n = 7)

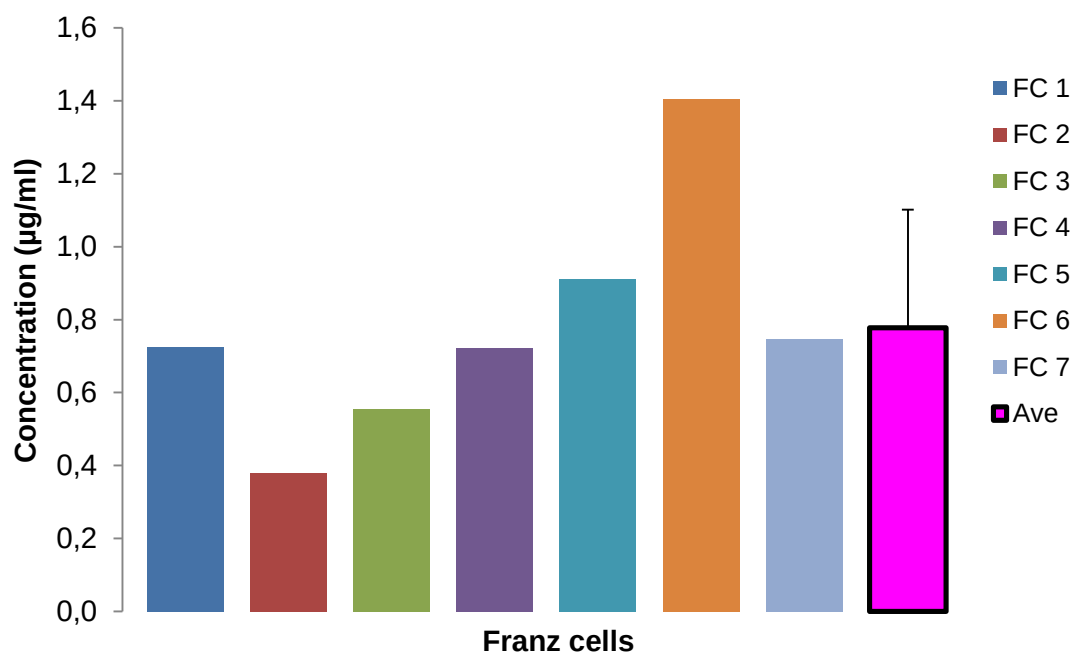


Figure E.38: Oestriol concentration (µg/ml) from the **(EOE)** present in the SCE (n = 7)

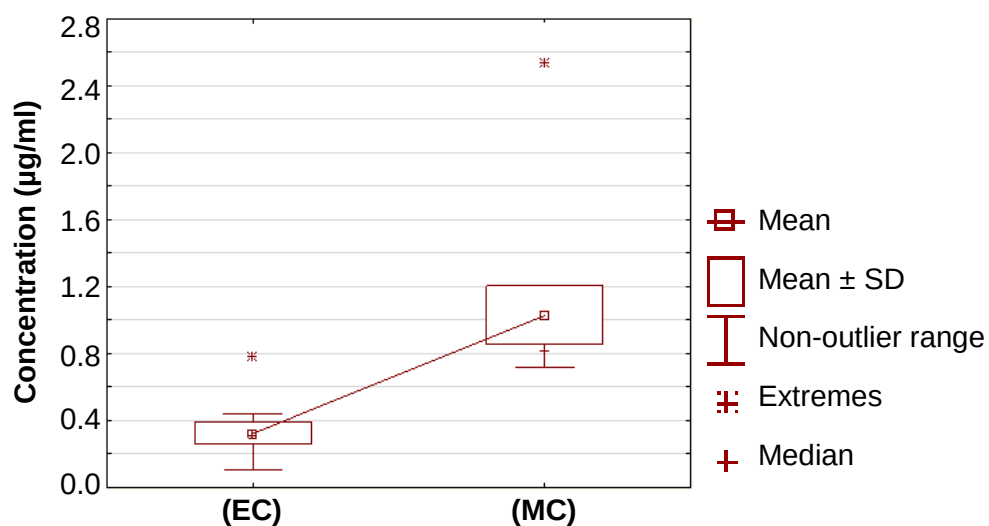


Figure E.39: Box-plot indicating the mean and median concentration (µg/ml) of oestriol present in the SCE from the Idexis products

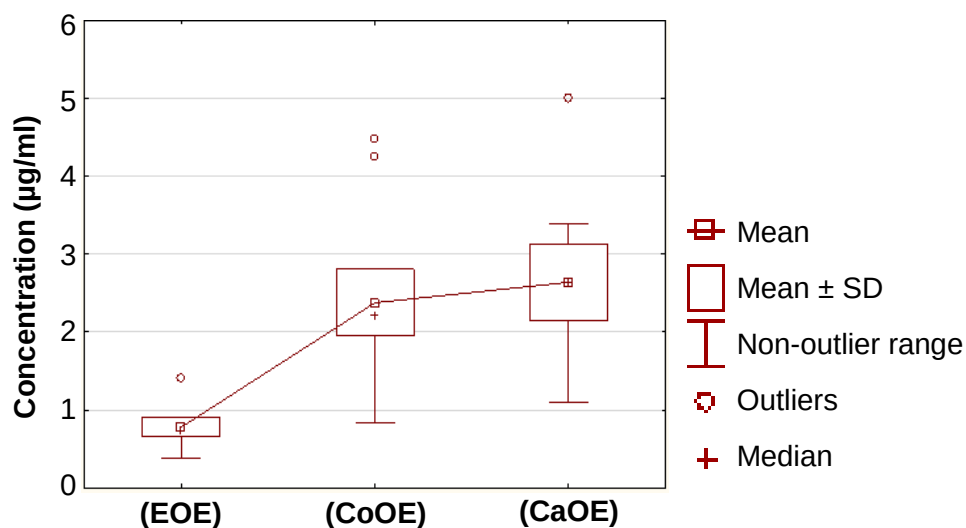


Figure E.40: Box-plot indicating the mean and median concentration (µg/ml) of oestriol present in the SCE from the **(NOE)**

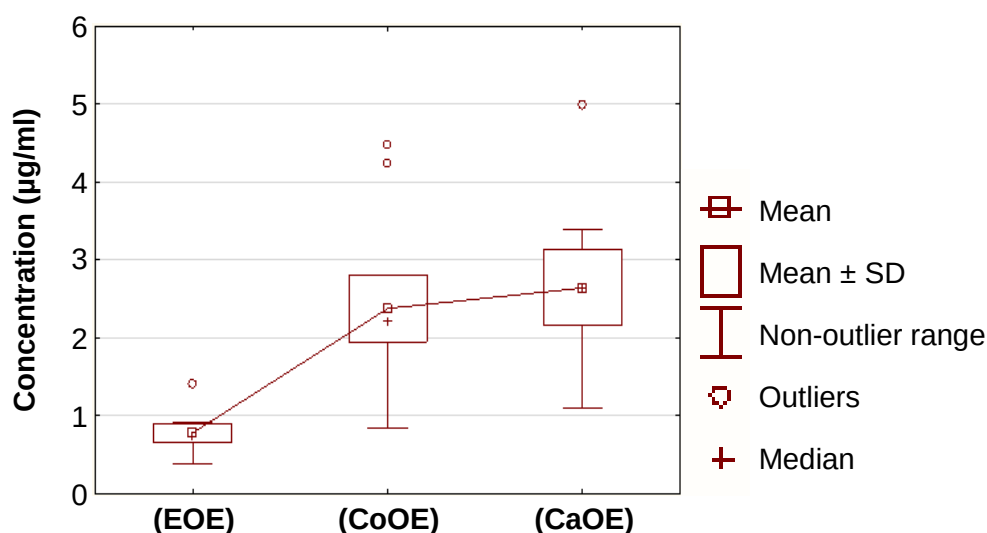


Figure E.41: Box-plot indicating the mean and median concentration (µg/ml) of oestriol present in the SCE from the **(FS)** and the **(NOE)**

When comparing the Ilex products, the **(MC)** obtained the highest median concentration (0.811 µg/ml), followed by the **(EC)** (0.314 µg/ml) and lastly, the **(FS)** (for which no oestriol concentration was quantified within the SCE after tape stripping was performed). The physicochemical properties of oestriol (MW = 288.4 g/mol, log P = 2.4) (Moffat *et al.*, 2004:1350-1351) favours skin penetration (Naik *et al.*, 2000:319); therefore, it was expected that the lipophilic hormone would diffuse into the lipophilic stratum corneum, but then partition slowly into the hydrophilic viable epidermis (Benson *et al.*, 2005:210). The low concentrations of oestriol from the **(MC)** and **(EC)** in the SCE could be due to slow diffusion into the ED (Benson *et al.*, 2005:210).

