Extemporaneously prepared astaxanthin capsules for improved systemic delivery

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ABSTRACT

Extemporaneous compounding of supplements or drugs has been practised since the beginning of pharmaceutical disease treatment approaches. In recent years extemporaneous compounding methods have been used for the formulation for a wide variety of drugs and especially for the formulation of natural supplements and remedies. The biggest concern with the practice of extemporaneous compounding is the lack of sufficient regulatory requirements, including safety and quality evaluations, and this lack of regulatory requirements may lead to severe drug/drug or drug/supplement interactions in patients. The use of supplements is increasing in popularity around the world and the use of these supplements, for example astaxanthin, may pose a risk to patients, mainly because the interactions and side effects of these supplements are not well documented.

The oral route of administration remains the most popular and convenient route of drug administration, however due to physical- and biochemical barriers, the absorption of these drugs may be compromised. With the safe use of different bioenhancers these absorption problems can be overcome. In previous studies the effectiveness of different bioenhancers, including *Aloe vera* leaf materials and piperine, have been proven to enhance the intestinal membrane permeability of drugs in *ex vivo* studies.

The purpose of this study was to compound different formulations for use in hard gelatin capsules containing the supplement astaxanthin in combination with different bioenhancers (i.e. *Aloe vera* gel; *Aloe vera* whole leaf extract and piperine) to enhance the permeability of astaxanthin across pig intestinal epithelial tissue using an *ex vivo* transport model. Each formulation was compounded by mixing the different bioenhancers and Pharmacel®, used as a filler, with astaxanthin and filling capsule shells with the different powder mixtures. One formulation contained only astaxanthin and Pharmacel® and was used as the control formulation. The compounded formulations were all evaluated in terms of astaxanthin content and also for dissolution characteristics of astaxanthin in combination with the different piperine concentrations. The permeability of astaxanthin across excised pig intestinal tissue, while combined in the different formulations, was evaluated using the Sweetana-Grass diffusion apparatus.

The release of astaxanthin from the formulations containing piperine showed that the inclusion of different concentrations of piperine had mediated an inverse concentration dependent decrease in astaxanthin release with increasing piperine concentrations. *Ex vivo* transport studies in both apical-to-basolateral and basolateral-to-apical directions, showed that the different bioenhancers, used in the different formulations, did increase the permeability of astaxanthin across the intestinal

epithelial tissue when compared to the control formulation, however the different concentrations of the bioenhancers did mediate variable effects on the extent of astaxanthin transport. The highest concentration of *Aloe vera* gel and -whole leaf extract exhibited similar astaxanthin transport than the control formulation in the apical-to-basolateral direction. However, the low and medium concentrations did mediate an increase in astaxanthin transport in the apical-to-basolateral direction when compared to the control formulation. Furthermore, the extent of astaxanthin transport in the basolateral-to-apical direction was higher than the control formulation in all instances, however the highest concentration mediated a less pronounced increase in astaxanthin transport. The transport of astaxanthin in combination with varying concentrations of piperine resulted in a concentration dependent decrease in the transport of astaxanthin with an increase in piperine concentrations in the apical-to-basolateral direction. This result is in accordance with the dissolution data which showed that astaxanthin dissolution was impeded when the piperine concentration in the formulations was increased. The results of this study showed that the addition of piperine to the capsule formulations mediated the most pronounced transport enhancing effects in combination with astaxanthin followed by *Aloe vera* whole leaf extract and then *aloe vera* gel. Although this study showed positive results, additional studies are recommended to further investigate the effects of the selected bioenhancers on astaxanthin membrane permeation.

Key words: Extemporaneous compounding, astaxanthin, bioenhancers, *Aloe vera* leaf extract, *Aloe vera* gel, piperine, membrane permeation, *ex vivo.*

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CHAPTER 1: INTRODUCTION

1.1 Background and justification

1.1.1 Pharmaceutical or extemporaneous compounding

Extemporaneously compounded medication and supplements can be described as preparations that are prepared, mixed, packed and labelled according to a prescription for a specific individual (Aquilina, 2013). Extemporaneously compounded preparations allow access to medication that is required for specific patients, which is not commercially available (Cook *et al.,* 2007). Access to "special" dosage forms are sometimes required in certain population groups such as infants, children and the elderly. These dosage forms are usually not commonly available in pharmacies and must be prepared on small scale for a specific patient, according to a specific prescription (Gudeman *et al.*, 2013; Nahata & Allen 2008).

When prescriptions stipulate extemporaneously prepared formulations, it is important to consider the physical and chemical properties of the active ingredients and excipients that are being used in the formulation. It is important to make sure that the product is stable (chemically and physically), that it produces the desired therapeutic outcomes, is easy to administer, and has favourable organoleptic properties. The main concern with most of these extemporaneously prepared formulations is that they have not been subjected to safety-, stability-, bioavalibility-, pharmacokinetic- and/or efficacy studies (Nahata & Allen 2008*).*

A variety of ingredients are used in extemporaneously prepared medication and dietary supplements. These ingredients include vitamins, minerals, amino acids, botanicals, herbs and enzymes and are available in a variety of preparations, including, powders, capsules, tablets, liquids and even energy bars (NIH, 2011). Recent increases in availability and popularity of these supplements and complementary health care products created an environment where miscommunication between care givers and patients have become inevitable. Health care practitioners must be able to distinguish between these products and the dangers of the use of these products (Winterstein & Storrs, 2001).

1.1.2 Supplement usage

A supplement or dietary supplement is defined as a product that is intended to complement a diet. It contains one or more of the following ingredients: vitamins, herbs, minerals, and/or amino acids. It can also be described as a concentrate, constituent, extract or combination of any of the above-mentioned ingredients (Onel, 2005; Radimer, 2000).

The main reason for the use of supplements is generally not well defined, but the most popular reasons include the improvement of physical health and mental well-being, while many patients who work long hours and are under considerable physical and mental stress also take supplements to help them cope with stressful situations (Parry *et al.*, 2017; Van der Horst *et al.,* 2011). Other common reasons for the use of supplements include the improvement of lifestyles, doctor's recommendation, enhancing performance and energy levels, and also to treat specific health conditions (Lieberman *et al.*, 2015).

The use of supplements alone or in conjunction with Western medication, may elicit unpredictable/undocumented interactions and as a result also side effects. Many patients self-medicate with these substances (while also using Western and other medication) without consulting healthcare practitioners regarding the possibility of dangers in the form of side effects and adverse reactions (American Cancer Society, 2015).

Astaxanthin, also known as 3,3´-dihydroxy-β,β-carotene-4,4´-dione, is a lipid soluble ketocarotenoid. Although this red carotenoid is used as pigmentation in food industries and aquaculture, it is also being used more regularly in the pharmaceutical and nutritional industries as a dietary supplement due to its antioxidant properties. Furthermore, it is also used as an anti-inflammatory agent, immunostimulant and in cosmetics for the protection of skin against the damaging effects of ultraviolet radiation (Higuera-Ciapara *et al.,* 2004; Juan *et al.,* 2008; Kobayashi *et al.,* 1997; Naguib, 2000; Odeberg *et al.,* 2003).

Astaxanthin is naturally found in plants and microalgae such as *Haematococcus pluvialis* (Chlorophyceae), *Chorella zofingiensis* and *Chlorococum* species. It is also present in salmon, trout, red sea bream, shrimp, lobster and fish eggs as well as in some bird species such as flamingos and quails. However, not all of these animals can synthesize astaxanthin from other carotenoids and needs to acquire astaxanthin from their diets (Higuera-Ciapara *et al.,* 2004; Juan *et al.,* 2008; Kobayashi *et al.,* 1997; Naguib, 2000; Odeberg *et al.,* 2003).

Unfortunately, the oral bioavailability of astaxanthin is low due to limited dissolution in gastrointestinal fluids. Absorption is further limited, at high doses, by a saturated volume of incorporation into bile micelles - formed during lipolysis and in turn facilitates the absorption of lipophilic compounds (Odeberg *et al.,* 2003). The recommended extravascular dosage of astaxanthin is 4 mg daily, although higher doses of up to 40 mg daily has been reported to be well tolerated (Odeberg *et al.,* 2003).

1.1.3 Beneficial pharmacokinetic interactions

Interactions between drugs and other components used in formulations may often mediate a reduction or enhancement in the therapeutic activity of co-administered compounds. These interactions are usually unintentional and undesirable, however, in some instances it may be feasible to intentionally elicit pharmacokinetic interactions with the intention to attain a beneficial therapeutic outcome (Edwards, 2012). Drug interactions occur when the toxicity or effectiveness of drugs or supplements are altered after co-administration of other substances (Dresser *at al.,* 2000). Beneficial pharmacokinetic interactions include the inhibition of drug metabolism, which in turn can increase drug bioavailability; lower the rate of drug clearance; reduce the variability in plasma concentrations and prolong drug half-life; inhibition of metabolic enzymes and P-glycoprotein (P-gp), leading to the inhibition of efflux and increasing permeability across the intestinal lumen; the opening of tight junctions, reducing the transepithelial electrical resistance (TEER) and thereby increasing drug permeability (Edwards, 2012; Hayeshi *et al.,* 2006; Sonaje *et al.,* 2012).

Improved systemic bioavailability after oral or transmucosal (including nasal, sublingual, buccal and rectal) administration provides needle-free therapy for patients, thus increasing patient acceptance and compliance and improved control of intended and unintended medication actions. The normal daily dose size and frequency of administration may also be reduced, which may promote patient compliance and reduce the overall treatment costs (Aungst, 2012; Edwards, 2012).

Bio-enhancers are agents capable of enhancing the bioavailability and bio-efficacy of specific drugs with which it is co-administered. Ideally, bio-enhancers should not have any pharmacological activity themselves in the doses used for enhancing the effect or bioavailability of other drugs (Atal *et al.,* 2010). *Aloe vera* gel, *Aloe vera* whole leaf extract and piperine are examples of bio-enhancers, which specifically act as drug permeation enhancers or drug absorption enhancers (Atal *et al*., 2010; Beneke *et al*., 2012, Gerber *et al*., 2019). The aim of using absorption enhancers is to enhance the membrane permeation and consequently also the bio-efficacy of drugs and/or any other active compounds such as supplements (Atal *et al.,* 2009).

1.1.3.1 *Aloe vera* gel and whole leaf materials

Aloe vera gel is a colourless substance that is contained in the inner part of the fresh leaves of the *Aloe vera* (L.) Burm. f. plant. *Aloe vera* gel is used in the food industry as a functional food and as an ingredient in other products such as beverages and health drinks. Cosmetic industries use the gel for topical treatment of wounds, psoriasis and even genital herpes and the production

of lotions, soaps, shampoos, facial cleansers and creams (Beneke *et al.,* 2012; Chen *et al.,* 2009; IARC Monographs). The gel has also been studied for the treatment of patients with diabetes and the healing of gastric ulcers (Chen *et al.,* 2009).

Whole leaf extract is obtained from the complete leaves, from which the lignified fibres were removed by means of filtration and which is usually treated to reduce the content of the latex anthraquinones. The whole leaf extract is used in powder form to produce capsules for herbal remedies and has been studied for potential treatment in cancer patients and acquired immunodeficiency syndrome (Boudreau *et al.,* 2013; Chen *et al.,* 2009). The promotion and advertisement for the use of *Aloe vera* whole leaf is mostly as herbal remedies for detoxification, constipation, better digestion and reducing the risk of getting sick (Boudreau *et al.,* 2013).

The most common mechanism of action of *Aloe vera* gel and whole leaf extract in terms of drug absorption enhancement has been reported to be related to its ability to reduce the TEER across biological membranes, thus opening of tight junctions between adjacent epithelial cells and thereby enhancing paracellular permeability (Beneke *et al.,* 2012; Chen *et al.,* 2009; Haasbroek *et al*., 2019).

1.1.3.2 Piperine

Piperine is an alkaloid attained from *Piper longum* (Piperaceae), commonly known as black pepper, and is used as a flavouring agent in food (Zhao-Hui *et al.,* 2018). In the pharmaceutical industry, it is used for its anti-inflammatory, anti-hypertensive, antioxidant, anti-microbial, anti-parasitic, anti-depressant and hepatic protective effects (Baspinar *et al.,* 2018; Di *et al.,* 2015). Piperine is one of the most potent natural bio-enhancers and its effects are mainly attributed to the formation of polar complexes with compounds or by the inhibition of hepatic and gut metabolism (including UDP-glucuronyltransferase activities, arylhydrocarbon hydroxylase and ethylmorphine-*N*-demethylation) (Di *et al.,* 2015). Piperine is a potent inhibitor of the primary metabolizing enzyme CYP3A4 and the P-gp efflux transporter, which results in an increase drug absorption across biological membranes (Atal *et al.,* 2010; Baspinar *et al.,* 2018; Zhao-Hui *et al.,* 2018; Gerber *et al*., 2019).

1.1.4 Use of capsules for extemporaneous preparations

A capsule is a solid dosage form that is either made from gelatine or other materials that are suitable for production and handling of medicinal or supplement products. The gelatine shells are filled with medicines or supplements and excipients to produce a unit dosage and is mainly used for oral consumption. Different types of capsules are available, namely gastro-resistant capsules, modified-release capsules, cachets, hard and soft capsules. Hard capsules consist of two pieces

of shells that fit together, with the shorter section fitting over the longer section and contains mainly solid formulations such as powders. Soft capsules only consist of one piece and contains mainly liquid formulations (BP, 2018; Jones, 2018).

Hard capsules are available in different sizes that are described according to a numbering system varying from nr 000 to nr 5, which describe capsules from the largest to the smallest sizes. Hard capsules can be filled with a variety of materials and excipients, which include dry solids such as powders, pellets, granules and tablets, or semi-solids such as thermos-softening mixtures, thixotropic mixtures and pastes, or non-aqueous liquids (Jones, 2018).

Capsules are usually used in extemporaneous preparations as requested on prescriptions for small scale preparation of products for older children or adolescents, due to smaller children and infants being unable to swallow solid formulations (Nahata & Allen 2008).

Capsules containing drugs that are registered at the medicinal authority should comply with minimum Pharmacopoeial requirements such as mass variation (to indicate uniform filling); disintegration, release of the active contents in a form that is available for absorption (i.e. dissolution), content assay and stability requirements (BP, 2018). The types of excipients mostly used in capsules include diluents, lubricants, solvents, glidants, wetting and disintegrating agents and stabilizers (BP, 2018; Jones, 2018).

1.1.5 Dissolution studies

Dissolution studies play a significant role in the process of pharmaceutical manufacturing of drugs and drug products and form part of mandatory quality control measures (Anand *et al.*, 2011). Successful membrane permeation of a drug is only possible if the drug is in its dissolved state at the target area of absorption. Dissolution will be the rate-limiting step to absorption if the solubility of the specific drug is limited in the gastrointestinal aqueous fluids and will dictate the rate and the extent to which the drug becomes available in the bloodstream. The link between oral bioavailability and dissolution suggests that dissolution studies are required to determine the pharmaceutical availability of oral drug products such as tablets, suspensions, granules, pellets and capsules. In most instances, there is a significant correlation between the rate and extent of drug dissolution and the resultant plasma drug concentration (Freire *et al.,* 2018). The parameters used to define oral drug absorption (i.e. solubility and membrane permeability) are used as the basis of a classification scheme referred to as the Biopharmaceutics Classification System (BCS). The BCS classifies drugs in one of four groups or classes according to their solubility in water and permeation across biological membranes. The four classes can be defined as Class I – High solubility, High permeability; Class II – Low solubility, High permeability; Class III – High solubility,

Low permeability; and Class IV – Low Solubility, Low permeability as schematically illustrated in Figure 1.1 (Dahan *et al.,* 2009; Amidon *et al.,* 1995).

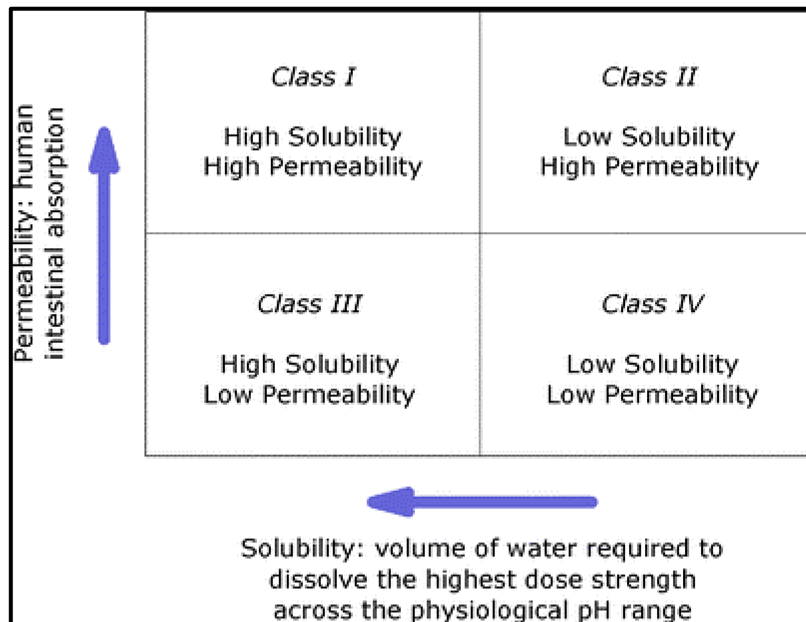


Figure 1-1: Schematic illustration of the Biopharmaceutics Classification System. Drugs are classified by the system according to their solubility and membrane permeability properties (Amidon *et al.,* 1995)

1.1.6 Models for the evaluation of drug transport

Models to evaluate the transport (or absorption) of drugs across the gastrointestinal epithelium can be divided into five broad groups. These groups are based on whether the studies are conducted in living organisms (i.e. *in vivo* and *in situ*), outside living organisms (i.e. *in vitro* and *ex vivo*) or with computer and other simulations (i.e. *in silico*) (Zhang *et al.*, 2012).

In situ studies are performed on the organ(s) of living organisms. These studies can be performed on human or animal subjects. There are different techniques that can be used to conduct these studies on isolated organs that are attached to a living organism such as intestinal perfusion or closed loop perfusion setups (Holmstock *et al.*, 2012; Maske *et al.*, 2018).

In silico studies are performed using computer software programs that have been developed to predict the pharmacodynamic and pharmacokinetic properties of drugs based on simulations. Software programs that are used for *in silico* investigations include MetaDrug™ and ACD/Percepta™ (da Cunha *et al.*, 2016).

In vitro studies are performed on cell cultures grown in flasks that are incubated in an environment that simulate conditions in the body, e.g. in an incubator. Organs or tissues that have been

removed (excised) from a specific animal, for example, intestinal tissues removed from slaughtered pigs can also be described as *in vitro*, but the term *ex vivo* can be used interchangeably in this instance. An example of one of the techniques used in these *in vitro/ex vivo* studies, is the removal of the intestinal tissue and mounting it on diffusion chambers, like the Sweetana-Grass diffusion chamber apparatus (Shikanga *et al.*, 2011). Another definition for *ex vivo* studies is the performance of experiments on living organisms, where the organ or tissue is removed and investigated after a drug has been administered to the live subject (e.g. for enzyme induction) (Zhang *et al.*, 2012). In terms of drug permeation, *in vitro/ex vivo* studies are performed on excised tissue that are mounted in a diffusion apparatus (Pund *et al.*, 2013; Reis *et al.*, 2013).

In vivo studies are performed on living organisms, which include animals and/or humans. Subjects that are involved in pharmacokinetic studies are usually injected with the compound of interest, after which samples, such as plasma, blood or other samples, are taken. Various routes of administration may be used for *in vivo* studies including oral, subcutaneous, transdermal, intraperitoneal, continuous infusions, intrathecal (Hidalgo, 2001; Linert *et al.*, 1999; Perzborn *et al.*, 2005; Zhang *et al.*, 2012).

1.1.7 Research Problem

Extemporaneous compounding is required when certain medicinal preparations are not commercially available. While the compounding of medication and supplements provides the patient with the required preparation, there are some concerns about the quality, effectiveness and safety of these preparations and the quality of these products (Cook *et al.,* 2007).

In terms of the quality of extemporaneously compounded preparations, the content of the active ingredient (assay), the rate and extent of release of the active ingredient (dissolution) and the delivery of the active ingredient into the systemic circulation after oral administration (bioavailability) are major concerns. There are currently no requirements in terms of these aspects for extemporaneously compounded preparations and therefore many compounding pharmacies prepare supplements for health improvement without testing the quality of the products. This study investigated this research problem in terms of extemporaneously prepared hard capsules containing astaxanthin together with selected drug absorption enhancing agents and evaluating these capsule formulations by means of delivery across excised intestinal tissues.

1.1.8 Aims and Objectives

1.1.8.1 Aims of this study

The primary aim of this study was to prepare and evaluate different extemporaneously compounded astaxanthin formulations in the form of hard gelatine capsules. A secondary aim was to improve the intestinal membrane permeation of astaxanthin across the intestinal epithelium by incorporating selected bio-enhancers into hard gelatine capsule formulations.

1.1.8.2 Specific objectives

In order to achieve the aims stated above, the specific objectives were to:

- Conduct a literature study concerning extemporaneous compounding and the frequency of supplement use by the general population;
- Validate a high-performance liquid chromatography (HPLC) methods for the analysis of astaxanthin content in all the experimental samples;
- Prepare extemporaneously compounded capsules containing the chosen supplement astaxanthin in combination with selected bio-enhancers, namely, *Aloe vera* gel, *Aloe vera* whole leaf extract and piperine;
- Conduct an assay to determine the astaxanthin content in each of the capsule formulations;
- Determine the release of astaxanthin from the different capsule formulations by means of dissolution studies;
- Conduct bi-directional transport studies for astaxanthin from the different capsule formulations across excised pig intestinal tissues mounted between the half cells of a Sweetana-Grass diffusion chamber apparatus;
- Measure changes in trans-epithelial electrical resistance (TEER) of the excised pig intestinal tissues exposed to the different formulations in order to determine modulation effects on the tight junctions;
- Process and interpret the transport data of the bi-directional transport studies and to calculate the apparent permeability coefficient (P_{app}) and efflux ratio (ER) values; and
- Perform a statistical analysis of the data, using a one-way repeated analysis of variance (ANOVA) and an appropriate post-hoc test to calculate p-values to determine if any statistically significant differences were evident.