When comparing the **(NOE)**, the **(CaOE)** obtained the highest median concentration (2.641 µg/ml) within the SCE, followed by the **(CoOE)** (2.207 µg/ml) and lastly, the **(EOE)** (0.725 µg/ml). Fatty acids in natural oils are classified as chemical penetration enhancers as they disrupt the skin barrier function by means of fluidisation and perturbation of the stratum corneum, which in turn, enhances the permeability of actives across the skin (Correa *et al.*, 2014:39; Haque & Talukder, 2018:174; Kezutyte *et al.*, 2013:3). Castor oil is rich in fatty acids, in particular ricinoleic acid (Naik *et al.*, 2018:640), which could have attributed to the high accumulation of the **(CaOE)** in the SCE. However, not all fatty acids are equal in regard to their penetration enhancing effects. A study conducted by Viljoen *et al.* (2015:2049) found that lower API concentrations were obtained in the stratum corneum with formulations containing coconut and emu oil, respectively, which correlates with the results from this study (although the difference in concentrations between **(CaOE)** and **(CoOE)** did not differ much).

When comparing the complex **(FS)** with the simple **(NOE)**, the **(CaOE)** obtained the highest median concentration, followed by the **(CoOE)**, **(EOE)**, and lastly, the **(FS)** for which no oestriol

concentration was detected in the SCE. The difference in the median concentrations could be attributed to the amount of oestriol release from the vehicle. The recovery of oestriol from the **(FS)** was merely 34.97% (see Section D.3.1). The low recovery% could be due to the incorporation of polymers, whether synthetic or natural, which have been widely used for controlled drug release (Brannon-Peppas, 1997). Prolonged drug release can be utilised to minimise adverse effects (Patel *et al.*, 2016:185). Polymeric nanoparticles have been effectively used to deliver acyclovir, quercetin and retinol topically (Kim *et al.*, 2006:139; Hasanovic *et al.*, 2009: 1613; Tan *et al.*, 2011:1621). Lastly, the high concentrations of oestriol in the SCE observed with the **(NOE)** could be attributed to the penetration enhancing effects of the natural oils.

E.3.3.2 Epidermis-dermis concentration

Figures E.42 – E.47 show the results of the average oestriol concentration obtained in the ED from the respective Idexis products and the **(NOE)** after tape stripping was completed.

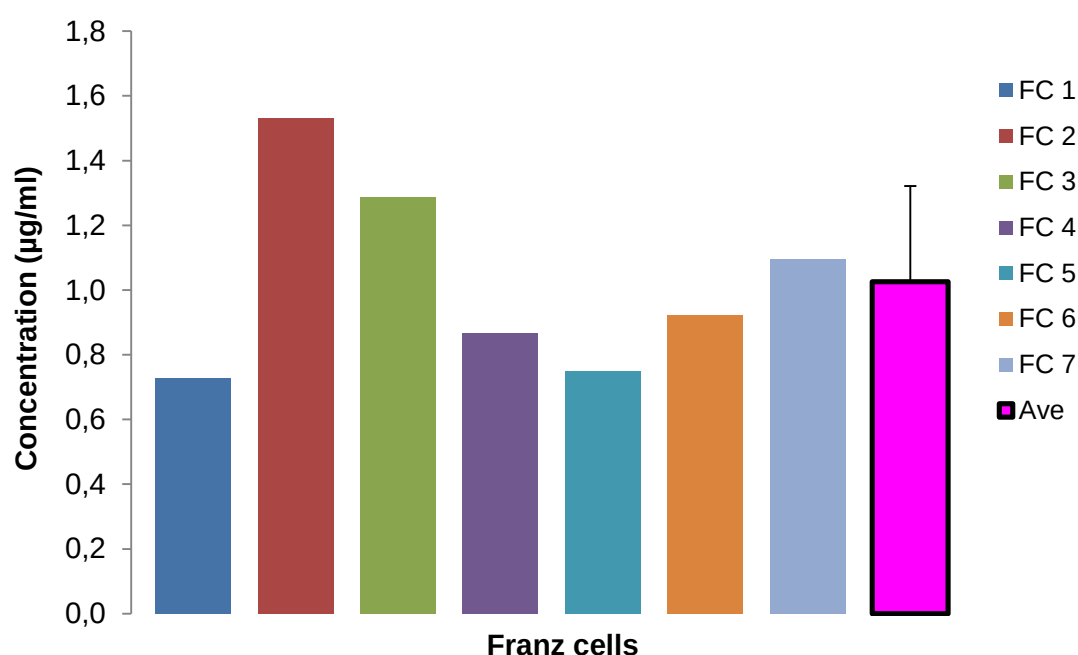


Figure E.42: Oestriol concentration (µg/ml) from the **(FS)** present in the ED (n = 7)

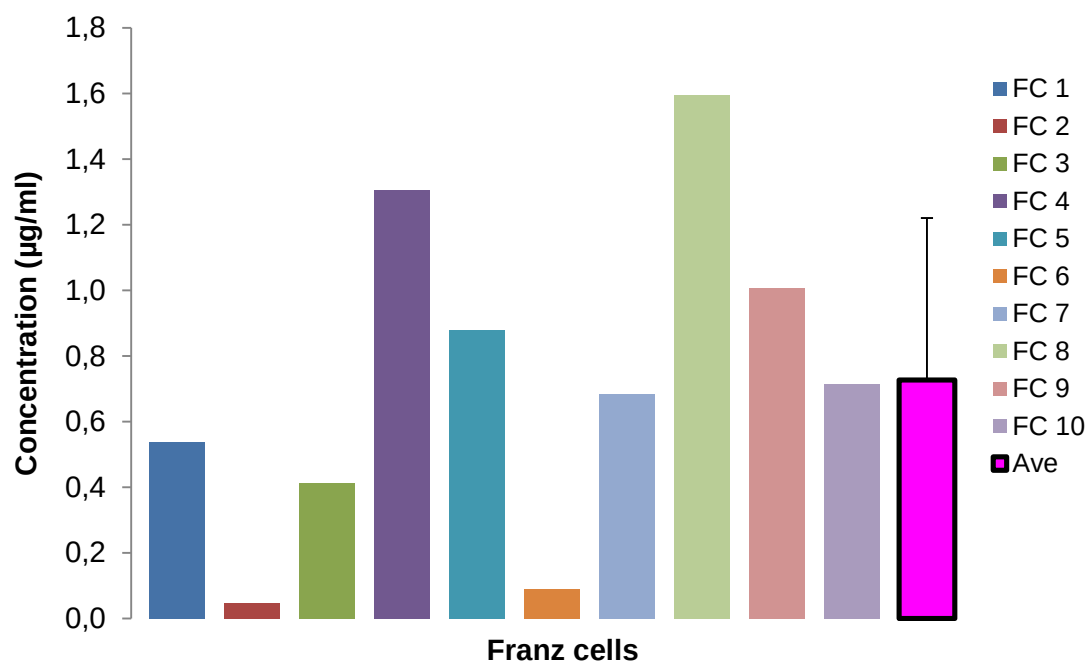


Figure E.43: Oestriol concentration (µg/ml) from the **(EC)** present in the ED (n = 10)