1.1.9 Ethics regarding the research

An application to use excised pig intestinal tissue was approved by the North-West University's ethics Committee (AnimCare) (ethics certificate no: NWU-00025-15-A5). Pig intestinal tissue was obtained from the local abattoir in Potchefstroom where pigs are slaughtered on a regular basis for meat production purposes. By using tissue from pigs that are slaughtered for meat production and not for research purposes complies with the three R principle (Refine, Replace and Reduce).

Waste management was handled according to a Standard Operating Procedure regarding biological waste control at the North-West University, (Pharmacen_SOP001_v02_Biological waste management), which was approved by the Ethics committee (NWU-00369-16-A1).

1.1.10 Layout of dissertation

Chapter 1 provides a brief project background, specific aims and objectives of the study and motivates why the study was conducted. Chapter 2 entails an extensive literature review on the relevant aspects of the study and explains the study on more detail. Chapter 3 provides the experimental methods that were employed, and materials used during the study. The experimental results are presented and discussed in detail in chapter 4 and the final conclusions and recommendations for future studies are presented in chapter 5.

CHAPTER 2: EXTEMPORANEOUS COMPOUNDING OF SUPPLEMENTS AND THE IMPROVEMENT OF THE MEMBRANE PERMEATION OF SUBSTANCES

2.1 Introduction

Extemporaneous compounding can be defined as small-scale manufacturing, packaging and labelling of medicinal and supplement products in compliance with a prescription, order or initiative based on the relationship between the patient, practitioner, pharmacist or compounder (Giam *et al.,* 2011; Jaksic *et al.,* 2012). Access to speciality drug/supplement products is an important aspect to consider when aiming to provide good quality healthcare services, and despite the vast variety and continuously increasing amount of industrially manufactured pharmaceutical products in recent years, compounding of specific products are still very often required. Although certain medicines are commercially available, the dosage forms may not always be suitable for specific population groups such as children and elderly patients and in these cases, extemporaneous compounding of unavailable products and dosage forms are needed (Masupye *et al.,* 2015; Todorova, *et al.*, 2016).

Supplement products can be defined as products that contain botanical components such as herbs, and non-botanicals such as minerals, metalloids, amino acids, vitamins and microbial products, Asian herbal medication and traditional remedies (Palmer *et al.,* 2003). Supplement availability, popularity and use have increased immensely over the past years. Although the reason behind the use of these products can't be well defined, reasons may include improved immunity, greater health benefits, better performance, increased energy levels and to support nutrient deficient diets (O'Dea, 2003; Winterstein & Storrs, 2001).

The spike in supplement use has led to an increase in the compounding of various supplement products and it is important that these products should be subjected to specific safety and quality evaluations (O'Dea, 2003; Winterstein & Storrs, 2001). There are numerous advantages associated with the process of compounding either supplement or medicinal products. Some of these advantages include the opportunity to customize and prepare formulations for the specific needs of patients in terms of drug/supplement content and dosage form requirements (Todorova *et al.,* 2016). Multiple active ingredients and absorption enhancers can be combined to create products, which may potentially exhibit more therapeutic benefits to the patient as compared to standard formulations (Masupye *et al.,* 2015). Furthermore, products that are not commercially available can be manufactured for patients and these products can also in most cases be sold at lower prices (Masupye *et al.,* 2015; Todorova *et al.,* 2016). However, the disadvantages of these

extemporaneously compounded products should be considered to prevent potential harmful effects to the patient. For instance, the lack of applying acceptable manufacturing and formulation processes and principles as well as ensuring stability of these products can pose a risk to patients and cause serious side effects or even death (Masupye *et al.,* 2015). The combination of the specific excipients and active ingredients in formulations may be prone to chemical interactions, which may compromise stability and quality of the final product. Other risk factors to consider include content uniformity issues, chemical purity of ingredients, and elicitation of adverse reactions due to wrong doses, potential herb-drug interactions and the efficiency of the manufacturing process of these products (Doogue & Thynne, 2013; Winterstein & Storrs, 2001). Due to the fact that extemporaneously compounded supplement products are not subjected to mandatory safety and quality evaluations, there is very little information available regarding performance in terms of active ingredient delivery and efficacy (Palmer *et al.,* 2003; Ohnishi *et al.,* 2003).

2.1.1 Astaxanthin

Astaxanthin is a compound that forms part of the carotenoid family. Carotenoids are generally lipophilic compounds but may also exhibit increased affinities for water interfaces if they contain polar hydroxyl or keto functional groups. Carotenoids can be divided into two main groups, namely carotenes and xanthophylls. Carotenes include beta-carotene and lycopene, whereas xanthophylls include astaxanthin, lutein and canthaxanthin. Xanthophylls exclusively have hydroxyl groups attached to the ring structures at the ends of the linear, unbranched carbohydrate molecule. Astaxanthin contains two hydroxyl groups and this structural attribute may account for its greatantioxidant activity and health benefits in humans (Capelli *et al.,* 2013; Liang *et al.,* 2009). Figure 2.1 illustrates the planar structure of ataxanthin.

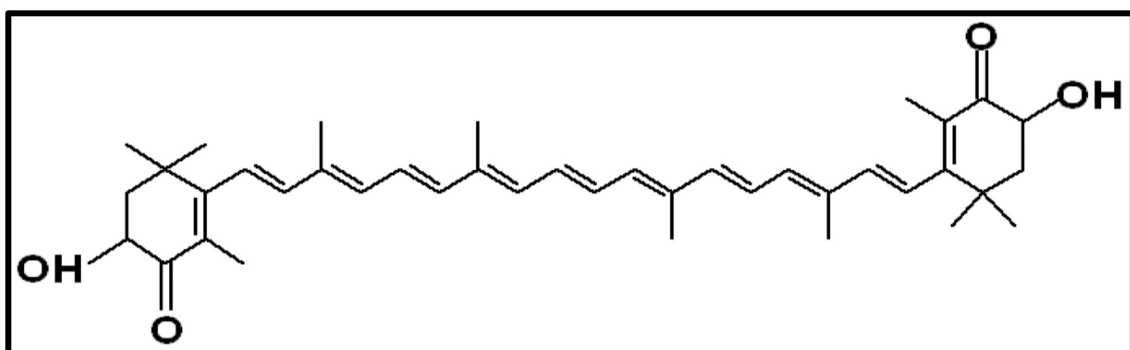


Figure 2-1: Planar structure of astaxanthin (Kidd, 2011)

Astaxanthin (3,3´-dihydroxy-β,β-carotene-4,4´-dione) is synthesised in plants and microalgae such as *Haematococcus pluvialis* (Chlorophyceae), *Chorella zofingiensis* and *Chlorococum*

species (Ambati, 2014; Juan *et al.,* 2008; Liu, 2016). It is abundant in nature, particularly in the marine environment in the flesh of salmonids, shrimp, lobsters and crayfish, giving them the distinctive pink-reddish colour of their flesh. Since these animals can't produce astaxanthin from other carotenoids, this supplement must be ingested via their diet (Juan *et al.,* 2008; Orosa, 2005).

This red-pigmented carotenoid is used in a vast number of industries, but the major market for astaxanthin is as a source of pigmentation in aquaculture, either from natural sources or synthetically produced. However, the use of this specific supplement is growing tremendously in the pharmaceutical, nutritional and even cosmetic industries for a variety of clinical benefits. Astaxanthin exhibits high antioxidant activity, thus having a powerful and beneficial effect on oxidative damage-related illnesses, such as age-related macular degeneration, cancer, hypertension and obesity. Astaxanthin can therefore be used to prevent these diseases and other diseases such as ischemic diseases, atherosclerosis and diseases associated with inflammation (Kidd, 2011; Liu, 2016; Naguib, 2000). Other uses for astaxanthin include its use in anti-aging and UV-protective agents to improve skin health, as well as acting as an immune system modulator and an agent to combat brain-aging (Capelli *et al.,* 2013; Nakagawa, 2011). According to a study by Otton (2010), astaxanthin can be used as a prophylaxis or for the recovery of lymphocyte dysfunctions in diabetic patients.

Astaxanthin was first sold in the late 1990s in its natural form as a dietary supplement. This supplement is marketed and consumed as a functional food and is commercially available in various dosage forms such as capsules, tablets, syrups, creams, oils, soft gels, granulated powders, biomass and even health promoting beverages (Ambati, 2014; Liu, 2016). The recommended daily supplement dosage for astaxanthin is 4 mg, even though higher doses of up to 40 mg has been reported to be well tolerated (Kidd, 2011; Odeberg *et al.,* 2003).

2.1.2 Extemporaneous compounding of supplements and other compounds

The extemporaneous compounding of products, containing either drugs or supplements, is a practise that has been around since the beginning of the pharmacy practise itself. In the 1920s, more than 80% of all prescriptions required extemporaneous compounding and the trend continued for many years until the advent of modern pharmaceutical manufacturing in the 1950s and 1960s where labour-intensive compounding became the norm. In the 1990s, the wellness movement grew immensely, and some pharmacists saw this as an opportunity to advertise and sell "all natural" products with claims of improved well-being and low risk to the user, as well as products that were more effective than commercially available products (Boodoo, 2010).

Compounding can be defined as the small-scale preparation or manufacturing of products for a patient with specific needs, which involves the preparation, mixing, homogenisation, assembling, packaging and labelling of a product according to a licensed practitioner's prescription. This type of preparation, or rather compounding, of products may involve the modification of a product that is either commercially available or derived from basic, natural and pure products (Giam *et al.*, 2011; Gudeman *et al.*, 2013; Jaksic *et al.*, 2012; Todorova *et al.,* 2016).

2.1.2.1 The need of extemporaneous compounding

Extemporaneously compounded products can be prepared by reconstructing or manipulating products that are commercially available, for example, using a tablet or capsule to make a liquid/solution for oral use, or by combining different ingredients in a specific way (Todorova *et al.,* 2016). There is a global lack of licensed medicines and appropriate dosage forms and strengths of certain drugs for specific groups of patients such as children and elderly patients. This lack of availability of products has sparked the fast-growing practise of extemporaneous compounding of products (Boodoo, 2010; Masupye *et al.,* 2015). According to Gudeman *et al.,* (2013), extemporaneously prepared products play a valuable role in patients with unique and specific medical needs by providing medication that cannot be obtained commercially.

Extemporaneously compounded products are mainly used in paediatrics, where the required strength and suitable dosage form is not available for these patients. Extemporaneous compounding is also used for elderly patients that have trouble swallowing; hospices that need medication in higher strengths or in alternative dosage forms; rare diseases that need specific treatment; allergy or sensitivity to preservatives or excipients; compliance problems and when alternative routes of administration are needed (Masupye *et al.,* 2015; Nahata & Allen 2008; Todorova *et al.,* 2016). The main advantage of extemporaneous compounding of products is the ability to serve the needs of individual patients by preparing customized products to cater for patient specific needs (Todorova *et al.,* 2016).

Other extemporaneously prepared products that are relevant in the health care sector includes alternative and complementary medicines such as homeopathic remedies, Chinese herbs, and Ayurvedic medicines (Berman, 1995; Doogue & Thynne, 2013).

2.1.2.2 The dangers of extemporaneous compounding

Proper formulation of products and different dosage forms is dependent on the physical, chemical, biological and therapeutic characteristics of the substances and ingredients used to formulate a product. All the ingredients used must be compatible with one another to ensure a product that

is safe, stable, efficient, palatable and easy to administer to a patient and must be well tolerated (Nahata & Allen 2008).

Extemporaneous compounding is a high-risk activity due to insufficient regulatory requirements in terms of safety and quality evaluations and pose a greater risk of causing drug interactions in comparison to licenced, commercially manufactured, medicines (Lowey *et al.,* 2008). Unlike manufactures, compounding facilities or pharmacies are not obligated to perform stability and other safety studies and tests on their products, or to report adverse events to institutions like the Food and Drug Administration (FDA). These products are not subjected to Good Manufacturing Practice (GMPs) regulations, and concerns have been raised regarding the stability, storage, expiry dates, quality and the compatibility of the ingredients in these compounded products (Cook *et al.,* 2007; Gudeman *et al.,* 2013). There are five general risks for patients associated with the use of extemporaneous compounded products, including sub potency, super potency, overmedication and contamination (Boodoo, 2010).

Extemporaneously prepared products are usually given to vulnerable patients in hospitals and in the community. These patients include neonates, children, elderly patients, and patients with feeding tubes, stroke victims and patients that are on other chronic medication (Lowey *et al.,* 2008). The lack of proper safety and quality evaluation and variations in product formulae may pose a great risk to these patients. Furthermore, the different excipients and other ingredients, such as the actives used, may differ greatly in these products and can affect the stability of compounded products and may potentially cause severe adverse reactions in patients (Masupye *et al.,* 2015).

Compounding is usually performed on small scale to satisfy the immediate need of a certain patient but can also be performed on a larger scale to provide a number of dosage units which can be stored and distributed later (Cook *et al.,* 2007; Jaksic *et al.,* 2012). When products are produced on a larger scale, more errors and adverse effects may occur but due to a lack of regulatory requirements some manufacturers choose to ignore these issues and are only interested in increasing their profits (Boodoo, 2010; Gudeman *et al.,* 2013).

2.1.2.3 Extemporaneously compounded products

Compounded products were traditionally simple dosage forms, however, nowadays these compounded products are more complex and are causing increasing concern (Feldschuh, 2008). Some products are still being compounded in small quantities based on the specific requirements of individual patients, however, it has become common practice amongst small scale manufacturers to produce larger quantities for distribution purposes. These products include

liquid dosage forms such as suspensions and syrups, tablets, capsules, parenteral and rectal preparations, including suppositories (Gudeman *et al*., 2013; Nahata & Allen 2008). Preparations can either be made from commercially available products or from pure and/or natural ingredients (Giam *et al*., 2011).

2.1.2.3.1 Oral liquids

Liquid dosage forms, such as suspensions and solutions, can be defined as aqueous formulations with favourable palatability characteristics. Flavourings, colourants and sweeteners are usually added to these formulations to enhance the appearance and taste of these products (Murdan, 2018). Liquid oral solutions are usually popular among paediatric and elderly patients, as well as patients who are unable to swallow solid dosage forms due to illness or disability (Allen, 2008; Haywood *et al.,* 2013). According to Brion *et al.* (2003) and Giam *et al.* (2011), suspensions and solutions, such as syrups, can be prepared from tablets or capsules that are already available or from natural and pure ingredients, without official standards, and can be obtained from different sources and suppliers. Not all these ingredients, or rather products, are sold for medicinal purposes or for the preparation of medication.

The preparation of these dosage forms entails the crushing of tablets and/or the opening of capsules that are commercially available and dissolving the powder and other excipients in a suitable solvent or suspending these powders with other excipients. Natural ingredients can also be used in conjunction with selected excipients to create suitable products (Brion *et al.,* 2003; Haywood *et al.,* 2013). Before any preparation can be compounded, a suitable vehicle for the specific preparation must be chosen, taking into consideration the physico-chemical characteristics of the active ingredient. These liquid preparations may contain excipients such as antioxidants, antimicrobial preservatives, colourants, and/or agents for dispersing, suspending, emulsifying, buffering, wetting, thickening, solubilising, sweetening and flavouring (BP, 2018).

Suspensions are prepared by wetting the drug (active) powder and adding purified water or oil and other excipients to the formulation while mixing continuously until a uniform mixture is obtained. After the powder is wetted, the dispersion medium, containing flavourings, colourants and preservatives, is added to the powder mixture in small quantities and mixed thoroughly. A small amount of the vehicle is then added to the mixture to form the suspension. A portion of the vehicle is then also used to wash the remaining ingredients from the mixing equipment and the suspension is then made up to volume (USP, 2018).

Solutions, which are commonly referred to as syrups, are oral solutions containing high concentrations of sugars, such as sucrose or diabetic-friendly syrups, hydrogenated glucose,

mannitol, xylitol or sorbitol (Murdan, 2018). Syrups are prepared by dissolving the ingredients in a suitable solvent or mixture of solvents. Sweetening, flavouring or aromatic agents are added to the mixture, as well as viscosity enhancers to prepare a product with favourable organoleptic properties (BP, 2018; USP, 2018).

There are several advantages associated with the use of solutions such as better drug absorption since the drug (active ingredient) is already in solution and is immediately available for absorption. Furthermore, solutions can provide uniform doses and specific volumes can be measured accurately, thus providing a larger range of doses that can be administered. Oral solutions can be swallowed easily, making administration less troublesome in patients such as children and elderly patients whom may find it difficult to swallow. However, there are also some disadvantages associated with the use of these products, including that many drugs or products are unstable in solution and some ingredients may be insoluble in water and can make the process of preparing a homogenous solution challenging. Compared to solid dosage forms, liquids are packed into bigger and bulkier containers and can be more inconvenient for patients to carry around (Murdan, 2018; USP, 2018).

2.1.2.3.2 Tablets

According to the British Pharmacopeia (2018), tablets can be defined as "solid preparations that contain a single dose of an active ingredient" and are obtained by compressing uniform amounts of particles or using other manufacturing techniques (Alderborn & Frenning, 2018). Unvarying volumes of active substances together with excipients are mixed and compressed together to produce tablets. Other techniques, such as extrusion, freeze-drying or moulding, can also be used to produce tablets (BP, 2018; USP, 2018).

The most common route of drug administration is the oral route, and tablets in various forms are most commonly used to target this administration route. The different forms of oral tablets on the market include coated, uncoated, chewable, soluble, effervescent, gastro-resistant, modified release, dispersible and oro-dispersible tablets, and oral lyophilisates (Alderborn & Frenning, 2018; BP, 2018; USP, 2018).

Various excipients are used to formulate tablets, such as diluents (fillers), binders, lubricants, glidants, anti-adherents, super-disintegrants, disintegrants, viscosity enhancing agents, and anti-caking agents. It is important to choose the correct excipients for the specific formulation being prepared (Jivraj *et al.*, 2000; Varma, 2016).

Tablets can be compounded by either granulation or direct compression methods. Direct compression is a time- and cost-effective method to use due to the simplicity of the manufacturing

process. The active substances and excipients are mixed together using a mixer (in a double cone mixer or in a high-shear mixer) and then compressing uniform quantities of the mixture into tablets using a tablet press. The main advantage of direct compression is that the process is cost-effective, and the cost of the product can be reduced. No heat or water is present in the manufacturing process; thus, product stability and drug dissolution can be ensured. There is, however, some disadvantages associated with the use of direct compression methods, some of which include the need for special binders and fillers that can be more expensive; a large number of quality tests are required before processing and the use of large particles may not always produce homogenous mixtures and may also lead to segregation and dose variation (Alderborn & Frenning, 2018; USP, 2018).

Granulation of powders may also be used as an alternative method to compound tablets. There are a variety of granulation methods, but the most effective method is wet granulation. Wet granulation involves the mixing of the dry ingredients with a granulating liquid in order to form a moist granular mass. This mass is dried and sized using either a hammer mill or by pressing the granulate mixture through a screen coupled with an oscillating granulator. The resultant granules are then dry mixed with the remainder of the excipients and compressed into tablets. This is a useful technique to achieve uniform blends of drugs with low doses and to enable the wetting and dissolution of drugs with poor solubility characteristics (Alderborn & Fenning, 2018; USP, 2018).

After manufacturing, the tablets may also be enteric/sugar coated if necessary, to protect the ingredients from air, moisture or light and to mask unpleasant tastes and odours. This may also help to improve the appearance of the tablet and to reduce dustiness (USP, 2018).

2.1.2.3.3 Capsules

The USP (2018) defines capsules as solid dosage forms that consist of a shell that is filled with a drug substance, with or without excipients. To produce a capsule as dosage form, these ingredients can be filled into hard or soft capsule shells. Capsule shells are mainly made of gelatine but can also be manufactured in combination with other substances such as glycerol or sorbitol if necessary, to adjust the consistency of the capsule. Other possible excipients include surface-active agents, antimicrobial preservatives, sweeteners, colourants, flavouring agents, and opaque fillers. Capsule shells may also have surface markings for identification purposes (BP, 2018).

Capsules are mostly aimed at oral administration and can be divided into several dosage form categories namely hard, soft, gastro-resistant, modified-release capsules and cachets. The contents of capsules may be solids, semi-solids, or liquids, depending on the type of shell being used (BP, 2018; USP, 2018).

Hard capsules are solid, single-dose preparations that can be filled with dry solids, such as powders, pellets, granules and/or mini-tablets. Semi-solids such as thermos-softening mixtures, thixotropic mixtures and pastes; or non-aqueous liquids may also be incorporated into hard capsules. These capsules usually consist of two cylindrical sections, a “body” and a “cap” part. Both the body and cap are open at one end and closed and rounded at the other end. The substances used in the capsule formulation are filled into one of the sections and the other section is slipped over this part. There are various sizes of capsules available, which range from 000 to 5 (BP, 2018; Jones, 2018; USP, 2018).

Formulation ingredients used for filling into capsules usually consist of a combination of active substance and excipients. The most common excipients used in powder-filled capsules include diluents, lubricants, glidants, wetting agents, disintegrants and stabilizers. Powder formulations should be evaluated to ensure that they exhibit adequate flow properties and its particles should neither have adhesion nor cohesion attributes. Capsules can either be filled manually or with the aid of capsule-filling machines. The most significant difference between these two filling methods is the way in which the capsule content is determined (BP, 2018; Jones, 2018).