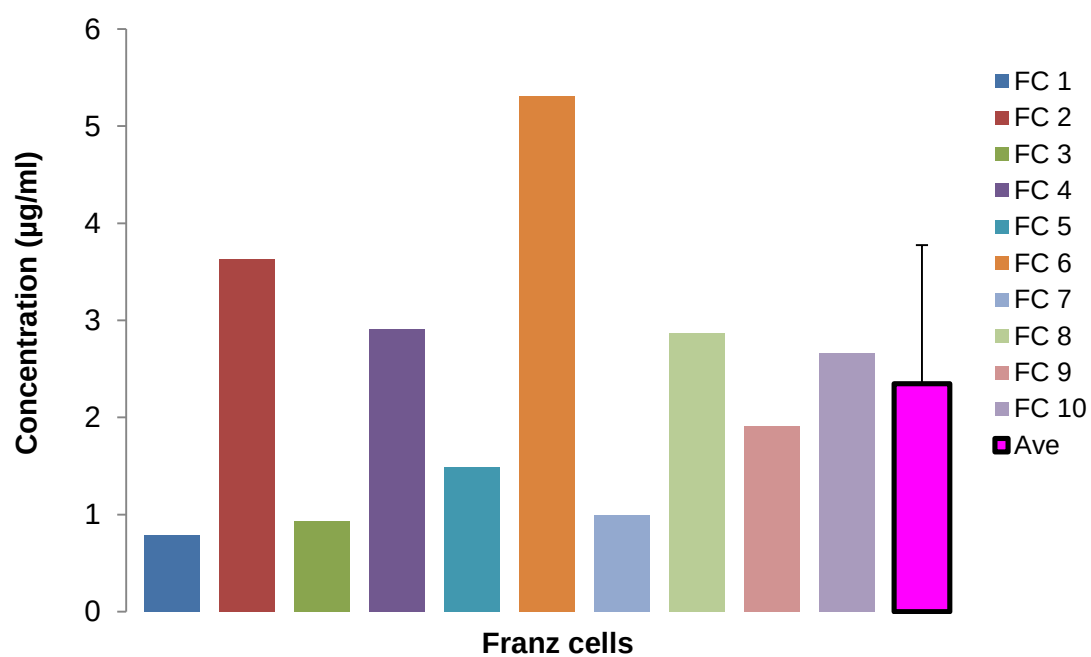


Figure E.44: Oestriol concentration (µg/ml) from the **(MC)** present in the ED (n = 10)

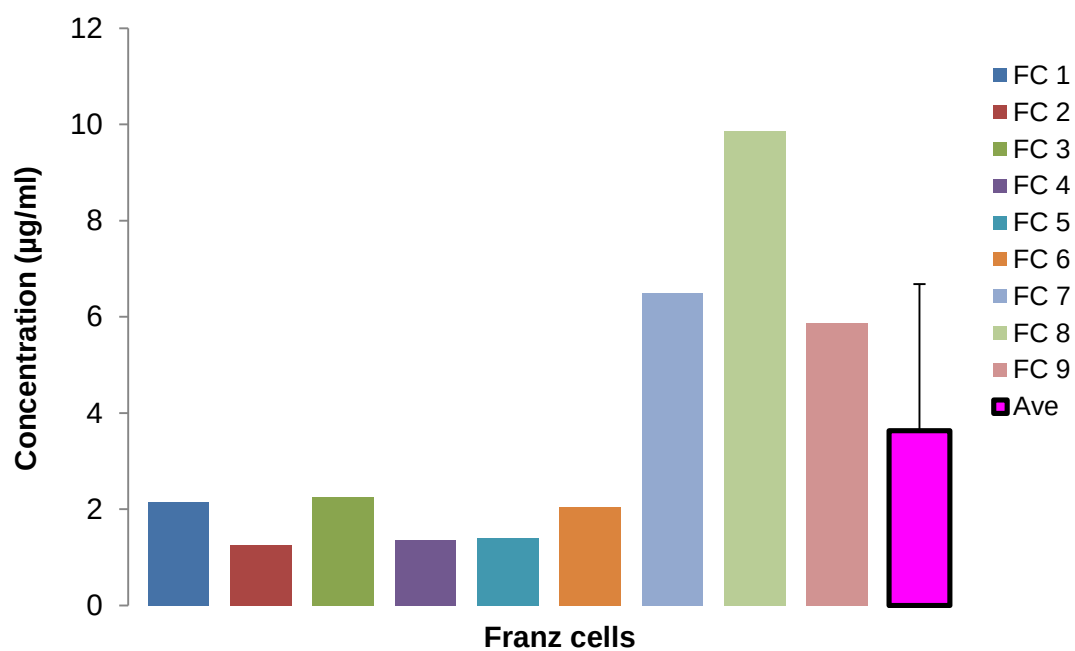


Figure E.45: Oestriol concentration (µg/ml) from the **(CoOE)** present in the ED (n = 9)

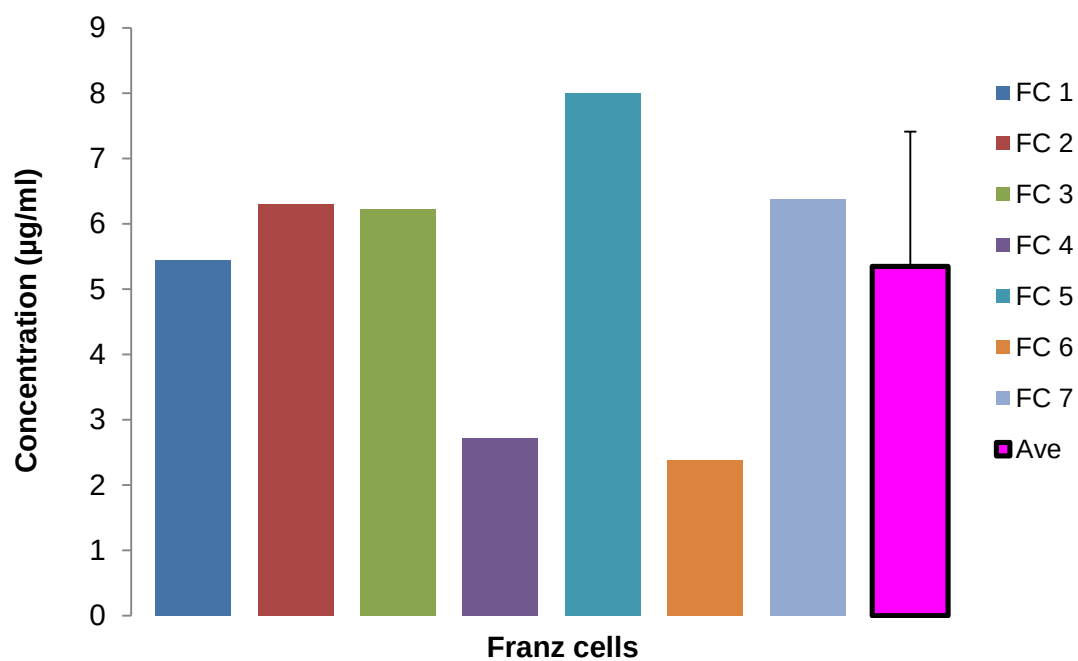


Figure E.46: Oestriol concentration (µg/ml) from the **(CaOE)** present in the ED (n = 7)