Bench-scale filling is primarily used in hospital pharmacies or for special prescriptions where relatively small quantities of capsules (50 – 10 000) are required. The equipment used for this method is quite simple and consist of plastic or stainless-steel plates with holes of specific sizes drilled into them. These plates can accommodate 30 to 100 capsules at a time, which are placed into these holes and the bodies of the capsules are then secured in place mechanically followed by the removal of the caps. The capsule bodies are then filled with the drug/excipient mixture until full and the caps are then placed over the bodies using manual pressure to seal the capsule units (Jones, 2018).

Industrial-scale filling machines are available in various shapes and sizes and can be semi- or fully automated. This method is used when relatively larger quantities of capsules (e.g. 3 000 – 150 000 per hour) are manufactured. These machines can work in a continuous motion or it can be stopped to perform additional actions before commencing to the next filling process or a different set of capsules. Filling and dosage metering of capsules can be divided into dependant- and independent systems. Dependent dosing systems use the capsule body directly to measure

the powder required, while independent dosing systems entail that the powder is measured separately in a measuring device before filling the capsules (Jones, 2018).

All formulations used for filling capsules must meet some basic requirements, namely:

- The formulation characteristics must ensure uniform filling of the capsules;
- Compatibility of all the formulation ingredients must be confirmed to ensure chemical stability of the final product;
- Active contents must be released in a form that is available for absorption; and
- Pharmacopoeial and regulatory authority requirements must be met (Jones, 2018).

2.1.2.3.4 Parenteral preparations

Murdan (2018) refers to parenteral dosage forms as dosage forms for injectable routes of administration. These products are commonly injected into the veins or muscles, into and/or under the skin and can also be administered into joints, arteries, joint fluid areas, spinal fluid and the spinal cord, and even into the heart. Parenteral products must be sterile and free of any pyrogens.

Most patients prefer oral tablets or liquids, which can be swallowed, or creams, ointments or transdermal patches that can be applied to the skin over invasive type of dosage forms such as injections administered by means of needle prick. Pharmaceutical companies also prefer to manufacture tablets or liquids instead of parenteral products due to the fact that parenteral products are much more expensive to manufacture. Since parenteral products need to be sterile, highly specialized and controlled environments are mandatory to manufacture these products, making the production costs much higher compared to that of tablets or liquids. However, in some cases, medicines need to be administered parenterally due to the fact that some drug molecules are prone to extensive enzymatic degradation and/or pre-systemic metabolism in the gastrointestinal tract before absorption. Injections may either provide systemic effects or localized effects, if administered to a specific anatomical area or organ system. Furthermore, intravenous injections enable the delivery of drug molecules directly into the circulatory system, which is crucial in emergency situations (Lowe, 2018).

One of the most prescribed parenteral preparations is parenteral nutritional solutions. Parenteral nutrition solutions provide patients with essential nutritional requirements, such as proteins, carbohydrates, fat (lipids), and essential vitamins and minerals, including sodium, potassium, calcium, magnesium, acetate, chloride, phosphate, trace elements, water-soluble vitamins and fat-soluble vitamins (BP, 2018).

All materials and ingredients used for the preparation of parenteral products need to comply with specific pharmacopoeial standards, including the monographs for substances intended for pharmaceutical use and pharmaceutical preparations. All preparations should be manufactured under aseptic conditions; no processes may be performed in uncontrolled environments; and processes should be automated or at least semi-automated (BP, 2018). In some cases, parenteral products may require the addition of extra excipients for example to ensure isotonicity with regard to blood; to adjust the pH; increase solubility and stability of a product; to increase the shelf-life or act as a preservative. The excipients should not affect the action of the substances or cause any side effects or toxicity (BP, 2018; Lowe, 2018).

2.1.2.3.5 Rectal preparations

The oral route is the most commonly used route to administer drugs, however, in some cases alternative routes are needed to administer drugs. One of the alternative routes that can be used is the rectal route. This route of administration is used when patients are unable to swallow oral medication due to several reasons, including nausea and/or gastrointestinal tract problems. Rectal preparations are also used in either young or very old or even mentally impaired patients. Additional reasons for using the rectal route of administration may include the delivery of specific drugs which are not suitable for oral administration, or in cases where localized treatment of the rectum is required (BP, 2018; Dodou, 2018).

Various rectal products are available on the market, including suppositories, emulsions, rectal capsules, rectal solutions and suspensions, rectal foams, semi-solid rectal preparations and rectal tampons. The most common of these dosage forms, is the suppository (Dodou, 2018). Suppositories are single-dose preparations made in the appropriate size, volume and consistency for rectal administration. These preparations are made in different sizes, varying from 1 - 4 g, but weighs mostly about 2 g for adult use and are point shaped at one or both ends. They contain one or more substances and are usually made from cocoa butter or various other vegetable oils, such as palm kernel oil or coconut oil that has been modified by esterification, hydrogenation or fractionation, to obtain products with different melting points and compositions. Finely divided drug substances are incorporated into the oil by either melting the oil or by incorporating it into the solid oil mass and shaping in into the appropriate forms (Dodou, 2018; USP, 2018).

In some formulations, additives, such as hardening agents, viscosity-increasing additives, surface-active agents and lecithin, are added. The additives manipulate the melting point of the suppositories, the release rate of the active substance and enhance the flow properties of the dispersion (Dodou, 2018).

The advantages of the rectal route include safe and painless administration and removal of the dosage form; prevention of degradation of drugs in the gastrointestinal tract; prevention of first-pass hepatic elimination of high clearance drugs; administration of larger and smaller doses; control of drug action by using a suitable formulation; administration of drugs to terminally ill and elderly patients (over a longer period) and children. It is a simple way to administer drugs and it can be self-administered by the patient or by unskilled healthcare workers. It is especially a useful administration route for patients that are nauseous or are vomiting. However, there are also some limitations to this route, such as the low acceptability and compliance of patients; the upward movement of the dosage form which can increase first-pass metabolism; leakage of the dosage form; problematic insertion of the suppository; and low drug absorption from suppositories, compared to other administration routes, such as intramuscular or sub-lingual administration (Dodou, 2018).

2.2 The gastrointestinal tract and drug absorption

2.2.1 The structure and function of the gastrointestinal tract

The gastrointestinal tract (GIT) forms part of the digestive system of humans and mammals and is the main site of absorption of orally administered drugs, while also acting as a barrier for the absorption of unwanted substances (and drugs in some instances) (Zhang & Benet, 2001). The most important functions of the GIT include the absorption of nutrients such as sugars and amino acids, digestion of food and preventing the entry of toxins, pathogens, harmful compounds and bacteria. It also aids in the removal of unabsorbed and undigested compounds (Daugherty *et al.,* 1999; Versantvoort *et al.,* 2000).

The GIT is made up of hollow organs that are conjoint from the mouth to the anus. These organs include the mouth, oesophagus, stomach, small intestine, large intestine and the anus, separated specifically into areas for digestion and absorption. The GIT wall is mainly made up of four different layers, namely the mucosa, muscles, submucosa and serosa. The different regions of the GIT are also supplied with blood by arteries and drained by veins, all supported by the mesenteric plexus (DeSesso & Jacobson, 2012; Liao *et al.,* 2009; Sjögren *et al.,* 2014).

The GIT can further be sub-divided into a primary (also preparative) storage area (the mouth and stomach), secretory and absorptive area (the mid gastrointestinal area), a water retrieval area (ascending colon) and an area where waste products are stored (sigmoid and descending colon) (Sjögren *et al.,* 2014).

2.2.2 Absorption mechanisms of the gastrointestinal tract

The movement of molecules, electrolytes, vitamins and water from the intestinal fluid across the digestive epithelia into the blood, is known as absorption (Martini *et al.,* 2014). The two main pathways of transport across the GIT is transcellular and paracellular transport. The transcellular path mechanisms are divided into passive diffusion, carrier-mediated transport and endocytosis (Ashford, 2018).

The transport of materials via the paracellular pathway involves the conveyance of materials through the aqueous intercellular spaces between cells, and not across them. The cells are joined together by tight junctions, fitting closely together on the apical side of the membrane. The "tightness" between these tight junctions varies among the different epithelia found in the body. The surface area of the intestinal epithelia is occupied by about 0.01% of intercellular spaces and the paracellular pathway of absorption becomes less prominent further down the length of the intestinal tract, as the size and number of the pores between the cells are reduced. As a result, the absorptive epithelia of the small intestine are leakier than other epithelia (Ashford, 2018; Liu *et al.,* 2009). This pathway is also useful for the transport of water-soluble, high-molecular weight compounds and ionized drugs. The paracellular pathway comprises a combination of two processes namely a convective (solvent drag) component (rate at which the compound is carried across the epithelia via water flux) and a diffusive component (Hayashi *et al.,* 1999; Liu *et al.,* 2009).

Smaller lipophilic drugs with relatively low molecular weight are absorbed via passive diffusion as the preferred transport route. Passive transcellular diffusion may be described as the process where drug molecules pass through membranes from a high concentration in the lumen to a lower concentration in the blood. The low concentration in the blood is maintained by blood flow (sink conditions), which evacuates absorbed drug molecules from the site of intestinal absorption (Liu *et al.,* 2009; Shargel *et al.,* 2012). No external energy is required during this process, thus making it a passive process. Three main factors govern the rate of transport, including the physico-chemical properties of the drug, the concentration gradient across the intestinal membrane, and the physiological nature/character of the membrane (Ashford, 2018; Shargel *et al.,* 2012).

Passive diffusion is the main absorption process for most drug molecules. After dissolution of the drugs in the aqueous fluids within the GIT lumen, the drug molecules partition into the lipoidal-like epithelial membrane. The drug molecules then diffuse across the epithelial cells through the gastrointestinal barrier into the blood of the capillary network (Ashford, 2018). Figure 2.2 presents a diagrammatic illustration of the processes involved in passive diffusion.

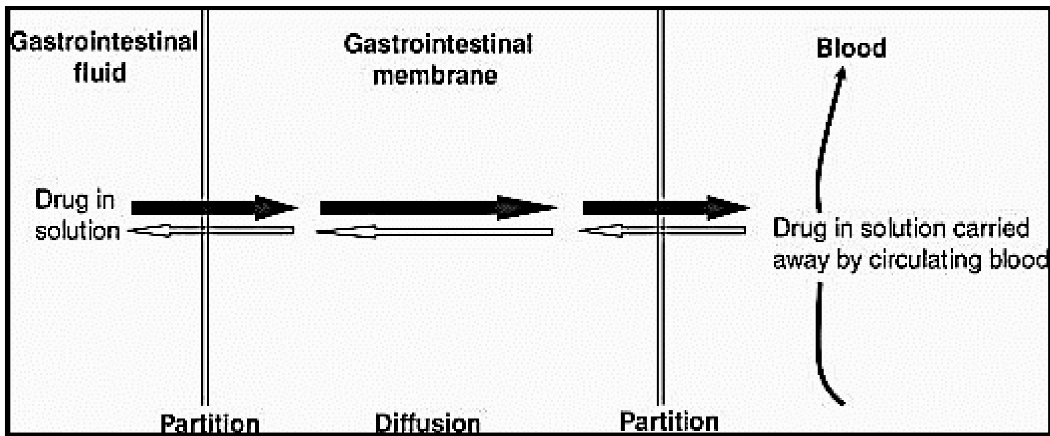


Figure 2-2: Diagrammatic illustration of the absorption process via passive diffusion (Liu *et al.,* 2009)

2.2.2.1 Carrier-mediated transport

Although most drugs are absorbed via passive diffusion, a selection of nutrients and other compounds may be absorbed via transcellular mechanisms known as carrier-mediated transport. Carrier mediated transport entail interactions between drug molecules and specific carriers or protein transporters. There are two types of carrier-mediated transport pathways present in the body, namely active transport and facilitated diffusion (Ashford, 2018; Liu *et al.,* 2009). Figure 2.3 is an illustration of the process of carrier-mediated transport.

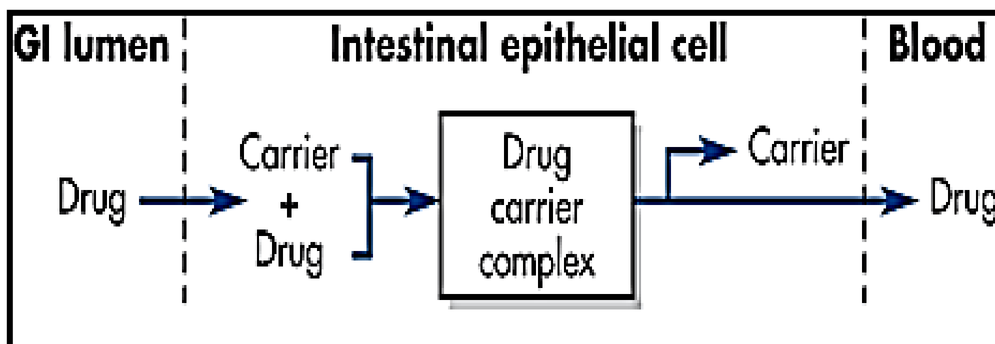


Figure 2-3: Graphic illustration of carrier-mediated transport (Shargel *et al.,* 2012)

2.2.2.1.1 Active transport

Unlike passive diffusion, active transport of molecules involves the participation of proteins, which are found in the apical cell membranes of columnar epithelial cells that take part in the absorption process. Drugs bind to a transporter protein and are transported across the membrane (Ashford, 2018).

The drug or compound binds to the carrier/transporter protein and forms a complex on the surface of the apical cell membrane. The drug-protein complex is then transported across the membrane followed by decomposition of the complex and subsequent release of the drug or compound on the other side of the membrane, while the carrier protein returns to its original position and is reactivated (Ashford, 2018). Carrier molecules are very selective for drug molecules and structural resemblances of drugs to natural substrates that are actively transported are more likely to be transported by the same carrier mechanism, thus competing with compounds for absorption on the site of the carrier. Since there is only a limited amount of carrier molecules available, carrier mediated transport may become saturated and in turn result in zero-order absorption kinetics (Shargel *et al.,* 2012).

During active transport, compounds are transported against the concentration gradient from a region with a low concentration to a region with a higher concentration, thus making it an energy consuming process (Liu *et al.,* 2009). The energy required for this process is either obtained from the hydrolysis of ATP or direct interaction with the carrier protein, or from the transmembrane sodium gradient and/or transmembrane electrical potential (Ashford, 2018). The process is also temperature dependent due to necessity of cellular metabolic energy which is required to mediate the process (Liu *et al.,* 2009).

2.2.2.1.2 Facilitated transport

In some cases where compounds are insoluble, or the molecules are too large to traverse biological membranes via membrane channels, the molecules may bind to a suitable receptor site on carrier proteins, after which the shape of the protein undergoes conformational changes to produce a drug-protein complex. The drug-protein complex is then transported across the membrane followed by decomposition of the complex and subsequent release of the drug or compound on the other side of the membrane, while the carrier protein returns to its original position and is re-activated (Ashford, 2018 ; Martini *et al.,* 2014).

Although facilitated diffusion is also classified as a carrier-mediated transport system, the drug or compound cannot move against the concentration gradient, but instead moves from a high concentration to a region with a lower concentration. This process is not energy dependent but is saturable and is selective to the structure of the drugs or compounds that it binds to. This may lead to competition between drugs and compounds with a similar structure (Ashford, 2018; Shargel *et al.,* 2012). Drugs or compounds transported by facilitated diffusion are transported down the concentration gradient at a much faster rate than would be expected for passive diffusion of molecules with a certain size and polarity (Liu *et al.,* 2009).

2.2.2.2 Endocytosis

Ashford (2018) defines endocytosis as the process where material or compounds are invaginated by the plasma membrane of the cell. The invaginations become strained off to form smaller intracellular vesicles that are bound to the membrane, which contain the invaginated materials. The invaginated material or compounds are then transported into the cells and transferred to other vesicles or lysosomes followed by subsequent degradation. Some material may escape the degradation process and migrate (transcytosis) to the basolateral surface of the cells and be released via exocytosis. Endocytosis requires energy (ATP) and endocytic vesicles are known as endosomes and the contents are isolated from the cytoplasm (Ashford, 2018; Martini *et al.*, 2014; Shargel *et al.*, 2012). Endocytosis can be divided into four subgroups, namely pinocytosis, receptor-mediated endocytosis, phagocytosis and transcytosis (Ashford, 2018).

2.2.2.2.1 Pinocytosis

The formation of endosomes filled with small droplets of extracellular fluid by pockets or in-cuppings are known as pinocytosis. Deep pockets, called caveolae, found on the surface of the plasma membrane, close up around the fluid and pinch off to form pinosomes. These "bubbles" are free in the cytoplasm of the cells (Martini *et al.,* 2014; Shargel *et al.,* 2012). This is a low efficiency process, which is not selective, and no receptor proteins are involved (Martini *et al.,* 2014).

2.2.2.2.2 Receptor-mediated endocytosis

Various cells in the body possess receptors on their surfaces. These receptors bind to ligands to form ligand-receptor complexes that form clusters, which are invaginated. Coated vesicles are formed from these clusters that "break" free from the membrane. Once the vesicles reach the cytoplasm of the cell, the vesicles become uncoated and their contents are released to endosomes. In the endosomes, the ligand-receptor complex is separated, and the individual ligands and solutes are delivered to pre-lysosomes and then to the lysosomes. The lysosomes are the final stage of the endocytic pathway, which are surrounded by a single membrane and contain digestive enzymes that are responsible for the degradation of bacteria and larger molecules (Ashford, 2018).

2.2.2.2.3 Phagocytosis

Phagocytosis is the engulfment of larger particles (500 nm) by cytoplasmic extensions, called pseudopodia. The pseudopodia "trap" the macromolecules or material, after which the membranes combine to form phagosomes. The vesicle then combines with other lysosomes and

the macromolecules or materials are digested by lysosomal enzymes. Most cells display active pinocytosis, whereas phagocytosis is only active in specialized cells, like macrophages. Macrophages protect tissue from cell debris, abnormal materials and engulfing bacteria (Ashford, 2018; Martini *et al.,* 2014).

2.2.2.2.4 Transcytosis

The transport of numerous macromolecules is facilitated by the process known as transcytosis. The macromolecules are transported through the cell and emitted on the other side. The vesicle that is formed, combines with the membrane to carry the material to the other side of the cell. This is the process of preference where the transport of larger proteins and certain vaccines are concerned (Shargel *et al.*, 2012).

2.2.3 Comparison between pig and human gastrointestinal tract anatomy

The use of animal models has a long history with disease and medicinal research. The selection of specific animal models depends on various factors, such as cost, availability, handling and functional or anatomical similarities to humans. Smaller mammals including rabbits, guinea pigs, rats and mice, are often used in research studies, because of the easiness of handling and reasonable costs. Despite these advantages, smaller animals differ from humans in terms of anatomical and physiological aspects (Rees & Alcolado, 2005; Sullivan *et al.,* 2001; Zhang *et al.,* 2012).

The use of larger animal species can be justified in certain cases where the harm versus benefit analysis indicate that the use of larger animal models is justified. Dogs, pigs and monkeys can be used based on various similarities in terms of physiology and anatomy of these animals and humans. The pig is becoming a more popular model in both medicinal and diseases related studies due to the many similarities in anatomy and physiology (Ziegler *et al.,* 2016). Both humans and pigs are omnivorous, thus their organs share common functions and features. The pig model offers a variety of advantages, including availability, similarity to human size, the opportunity to perform surgical procedures, longer life span than smaller animal species, various breeds, all-season breeding and large litter sizes. Furthermore, the protein and genome sequence homologies with humans are high. The resemblance with human immune parameters is higher than with smaller breeds (humans >80% vs mice <10%). It is also a cheaper and more ethically acceptable research model than primates (Meurens *et al.,* 2012).

A comprehensive comparison of the anatomy and physiology of the gastro intestinal tracts of humans and pigs is given in Table 2.1.

Table 2-1: Comparison of the anatomy and physiology between human and pig gastrointestinal tracts

Parameters	Region	Pig	Human
pH	**Stomach**	● Fasted state: 1.2-4.0 (Hossain *et al.,* 1990). ● Fed state: up to 4.4 (Merchant *et al.,* 2011).	● Fasted state: 1-3 (Koziolek *et al.,* 2015). ● Fed state: ≥6, depending on the volume and composition of the contents ingested (Koziolek *et al.,* 2015).
	Small intestine	● Fasted state: 7-8 (Sjögren *et al.,* 2014). ● Fed state: 4.7-7.2, depending on the region (Sjögren *et al.,* 2014).	● Fasted state: 6–7.5/8, depending on the different regions (Guerra *et al.,* 2012; Koziolek *et al.,* 2015). ● Fed state: 5-6.5, depending on the region (Persson *et al.,* 2005).
	Large intestine	● Fasted state: 6.1-6.6 (Merchant *et al.,* 2011). ● Fed state: N.a.	● Fasted state: 5.6-6.5 (cecum), 5.5-7.5 (ascending colon), 7.0-8.0 (descending colon) (Sjögren *et al.,* 2014). ● Fed state: N.a
Length (cm)	**Small intestine**	1500-2000 (DeSesso *et al.,* 2012).	680 (DeSesso *et al.,* 2012)
	Large intestine	350-380 (DeSesso *et al.,* 2012).	150 (DeSesso *et al.,* 2012).
Weight (g)	**Small intestine**	2310 (Patterson *et al.,* 2008)	1040 (Patterson *et al.,* 2008)
	Large intestine	1970 (Patterson *et al.,* 2008)	590 (Patterson *et al.,* 2008)

N.a – Not available

2.2.4 Barriers that may impede drug permeation in the gastrointestinal tract

Although there has been a major increase in the approval of pharmaceutical products that are intended for intravenous and subcutaneous administration, oral administration is still the most acceptable and used route of drug administration (Berben *et al.,* 2018; Ensign *et al.,* 2012). Unfortunately, oral administration of drugs can result in low bioavailability, erratic absorption, lack of dose proportionality and variations in inter- and intra-subject pharmacokinetics (Desai *et al.,* 2012).