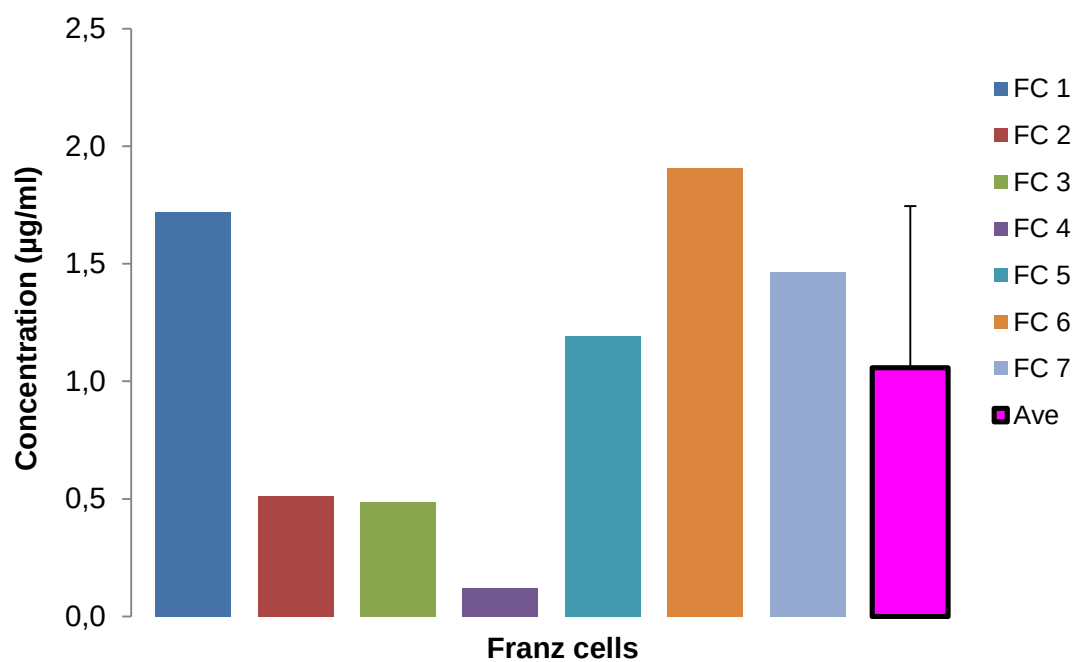


Figure E.47: Oestriol concentration (µg/ml) from the (EOE) present in the ED (n = 7)

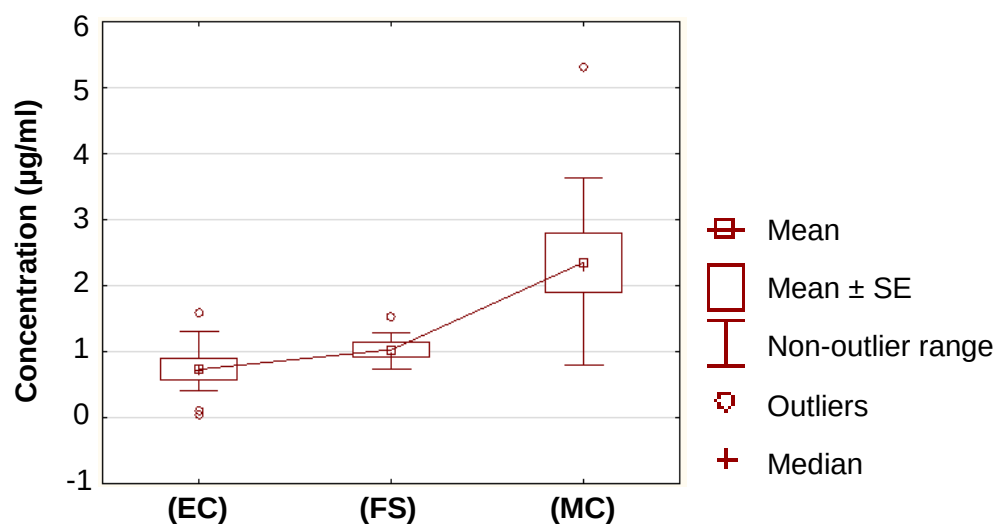


Figure E.48: Box-plot indicating the mean and median concentration (µg/ml) of oestriol present in the ED from the Idexis products

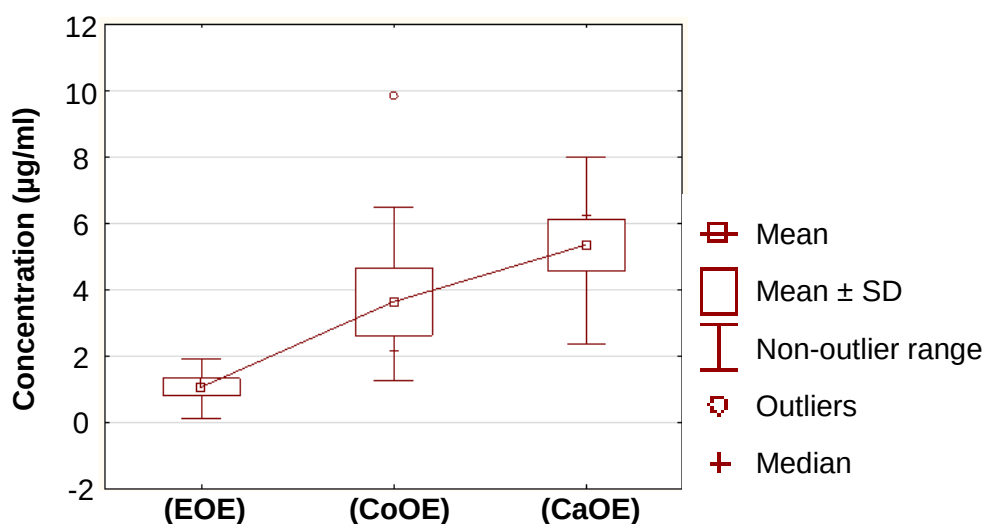


Figure E.49: Box-plot indicating the mean and median concentration (µg/ml) of oestriol present in the ED from the **(NOE)**

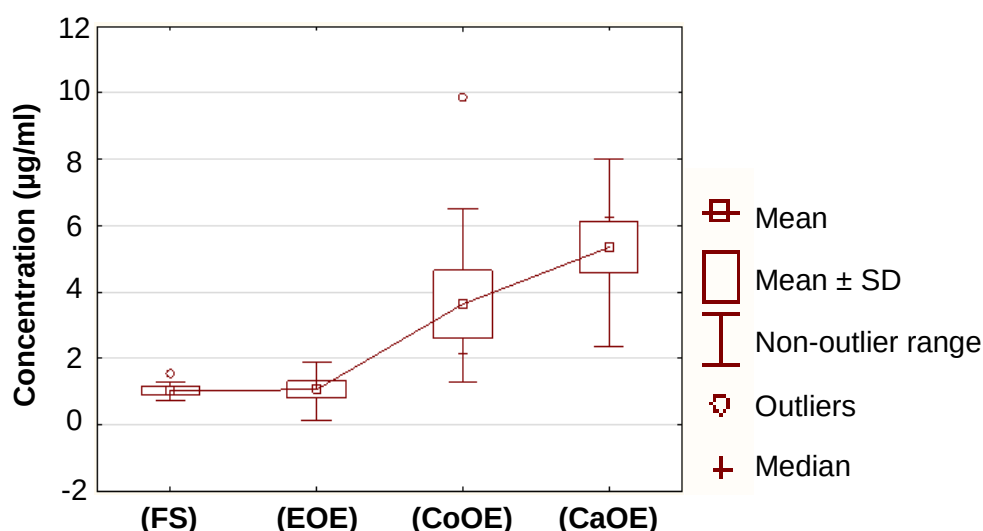


Figure E.50: Box-plot indicating the mean and median concentration (µg/ml) of oestriol present in the ED from the **(FS)** and the **(NOE)**

When comparing the median concentrations obtained in the ED for the respective Idexis products, the **(MC)** (2.281 µg/ml) obtained the highest median concentration, followed by the **(FS)** (0.921 µg/ml) and lastly, the **(EC)** (0.700 µg/ml). The viscosity of a formulation has been reported to influence the penetration depth of the incorporated API, where increasing viscosity resulted in slightly decreased penetration depths (Binder *et al.*, 2019:1). The viscosity of the formulations decreased in order of the **(MC)** > **(EC)** > **(FS)**, consequently, the lack of correlation between the viscosity and the penetration depth suggests that the viscosity of the respective Idexis products were not a primary factor contributing to the penetration depth of oestriol.

When comparing the respective **(NOE)** in terms of the median concentration quantified within the ED, the **(CaOE)** (6.222 µg/ml) obtained the highest median concentration, followed by the **(CoOE)** (2.143 µg/ml) and lastly, the **(EOE)** (1.191 µg/ml). The high median concentration displayed by the **(CaOE)** could be attributed to the use of castor oil, which contains high concentrations of ricinoleic acid (Naik *et al.*, 2018:640). Ricinoleic acid has been shown to penetrate effortlessly deep into the skin and increase the percutaneous absorption of other compounds (Barbosa *et al.*, 2018:86; Baynes & Riviere, 2004:15). The lower median concentration displayed by the **(CoOE)** in the ED could be attributed to its lipid composition. Lauric (C12:0), myristic (C14:0) and palmitic acid (C16:0) has a linear shape, which decreases their ability to disrupt the intercellular lipid region of the stratum corneum, resulting in decreased permeability of oestriol (Chi, 1995:270). Lastly, the lowest median concentration observed in the ED for the **(EOE)** could be due to the significantly higher concentrations of SFAs (i.e. palmitic and stearic acid) present in emu oil (see Table C.7). Longer chain SFAs have higher melting points, decreasing their solubility in the oil, and consequently, their ability of disrupting the stratum corneum lipid bilayer (Viljoen *et al.*, 2015:2052). Hence, the decreased penetration ability of the fatty acids present in emu oil could have contributed to the lower median concentration in the ED for the **(EOE)**.

When comparing the **(FS)** with the **(NOE)**, the **(CaOE)** (6.222 µg/ml) obtained the highest median concentration in the ED, followed by the **(CoOE)**, **(EOE)**, and lastly, the **(FS)** (0.921 µg/ml). The higher median concentrations in the ED displayed by the **(NOE)** could be attributed to the penetration enhancing effect of the fatty acids present in the selected natural oils (Correa *et al.*, 2014:39; Haque & Talukder, 2018:174; Kezutyte *et al.*, 2013:3). Lastly, the low median concentration obtained for the **(FS)** could be attributed to the low %recovery of oestriol from the vehicle (see Section D.3.1), resulting in a lower concentration gradient, and therefore, lower accumulation within the ED (Fu & Kao, 2010:430).

E.4 Statistical analysis

E.4.1 Membrane release studies

One-way ANOVA (single factor analysis) is an appropriate statistical test used to determine whether statistical significant differences exist between the means of two or more independent groups (Abell *et al.*, 1999:360; Kao & Green, 2008:162, 167). Statistical comparison of the flux values by means of one-way ANOVA revealed statistically significant differences ($p < 0.001$) between the Idexis products' means. The normal probability plot is a graphical tool used to evaluate whether or not a dataset is reasonably normally distributed (Chambers *et al.*, 1983:206; Filliben & Heckert, 2012). The normal probability plot illustrated that many points varied from the straight line. Hence, normality did not hold (Meyers *et al.*, 2013:68). Levene's

test for homogeneity of variance was performed to identify whether the variances of the independent groups were equal or approximately equal (Coolidge, 2000:244). The assumption of homogeneity of variances was met based on Levene's test, $F(2, 23) = 6.871$, $p < 0.005$. Note that Tukey's Unequal N HSD post-hoc test is a modification of the Tukey's HSD post-hoc test and used when the sample sizes are unequal. The statistically significant ANOVA was followed-up with a Tukey's Unequal N HSD post-hoc test to determine the significant differences between every possible pair of means of the three Idexis products (Lee & Lee, 2018:355). From Table E.7, it can be observed that all three Idexis products differed significantly from each other during the membrane release studies (as indicated by a, b and c).

Table E.7: Unequal N HSD post-hoc test performed on the Idexis products

Idexis products	Mean flux ($\mu\text{g}/\text{cm}^2\cdot\text{h}$)
(EC)	12.250 ^a
(FS)	16.339 ^b
(MC)	23.658 ^c

a, b and c: represent statistically non-significant homogenous groups

Statistical comparison of the mean flux values using one-way ANOVA revealed no significant differences ($p > 0.05$) between the means of the **(NOE)**. The normal probability plot showed that the data was approximately normally distributed as most points appeared to fall on the straight line. Also, the assumption of homogeneity of variances was satisfied based on Levene's test, $F(2, 27) = 1.713$, $p = 0.199$. Following the ANOVA, Tukey's Unequal N HSD post-hoc test was not performed. Therefore, no statistically significant differences exist between the **(CoOE)**, **(CaOE)** and **(EOE)**.

Statistical comparison of the flux values using one-way ANOVA revealed significant differences ($p < 0.001$) between the means of the **(FS)** and the **(NOE)**. The normal probability plot illustrated that the points deviated significantly from the straight line. Therefore, normality did not hold. The assumption of homogeneity of variances was not satisfied based on Levene's test, $F(3, 36) = 7.157$, $p < 0.001$. The Kruskal-Wallis test, also commonly referred to as the one-way ANOVA by ranks test, is a non-parametric test used to compare three or more independent groups on a dependant variable that is measured at an ordinal level (Chan & Walmsley, 1997:1755; Leon, 1998:259). Normality did not hold; therefore, the Kruskal-Wallis test was performed to investigate the differences between the **(FS)** and **(NOE)** medians. The test revealed a p-value of < 0.05 , which is indicative of significant differences between the **(FS)** and each of the **(NOE)** medians, as seen in Table E.8. Moreover, it can be observed that the respective **(NOE)** medians did not differ significantly.

Table E.8: Kruskal-Wallis test comparing the medians of the **(FS)** and **(NOE)**

Multiple comparison p-values (2-tailed); membrane flux Independent variable: Formulation				
Depended flux	(FS)	(CaOE)	(CoOE)	(EOE)
(FS)		0.00002	0.002560	0.00562
(CaOE)			1.00000	1.00000
(CoOE)				1.00000
(EOE)				

E.4.2 Skin diffusion studies

Statistical comparison of the amount per area diffused values using one-way ANOVA revealed significant differences ($p < 0.001$) between the Idexis products' means. The normal probability plot demonstrated that the points were not on a straight line. Therefore, normality did not hold. The assumption of homogeneity of variances was met based on Levene's test, $F(2,24) = 0.978$, $p = 0.390$. Logarithmic transformation was used to transform the skewed data distribution to conform more closely to normality (Feng *et al.*, 2014:105). Statistical comparison of the log amount per area diffused values using one-way ANOVA revealed significant differences ($p < 0.001$) between the Idexis products' means. The normal probability plot of the transformed data demonstrated a near to normal distribution, as most of the points followed the straight line with a small number of outliers. The assumption of homogeneity of variances between the mean log amount per area diffused values of the Idexis products was satisfied based on Levene's test, $F(2,24) = 1.039$, $p = 0.369$. The statistically significant ANOVA was followed-up with a Tukey's Unequal N HSD post-hoc test to determine the significant differences between all possible pairs of the mean log amount per area diffused values of the three Idexis products. From Table E.9, it can be observed there is no significant difference between the **(FS)** and **(EC)** (indicated by a); however, both products displayed a significant difference when compared to the **(MC)** (indicated by b).