There are several mechanisms in the GIT to protect humans against the invasion of harmful agents like toxins, antigens and pathogens. These mechanisms (physical and biochemical barriers) that protect humans, however, can also counteract the absorption of drugs that are administered orally (Hamman *et al.,* 2005).

2.2.4.1 Physical barriers limiting oral drug absorption

2.2.4.1.1 Tight junctions

Paracellular transport of drug molecules can be defined as the transport through intercellular spaces, between adjacent epithelial cells. However, near the apical end of the intercellular space, tight junctions form an impermeable, thick, belt-like structure that limits diffusion of drug molecules between epithelial cells (Hamman *et al.,* 2005; Schneeberger *et al.,* 2004). Tight junctions are occluding junctions and consist of a group of cytosolic and transmembrane proteins that interact with the cytoskeleton and play a role in the transduction of signals in more than one direction across cell membranes (Hamman *et al.,* 2005; Salama *et al.,* 2006).

Tight junctions have pores or fenestrae with dimensions estimated to range between 3 and 10Å. Tight junctions are selectively permeable to various hydrophilic molecules, such as certain drugs, ions and nutrients. It functions as both a gatekeeper and a barrier. The function as a gatekeeper controls the diffusion of solutes through the paracellular pathway and the barrier function, sustains polar distributions of plasma membrane proteins in both the basolateral and apical regions (Hamman *et al.,* 2005). Tight junctions can be modified by certain substances such as absorption enhancers (e.g. chitosan, *Aloe vera*.) which may lead to a reduction in the trans-epithelial electrical resistance and as a result enhance paracellular permeability of co-administered molecules (Salama *et al.,* 2006).

2.2.4.1.2 Gastrointestinal mucus layer and unstirred water layer

The mucus layer in the GIT is a complex network consisting of glycoproteins, serum and cellular macromolecules, lipids, cells, electrolytes and other cellular debris. It is the first barrier that nutrients and oral drugs encounter before entering the circulatory system. The transport of unknown pathogens, toxins and particulates are prevented from crossing the mucus layer. The high viscoelasticity and adhesiveness, coupled with other possible particle-environment interactions, can reduce or even prevent the transport of drug molecules (Crater & Carrier, 2010).

The unstirred water layer can be defined as a stagnant layer consisting of water, mucus and a glycocalyx layer which line the intestinal wall. The formation of the unstirred water layer is caused by an inability of the peristaltic movements of the GIT to achieve homogenous mixing of the luminal contents. The barrier function of the unstirred water layer is of less significance for actively and passively absorbed solutes, however, it may restrict the movement of larger molecules across the epithelial layer (Fagerholm & Lennernäs, 1995; Hamman *et al.,* 2005).

2.2.4.1.3 Efflux systems

Studies have shown that the barrier function of the intestinal mucosa cannot be effectively defined by the metabolic and physical barrier combination alone. Efflux transporter systems are present in the gastrointestinal epithelial and liver cells and can also be found in a variety of other human tissues including the adrenal glands, kidneys and brain. Efflux systems are also present in cancer cells and present a barrier for the absorption of chemotherapeutic drugs into tumours. P-glycoprotein (P-gp) is a membrane protein, which acts as an ATP-dependent efflux pump that causes a reduction in the intracellular/ transcellular flux or transport of a variety of drugs, thus causing a major barrier in the GIT which limits the absorption of substrate molecules. Furthermore, P-gp is considered to be the main causative element in the emergence of multi-resistance to drug treatment in several cell types (Hamman *et al.,* 2005; Pauletti *et al.,* 1997; Watkins *et al.,* 1997).

2.2.4.2 Biochemical barriers limiting oral drug absorption

The pH values of the different regions of the GIT differ from each other, which can cause the pH-dependent hydrolysis of drug compounds. Furthermore, the pH can influence the absorption of drug compounds in various ways since most drug-compounds are either weak acids or weak basic compounds and ionization is dependent on the pH of the solvent. It is well known that weak acids generally dissolve better in an area where the pH is relatively high, while for basic drugs the dissolution rate is generally higher in areas where the pH is lower than their pKa values (Song *et al.,* 2004). Bacteria located in the colon secrete enzymes, which may also mediate various

biochemical interactions, including glucuronidation, double bond reduction, decarboxylation, hydrolysis of esters and amides, and dihydroxylation and these reactions may also alter the pH of the intestinal fluids, which in turn may alter drug dissolution (Ashford, 2018; Hamman *et al.,* 2005).

The enzymes in the GIT are responsible for the degradation of dietary proteins to form very small sub-units that can be absorbed. Di- and tripeptides and amino acids are examples of these sub-units. Digestive processes can be catalysed by enzymes through hydrolytic cleavage of peptide bonds (by proteases), phosphorylation, (by kinases) and chemical modification (e.g. oxidation) (Hamman *et al.,* 2005). Peptides are often degraded in the lumen by proteases released from the pancreas into the lumen of the gastrointestinal tract. In addition, degradation of drugs can further be mediated by proteases associated with enterocytes in the epithelial cells, lysosomes and brush border membrane (Pauletti *et al.,* 1997). It is important that drugs intended for oral administration should be chemically stable and be able to withstand the abovementioned degradation reactions to ensure that a large fraction of the administered dose remain intact and reach the circulatory blood to elicit the desired therapeutic outcomes (Hamman *et al.,* 2005).

2.2.5 Models used to predict drug transport

2.2.5.1 *In vitro* and *ex vivo* techniques

The intestinal absorption characteristics of potential drug candidates may be assessed with the aid of *in vitro* methods. Animal tissues, including tissue from pigs, rabbits, dogs, monkeys, mice and rats can be used to perform studies related to biological membrane permeation characteristics of drug candidates. These intestinal tissue models primarily entail the use of epithelial cells obtained from animals to mimic the epithelial cells encountered in humans (Nunes *et al.*, 2016).

These excised animal tissue models can be used to explore the mechanism of absorption of drugs and nutrients from the GIT, however, the biggest concern with the use of excised animal tissue models is to ensure that the tissue remain viable for the duration of the study period. The tissue must be kept moist with appropriate buffer or biological solutions and a constant oxygen supply must be provided to keep the tissue viable (Balimane *et al.,* 2000; Nunes *et al.,* 2016).

There are different methods that can be used to employ animal tissues for *in vitro* transport studies including the everted gut sac technique or intestinal segments mounted in an Ussing chamber apparatus (Balimane *et al.,* 2000).

2.2.5.1.1 Everted gut sac technique

This technique has been used since the 1950's and was initially used for transport studies of sugars and amino acids. The studies were performed from the mucosal to serosal side, which was used to determine the absorptive properties of the tissue (Balimane *et al.,*2000; Grass, 1997). The desired intestinal region is removed and incubated in a medium that contains vitamins, minerals or drugs being studied and a medium that is favoured by the specific tissue. The intestinal tissue sacs are removed at different time points and analysed for the different elements in the incubation medium inside the sac. Oxygenation of the buffers during incubation and gentle shaking increases the viability of the tissue for up to two hours (Balimane *et al.,* 2000). The everted gut sac technique is useful in various areas of study including oral drug delivery and drug metabolism and absorption, it is also used to determine the paracellular transport of hydrophilic molecules and to determine the effect that enhancers may have on absorption (Barth *et al.,* 1999; Le Ferrec *et al.,* 2001).

The preparation of the tissue used in these studies is very important. Rats, for example, are starved for a certain period and euthanized just before the tissue is needed. The entire intestine is removed and flushed with a saline mixture at room temperature. After the excised tissue is placed in the oxygenated culture medium at a temperature of 37°C, it is everted over a glass rod and filled with the culture medium. The excised tissue is then cut and the ends closed off to form sacs of approximately 2 - 4 cm in length that are submerged in solutions of the test compound at different concentrations for predetermined time periods (Barth *et al.,* 1999).

The advantages of this model include that both passive and active transport of drugs can be studied, and drugs can potentially accumulate faster because of the relatively small volume on the serosal side of the everted sac. It is an uncomplicated, inexpensive, quick and reproducible technique. However, there are some disadvantages associated with the use of this model, which include the lack of blood flow and nerve supply, leading to a loss in viability that may cause morphological damage and can alter the experimental results (Balimane *et al.,* 2000, Barth *et al.,* 1999).

2.2.5.1.2 Ussing chamber

Transport studies using "sheets" of intestinal tissue excised from animals to determine or study drug absorption is a popular *ex vivo* research method. The device or apparatus used in these studies is known as the Ussing chamber (Balimane *et al.,* 2000).

Ussing chambers were invented in 1951 by Hans Ussing, a Danish scholar, to determine vectorial ion transport across the skin. This method has been used and applied to nearly every tissue that

can be found in the animal body, including intestinal-, airway-, exocrine- and endocrine-ducts, eye tissue and in studies using cultured epithelial cells (He *et al.,* 2013). The main application of this model is in the pharmaceutical field for studies pertaining to research related to permeability evaluation of drugs across biological membranes such as intestinal tissues. Furthermore, microelectrodes can be used to determine changes in intestinal cell membrane ion channels. The permeability of drugs can be measured with this method, based on the rate and extent of appearance of the drug on the mucosal side of the membrane and it is an ideal method to investigate region specific absorption of drugs. This model is also unique due to the fact that the TEER can be measured constantly for the duration of the entire study (Balimane *et al.,* 2000; He *et al.,* 2013).

Ussing chambers require the use of intestinal tissue, which is cut longitudinally to produce long mucosal sheets suitable for mounting in the Ussing chambers. These sheets are then cut into smaller sheets that fit over the opening of the adjoining chambers. The intestinal mucosa is clamped between the two chambers and the drug or compound under evaluation is then added to the donor compartment and the accumulation of the drug can then be determined in the receiver compartment at predetermined time intervals. The drug or compound can either be added to the mucosal or serosal side of the tissue, thus making it possible to determine the flux of the compound or drug from the mucosal-to-serosal (absorptive transport) or from the serosal-to-mucosal side (secretory transport). An equal rate of transport from the mucosal-to-serosal side and from the serosal-to-mucosal side may be attributed to passive diffusion, whereas a difference in the transport may likely be attributed to carrier-mediated transport. By measuring the TEER continuously during the study, the integrity and viability of the tissue can also be monitored (Barth *et al.,* 1999; Hidalgo *et al.,* 2001).

Human intestinal tissue can also be used in Ussing chambers, thus making it possible to compare permeability across tissues obtained from different species. The amount of drug or compound required for use in Ussing chambers is relatively small and the samples are analytically clean, thus simplifying quantitative analysis. However, some of the disadvantages associated with this method include the loss of tissue-viability during the experiments, lack of nerve and blood supply, and morphology and functionality changes of the transporter proteins during the cut of the tissue and the mounting in the chambers (Balimane *et al.,* 2000).