Table E.9: Unequal N HSD post-hoc test performed on the Idexis products

Idexis products	Mean log amount per area diffused values ($\log(\mu\text{g}/\text{cm}^2)$)
(FS)	0.350 ^a
(EC)	0.553 ^a
(MC)	0.979 ^b

a and b: represent statistically non-significant homogenous groups

A t-test is a parametric test used to determine whether a statistically significant difference exists between the means of two independent groups (SPSS Tutorials, 2019; Kim, 2015:540).

Therefore, a t-test was performed to compare the mean amount per area diffused values of the **(CaOE)** and **(CoOE)**. The t-test was associated with a statistically significant effect, $t(13.97) = 2.47$, $p = 0.027$. Logarithmic transformation was used to reduce the skewness of the data distribution. The normal probability plot illustrated that the points lay closer to the straight line, hence, the distribution of the **(CoOE)** and **(CaOE)** was closer to normality. A t-test was performed to compare the mean log amount per area diffused values of the **(CoOE)** and **(CaOE)** and revealed a statistically significant effect, $t(7.86) = 3.058$, $p = 0.016$.

Statistical comparison of the log amount per area diffused values using one-way ANOVA revealed significant differences ($p < 0.001$) between the means of the **(FS)** and the **(NOE)**. The normal probability plot illustrated that the points followed a straight-line pattern; therefore, the data was reasonably normally distributed. The assumption of homogeneity of variances between the mean log amount per area diffused values of the **(FS)** and **(NOE)** was met based on Levene's test, $F(2, 20) = 1.743$, $p = 0.201$.

Table E.10: Unequal N HSD post-hoc test performed on the **(FS)** and **(NOE)**

Formulation	Mean log amount per area diffused values ($\log(\mu\text{g}/\text{cm}^2)$)
(CaOE)	-0.804 ^a
(CoOE)	-0.366 ^b
(FS)	0.350 ^c

a, b and c: represent statistically non-significant homogenous groups

The statistically significant ANOVA was followed-up with a Tukey's Unequal N HSD post-hoc test to determine the significant differences between every possible pair of the mean log amount per area diffused values of the **(FS)**, **(CoOE)** and **(CaOE)**. From Table E.10, it can be observed that all three formulations differed significantly from one another during skin diffusion studies (as indicated by a, b and c).

E.4.3 Tape stripping

A two-way ANOVA was performed to determine the effect of the Idexis products and the skin layers (SCE and ED) on the accumulation of oestriol in the tape strips. The interaction between the Idexis products and the skin layers was nearly statistically significant ($F(1,36) = 3.18$, $p = 0.083$ (> 0.05) (i.e. no interaction). The normal probability plot illustrated that many points deviated from the straight line; therefore, normality did not hold. The assumption of homogeneity of variances was not met based on Levene's test, $F(3, 36) = 8.111$, $p < 0.001$.

A t-test was performed to compare the mean concentrations of the **(EC)** and the **(MC)** in terms of the SCE. The t-test was associated with a statistically significant effect, $t(11.439) = -3.768$, $p = 0.003$, which was then confirmed by the Mann-Whitney U test ($p < 0.001$). The normal

probability plot for the median concentrations of the IDEXIS products in the SCE illustrated that the data for the **(EC)** was normally distributed, whereas the data for the **(MC)** was skewed left (non-normal distribution).

One-way ANOVA was performed to determine the effect of the IDEXIS products and the ED during tape stripping studies, which yielded a statistically significant effect ($p < 0.05$). The normal probability plot showed that the data did not follow the straight line; therefore, normality did not hold. The assumption of homogeneity of variances was not satisfied based on Levene's test, $F(2, 24) = 7.694$, $p = 0.003$. Therefore, the Kruskal-Wallis test was performed to investigate the differences between the median concentrations in the ED for the IDEXIS products and showed a significant effect ($p < 0.05$).

Table E.11: Multiple comparisons following the Kruskal-Wallis test comparing the median concentrations in the ED for the IDEXIS products

Multiple comparison p-values (2-tailed); concentration Independent variable: Formulation			
Depended flux	(EC)	(FS)	(MC)
(EC)		0.829	0.003
(FS)			0.181
(MC)			

From Table E.11, it can be observed that only the median of the **(EC)** differed significantly from that of the **(MC)**, while the **(FS)** did not differ from any of the IDEXIS products.

Statistical comparison of the concentrations in the ED using one-way ANOVA revealed significant differences ($p < 0.001$) between the means of the **(FS)** and the **(NOE)**. The normal probability plot illustrated that the points did not follow the straight line, hence, normality did not hold. The assumption of homogeneity of variances was also not met based on Levene's test, $F(3, 26) = 8.663$, $p < 0.001$. The Kruskal-Wallis test was performed to investigate the differences between the median concentrations in the ED for the **(FS)** compared to the **(NOE)**. The Kruskal-Wallis test revealed a significant effect ($p < 0.001$); the median of the **(CaOE)** differed significantly from those of the **(FS)** ($p = 0.002$) and the **(EOE)** ($p = 0.005$), as seen in Table E.12.

Table E.12: Multiple comparisons following the Kruskal-Wallis test comparing the median concentrations in the ED for the **(FS)** and **(NOE)**

Multiple comparison p-values (2-tailed); membrane flux Independent variable: Formulation				
Depended flux	(FS)	(EOE)	(CoOE)	(CaOE)
(FS)		1.000	0.064	0.002
(EOE)			0.109	0.005
(CoOE)				1.000
(CaOE)				

The medians of the SCE and ED for the Idexis products and **(NOE)** were compared, respectively, using the Mann-Whitney U test, the nonparametric equivalent of the t-test, and provides the most accurate approximations of significance, especially when data is not normally distributed (Smalheiser, 2017:162).

Table E.13: Mann-Whitney U test comparing the medians of the ED and SCE for the Idexis products

Idexis Product	P-value
(EC)	0.064
(MC)	0.009

For the **(MC)** the median concentrations in the ED and SCE differed significantly ($p = 0.009$).

Table E.14: Mann-Whitney U test comparing the medians of the ED and SCE for the **(NOE)**

(NOE)	P-value
(EOE)	0.609
(CoOE)	0.659
(CaOE)	0.021

For the **(CaOE)** the median concentrations in the ED and SCE differed significantly ($p = 0.021$).

E.5 Conclusion

The profound effect that hormones have on the human skin has led to the formulation of various cosmetic products to meet the growing demand for youthful skin. The anti-ageing effects hormones have on the skin of postmenopausal women are well documented (Brincat *et al.*, 2005:110; Holzer *et al.*, 2005:633; Izykowska *et al.*, 2008:23; Kanda & Watanabe, 2005:6; Sator

et al., 2004:39; Schmidt *et al.*, 1994:25; Shin *et al.*, 2005:315). It is known that transdermal hormone delivery enters the blood circulation, initiating a systemic effect. Women diagnosed with cancer or who are at risk due to genetics cannot use HRT. Idexis aimed to formulate topical products that deliver female hormones to the target-site (i.e. ED) to exert an anti-ageing effect. The formulator incorporated polymers to inhibit systemic absorption, reducing the risk of breast cancer development associated with increased serum oestrogen levels (Yue *et al.*, 2010:1748).

The aim of this study was to investigate whether oestriol in the **(FS)**, **(EC)** and **(MC)** could only be delivered topically (hence, not transdermally). Due to the classified nature of this study, limited information with regard to the excipients, vehicle and formulation method was made available for the respective Idexis products. Hence, simple o/w emulsions were also formulated containing 2% oestriol and different natural oils **(NOE)** in order to compare the effectiveness of the polymers and vehicles used for the complex Idexis products to achieve topical delivery. Firstly, membrane release studies were performed, followed by skin diffusion studies and tape stripping for the respective Idexis products and the **(NOE)**.

From the membrane release studies, it was confirmed that oestriol was released from all six formulations. The simple **(NOE)** released significantly lower quantities of oestriol compared to the complex **(FS)**. This could be attributed to the lower viscosity of the **(FS)**, which facilitates the diffusion of the API molecules from the vehicle to the skin surface (El Gendy *et al.*, 2002:824). Consequently, the amount of oestriol available for skin absorption was greater for the **(FS)** compared to the **(NOE)**, which could be attributed to the higher amount of oestriol that diffused across the skin, and subsequently into the systemic circulation. Skin diffusion studies were performed after completing the membrane release studies.

From HPLC analysis it was confirmed that oestriol concentrations were quantified in the receptor phase samples for all the Idexis products; it can therefore be stated that the polymers used to retain oestriol within the skin and limit systemic absorption were ineffective. Oestriol concentrations were quantified in the receptor phase samples for the **(CoOE)** and **(CaOE)**. Consequently, the **(EOE)** was the only tested formulation that did not deliver oestriol transdermally. Significantly lower amounts of oestriol diffused through the skin from the **(NOE)** compared to the **(FS)**. Hence, lower quantities of oestriol were delivered transdermally from the **(NOE)** compared to the Idexis products. The difference could be attributed to the lower occlusion effect of the aqueous external phase of the **(NOE)** compared to the oily vehicle of the **(FS)** (Jiménez *et al.*, 2004:54). Another contributing factor could be the lower viscosity of the **(FS)**, as skin permeation increases with a decrease in formulation viscosity (Gallagher & Heard, 2005:191). After the receptor phases were extracted for HPLC analysis, tape stripping was performed.

The stratum corneum serves as the main diffusion barrier, allowing only certain API molecules to pass through (i.e. low MW and lipophilic) (Waghule *et al.*, 2019:1249). However, once it is overcome, delivery of the API within the skin layers without systemic exposure becomes challenging. It has been proposed that an API with good lipid solubility would be concentrated within the skin layers as increased lipophilicity facilitates reservoir formation (Cross *et al.*, 2003:759). Accordingly, oestriol concentrations were quantified in the SCE and ED during tape stripping for the **(NOE)**. The penetration enhancing effects of the fatty acids were confirmed, as higher concentrations of oestriol were quantified in the ED compared to the SCE for the **(CaOE)** and the **(EOE)**. This could be attributed to the relatively high concentrations of ricinoleic acid (C18:3) present in castor oil, which has been reported to penetrate deep into the skin (Barbosa *et al.*, 2018:86; Baynes & Riviere, 2004:15). Furthermore, linolenic acid (C18:2) present in emu oil could have increased the degeneration of the skin appendages (Viljoen *et al.*, 2015:2050). Emu oil is extremely rich in oleic acid in addition to being the only oil in this study that contains palmitoleic acid. A study conducted by Katsuta *et al.* (2005:1009) found that the application of oleic and palmitoleic acid induced scaling, which is indicative of abnormal epidermal differentiation. It is plausible that this alteration to the epidermis resulted in the higher accumulation of oestriol in the ED (in comparison to the SCE) for the **(EOE)**. For the **(CoOE)**, a lower concentration oestriol was quantified in the ED than in the SCE (although it did not differ much). This could be due to the higher concentrations of SFA present in coconut oil compared to the other two oils (Petrauskaitė *et al.*, 2000:582).

In contrast to what was expected, the **(FS)** did not exhibit a higher affinity for the lipophilic stratum corneum, as oestriol partitioned rapidly (within 12 h) into the hydrophilic viable epidermis. From visual inspection, it is clear that the **(FS)** is mainly composed of an oily base, which could have caused an increase in the percutaneous absorption of oestriol due to the effects of skin occlusion. According to Hafeez & Maibach (2013:82), the following can be concluded with regard to the effects of occlusion:

- occlusion increases the percutaneous absorption of numerous, but not all APIs;
- penetration can increase as the duration of occlusion increases, and
- occlusion appears to increase the penetration of highly lipophilic APIs more than that of highly hydrophilic APIs.

The increased diffusion rate shown by the **(FS)** could be a result of greater skin occlusion compared to the **(EC)** and **(MC)**. Occlusion increases the water content of the stratum corneum, which hydrates the polar head groups of the lipid bilayer, which in turn causes them to swell and disrupt the intercellular lipid domains (Murthy & Shivakumar, 2010:17). Furthermore, hydration increases the aqueous regions within the intercellular lipid domains, increasing the hydrophilic nature of the stratum corneum. As a result, this decreases the SCE distribution

coefficient, favouring the transport of APIs from the lipophilic stratum corneum to the hydrophilic viable epidermis (Nalenz, 2006:25).

For the **(NOE)**, lower concentrations of oestriol had diffused through the skin into the receptor phase compared to the amount that had been retained within the skin layers. Conversely, higher concentrations of oestriol were delivered transdermally from the Idexis products compared to the amount retained within the skin. The excipients used to formulate the respective Idexis products may have increased the fluidity of the stratum and solubilised the intercellular lipids of the stratum corneum, which disrupted the skin barrier function, and in turn, increased the permeability of oestriol across the skin (Bigucci *et al.*, 2014:1017; Yanase & Hatta, 2018:44). Due to the classified nature of this study, the excipients used are unknown; consequently, the extent of their penetration enhancing effects remains uncertain. However, it can be suggested that the excipients contributed towards the increased oestriol concentrations quantified in the receptor phase samples.

Considering all the data for the respective formulations, the following becomes evident:

1. When comparing the **(NOE)**, the **(EOE)** displayed the best drug release; however, it delivered oestriol the least to the SCE and ED. It can be proposed that there might not have been an adequate amount of oestriol available to be delivered transdermally. Relatively equal concentrations of oestriol were released from both the **(CoOE)** and **(CaOE)**. However, higher oestriol concentrations were delivered to the SCE, especially the ED (target-site) from the **(CaOE)**. Also, lower concentrations were delivered transdermally from the **(CaOE)** compared to the **(CoOE)**. Consequently, the **(CaOE)** displayed the best-targeted delivery with high oestriol concentrations in the ED, and a small amount that diffused across the skin to be delivered transdermally.
2. When comparing the Idexis products, the **(MC)** displayed the best drug release, as well as the best topical (SCE and ED) and transdermal delivery (2.1 times more than the **(EC)** and 4.4 times more than the **(FS)**). Both the **(EC)** and **(FS)** displayed good drug release, and although the delivery in the SCE (**(FS)** had no delivery) and ED was low, the transdermal delivery was considerably more than topical delivery for all three Idexis products. Thus, it can be stated that the polymers (systemic absorption inhibitors) were ineffective for the Idexis products. It could be suggested that Idexis might need to consider a basic formulation (such as the use of natural oils in o/w emulsions) that targets the ED more and delivers less transdermally.

For this study the following conclusions could be made:

- During membrane release studies, the highest median flux was obtained by the **(MC)** for the Idexis products and the **(EOE)** for the **(NOE)**.
- Comparing the complex **(FS)** and simple **(NOE)** in terms of flux after membrane release studies, the **(FS)** obtained the highest flux.
- During skin diffusion studies, the highest oestriol concentration quantified in the receptor phase was obtained by the **(MC)** for the Idexis products and the **(CoOE)** for the **(NOE)**.
- Comparing the complex **(FS)** and simple **(NOE)** in terms of the oestriol concentration quantified in the receptor phase after skin diffusion studies, the **(FS)** obtained the highest oestriol concentration.
- Within the SCE and the ED, the highest oestriol concentration was obtained by the **(MC)** for the Idexis products and the **(CaOE)** for the **(NOE)**.
- No oestriol concentration was quantified in the SCE for the **(FS)** and no oestriol was transdermally delivered from **(EOE)**.

Overall, it can be stated that the aim of topical delivery was not achieved by any of the formulations (except **(EOE)**), as oestriol was delivered transdermally from **(FS)**, **(EC)**, **(MC)**, and to a lesser extent from **(CoOE)** and **(CaOE)**.

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APPENDIX F:

GUIDE FOR AUTHORS: EUROPEAN JOURNAL OF PHARMACEUTICAL SCIENCES

INTRODUCTION

Manuscripts submitted to the journal are accepted on the understanding that: (1) they are subject to editorial review, (2) they have not been and will not be published in whole or in part in any other journal and (3) the recommendations of the most recent version of the Declaration of Helsinki, for humans, and the European Community guidelines as accepted principles for the use of experimental animals, have been adhered to. The European Journal of Pharmaceutical Sciences will, therefore, only consider manuscripts that describe experiments which have been carried out under approval of an institutional or local ethics committee.

Types of Paper

Research articles

The European Journal of Pharmaceutical Sciences publishes research articles in the multidisciplinary field of pharmaceutical sciences, with a focus on topics relevant for drug discovery and development.

More specifically, the Journal publishes reports on medicinal chemistry, pharmacology, drug absorption and metabolism, pharmacokinetics and pharmacodynamics, pharmaceutical and biomedical analysis, drug delivery (including gene delivery), drug targeting, pharmaceutical technology, pharmaceutical biotechnology and clinical drug evaluation.

The journal will typically not give priority to manuscripts focusing primarily on organic synthesis, natural products, adaptation of analytical approaches, or discussions pertaining to drug policy making.

Important other criteria for manuscript acceptance are conceptual novelty, scientific rigorousness of the experiments, relevance for a broad readership beyond the specific topic of the manuscript, and adherence to high ethics standards of experimentation. Research articles should comply with the format requirements set forth in the section "Article Structure below".

Review articles

The manuscript of a review article should be arranged as described for research articles but according to the following sections: title page, abstract and keywords (indexing terms, normally 3 – 6 items), Introduction, Specific sections determined by the author, Conclusions, Acknowledgements, References, Figure legends and Figures, Tables. Sections ranging from the Introduction to the Conclusions should be numbered. Subdivisions within a section should also be numbered within that section: 2.1., 2.2., 2.3. etc. All pages should be numbered consecutively, the title page being p.1.

Commentaries and Mini-reviews

One page suggestions for comprehensive reviews, commentaries or mini-reviews should be sent to the Editor-in-Chief at ejps@sdu.dk for consideration. Please see detailed information on commentaries and mini-reviews below.

Commentaries (Guidance)

The definition of a Commentary for EJPS is three-fold. Firstly, it can be an argued piece of provocative scientific writing purporting to take a balanced position on a controversial pharmaceutical science topic. A second option is for the author to approach the topic from a particular viewpoint on one side of an argument. A third option is to provide a topical update on a hot topic in Pharmaceutical Sciences and this can be more informative than controversial.

Commentaries will be commissioned by the editors in advance or invited from non-commissioned authors if they wish to initially submit a one page summary of the intended Commentary to the editors in advance. All manuscripts will be assessed by 2 – 3 independent referees.

The journal is looking for a stimulating and provoking essays, with referenced material, but without an extensive reference list. Commentaries can contain one summary figure and/or table and should have no more than 30 references to preferably recent peer-reviewed material. The word count should be approximately 2,000 words maximum. The commentary should have a short abstract summary of 150 to 200 words and 4 – 5 key words should be included, The text should be broken down into 4 – 5 numbered sections beginning with an Introduction and ending with a Conclusions section. A model of the structures is to be found in Eur. J. Pharm. Sci. 19, 1-11 by R.D. Combes.

Mini-review (Guidance)

Mini-reviews are thought provoking reviews of contemporary pharmaceutical research. Themes are as described in the Scope of the Journal section.

Mini-reviews will usually be commissioned by the editors in advance, but contributions are invited from non-commissioned authors if they wish to initially submit a one page summary of the intended review to the editors in advance. All manuscripts will be assessed by 2 – 3 independent referees.

The structure of the mini-review is as follows: a title page followed by a 200 – 300 word abstract with 4 – 5 key words. The text is then divided into numbered sections finishing with a Summary section. References should be kept to a maximum of 60 and should be mostly to recent peer-reviewed material. There is a combined maximum of 5 figures / tables. Authors are encouraged to submit their original unpublished work as part of the review if appropriate. The total length of the review should be a maximum of 4,000 words.

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Some abbreviations may be used without definition:

ADP, CDP, GDP, IDP	5'-pyrophosphates of adenosine
UDP	cytidine, guanosine, inosine, uridine
AMP etc.	adenosine 5'-monophosphate etc.
ADP etc.	adenosine 5'-diphosphate etc.
ATP etc.	adenosine 5'- triphosphate etc.
CM-cellulose	carboxymethylcellulose
CoA and acetyl-CoA	coenzyme A and its acyl derivatives
DEAE-cellulose	O-(diethylaminoethyl)-cellulose
DNA	deoxyribonucleic acid
EGTA	ethylene glycol-bis(β -aminoethyl ether)N,N,N',N'-tetraacetic acid
FAD	flavin-adenine dinucleotide
FMN	flavin mononucleotide
GSH, GSSG	glutathione, reduced and oxidized
Hepes	4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid
NAD	nicotinamide-adenine dinucleotide
NADP	nicotinamide-adenine dinucleotide phosphate
NMN	nicotinamide mononucleotide
Pi, PPi	orthophosphate, pyrophosphate
RNA	ribonucleic acid
Tris	2-amino-2-hydroxymethylpropane-1,3-diol

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kilogram	kg	millimole	mmol
gram	g	micromole	μ mol
milligram	mg	nanomole	nmol

microgram	μg	picomole	pmol
nanogram	ng	femtomole	fmol
mole (gram-molecule)	mol	equivalent	eq

Units of time

hour	h	millisecond	ms
minute	min	microsecond	μs
second	s		

Units of volume

litre	l	microliter	μl
milliliter	ml		

Units of length

metre	m	micrometer	μm
centimeter	cm	nanometer	nm
millimeter	mm		

Units of concentration

molar (mol/l)	M	nanomolar	nM
millimolar	mM	picomolar	pM
micromolar	μM		

Units of heat, energy, electricity

joule	J	volt	V
degree Celsius (centigrade)	°C	ohm	Ω
coulomb	C	siemens	S
ampere	A		

Units of radiation

curie	Ci	disintegrations per minute	dpm
counts per minute	cpm	becquerel	Bq

Miscellaneous

gravity	<i>g</i>
dissociation constant	<i>K_d</i>

median doses	LD ₅₀ , ED ₅₀
probability	<i>P</i>
routes of drug administration	i.v., i.p., s.c., i.m.
square centimeter	cm ²
standard deviation	S.D.
standard error of the mean	S.E.M.
Svedberg unit of sedimentation coefficient	S
Hill coefficient	<i>n_H</i>

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