2.2.5.2 *In vivo* methods

An *in vivo* method can be defined as a multi-factorial technique to investigate pharmacokinetics, which provides a combination of the effects of permeability, distribution, metabolism and excretion

of the drug or compound tested. This method is usually used to determine pharmacokinetic parameters, as well as to detect drug related toxicities (Zhang *et al.,* 2012).

In vivo studies are performed in whole living organisms, which include animals and/or humans. *In vivo* studies entail administration of a drug to test subjects using various dosage forms and routes of administration including oral, subcutaneous, transdermal, intraperitoneal and continuous infusions followed by blood sampling at pre-determined time points. The experimental samples are then analysed with the aid of validated analytical methods to determine the content of specific marker molecules in the samples to determine the rate and extent of drug/compound absorption in the test subjects (Hidalgo, 2001; Linert *et al.*, 1999; Perzborn *et al.*, 2005; Zhang *et al.*, 2012).

In vivo methods may also be used to investigate region specific absorption characteristics of drugs or other substances by making use of high frequency capsules or other similar systems. However, it is not possible to identify the specific mechanisms of absorption when using *in vivo* techniques. The perfusion technique, high frequency capsules, blood kinetics and local instillation are some examples of methods or techniques used in *in vivo* studies (Rouge *et al.,* 1996; Versantvoort *et al.,* 2000).

2.2.5.3 *In situ* methods

In situ perfusion methods and *in vivo* methods share certain similarities in terms of blood supply, clearance abilities and innervation characteristics. *In situ* methods are studies performed on different organs of animals or humans without the need to remove the specific organ used. These studies need little organ preparation, thus minimizing the risk of organ damage (Zhang *et al.,* 2012). These studies are both performed on living animals' or humans' organ(s). This method is often used in studies entailing absorption and/or permeability evaluations of compounds or drugs. Different types of *in situ* perfusion methods have been developed over the years, including single-pass perfusion, recirculating perfusion, closed-loop perfusion and oscillating perfusion techniques. These studies have been used extensively in animal models and have also found applications using human test subjects (Balimane *et al.,* 2000; Holsmstock *et al.,* 2012; Maske *et al.,* 2018; Versantvoort *et al.,* 2000).

Fasted animals are anesthetised, and the intestinal segment that will be used for the study is isolated and cannulated. The segment is then rinsed with an isotonic saline solution before being perfused with a solution containing the compound or drug. Specific time intervals are set for sample collection in order to attain steady-state conditions, and the perfusate is then collected and subsequently analysed. A large fraction of the compound or drug must be bioavailable to accurately determine the absorption of the compound or drug, thus making this method only

suitable for use with specific drugs (Balimane *et al.,* 2000; Barth *et al.,* 1999; Versantvoort *et al.,* 2000).

Disadvantages associated with the use of this method include that no information can be collected regarding the physiological state of the membrane; the rate of drug decrease in the perfusate may not always be representative of the amount of drug, which is absorbed in the vessels used for the studies; and a relatively large number of animals are required for experimental purposes to obtain accurate data (Barth *et al.,* 1999, Versantvoort *et al.,* 2000).

To circumvent the loss of too many animals using *in situ* methods, Poelma and Tukker developed a chronically isolated intestinal loop technique, which entails long-term isolation of an intestinal segment in the animal that makes it possible to perform cross-over studies without anaesthesia and without using additional animals (Versantvoort *et al.,* 2000).

2.2.5.4 *In silico* methods

In silico studies, also known as computer modelling, make use of computer reproductions to replace physical experiments. Computer programs have been developed to help conduct studies regarding the pharmacodynamic and pharmacokinetic properties of drugs. Software programs used for *in silico* investigations include MetaDrug™ and ACD/Percepta™ (da Cunha *et al*., 2016; Harrison *et al.,* 2004). *In silico* methods are potentially the way forward in limiting and replacing animal testing and it is a cost-effective alternative, especially in instances where research related to drug discovery and development is concerned (Harrison *et al.,* 2004).

2.3 Beneficial pharmacokinetic interactions

As mentioned before, oral dosing is the preferred route of drug administration because it is convenient to use, and patient compliance is usually much higher than with other routes. However, insufficient bioavailability of certain compounds can exclude the oral route as a way to administer these compounds. Low bioavailability can be caused by several factors, which may either be linked to the physico-chemical characteristics of the drug/compound itself or to physical/physiological characteristics of the site of absorption. Various formulation strategies have been developed to overcome some of these problems, for example, the inclusion of surfactants or fatty acids in the formulation, salt formation, complexation with cyclodextrins, nano-particle formulations, micronization, solid dispersions and co-administration of permeation enhancers (Kohli *et al.,* 2010).

2.3.1 Absorption enhancers

Absorption enhancement can be described as the technology that enables systemic delivery of poorly membrane-permeable compounds via non-injectable routes of administration (Aungst, 2012). Most patients prefer the oral route for drug administration, but low bioavailability of some drugs will result in poor efficacy of these drugs. The bioavailability of orally administered drugs may in some instances be improved with the co-administration of suitable permeation enhancers, which perform their actions via various mechanisms (Chen *et al.,* 2009, Desai *et al.,* 2012). Pharmacokinetic interactions are generally viewed as unwanted effects, however, if these interactions are employed strategically, it may be advantageous and could potentially improve the bioavailability of co-administered drugs and this may in turn benefit the patient (Edwards, 2012; Gerber *et al.*, 2018).

Various advantages may be gained from the inclusion of permeation enhancers/bio-enhancers in dosage forms and the addition of these enhancers may help to increase the bioavailability and bio-efficacy of co-administered active substances. Other advantages associated with the use of permeation enhancers include the potential increase in drug half-life, reduced variability in plasma drug concentrations; reduced dosage; and lower costs for patients, insurance companies and other institutions that are responsible for the payment of medicines, thus making medication more accessible in countries with limited funds for health care (Atal & Bedi, 2010; Edwards, 2012).

Permeation enhancers are substances, either from a natural source or synthetically prepared, that can enhance the bioavailability, efficacy and safety of co-administered drugs or other substances (Ajazuddin *et al.,* 2014; Atal & Bedi, 2010; Shanmugam, 2015). Most permeation enhancers may exhibit a range of pharmacological actions, which can be concentration dependent in nature. However, ideally permeation enhancers must be able to enhance the bioavailability of co-administered active substances without exerting any other pharmacological effects at the concentration used for permeation enhancement purposes (Shanmugam, 2015).

Some examples of herbal permeation enhancers include piperine, quercetin, glycyrrhizin, naringin, genistein, curcumin, sinomenine and niaziridin (Ajazuddin *et al.,* 2014). The permeation enhancement mechanisms of bioenhancers are schematically presented in Figure 2.4.

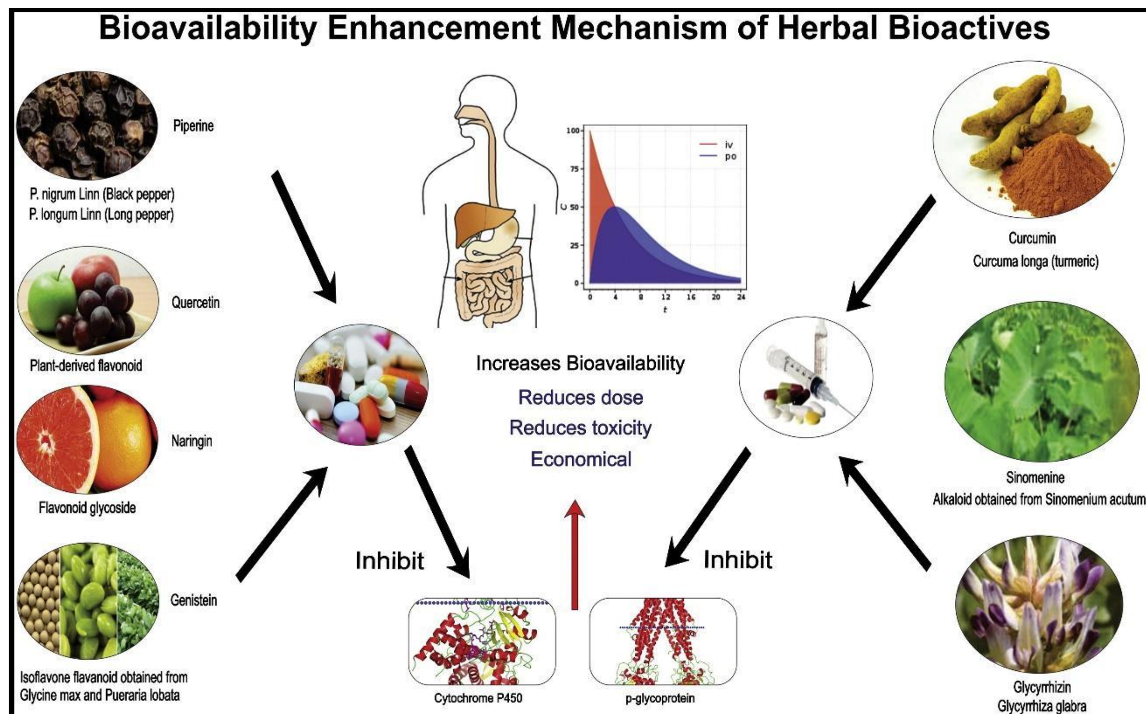


Figure 2-4: Selected natural permeation enhancers and their proposed permeation enhancement mechanisms (Ajazuddin *et al.,* 2014)

Permeation enhancers overcome the barrier function of the site of absorption with no or minimum damage to the tissue, thus allowing penetration across epithelial cells into the blood and lymph circulation (Chen *et al.,* 2009). A permeation enhancer is not limited to a single mechanism of action and some enhancers may elicit absorption enhancing effects via a combination of mechanisms of action (Shanmugam, 2015). Mechanisms of action of permeation enhancer may include, but are not limited to:

- Inhibition of enzymes, for example CYP1A1, CYP1B1, CYP3A4, CYP2E1 and CYP1B2, that are responsible for hepatic or gastrointestinal metabolism;
- Alteration of acid secretion (can increase or reduce acid secretion);
- Modification of blood supply to the gastrointestinal tract for enhanced absorption;
- Inhibition of P-gp related efflux transporters;
- Modification of membrane permeability (Atal & Bedi, 2010; Shanmugam, 2015).

Table 2.2 provides a list of drug absorption enhancers and their mechanisms of action.

Table 2-2: A summary of some absorption enhancers and their mechanisms of action that may make absorption enhancement possible

Absorption enhancer	Example	Mechanism of action
Plant materials/extracts	Aloe vera leaf materials Piperine	Allows paracellular transport by opening tight junctions which leads to a reduction in the transepithelial electrical resistance (TEER) (Beneke *et al.,* 2012; Haasbroek *et al.*, 2019) Supresses hepatic and gut metabolism by inhibiting CYP3A4 and P-gp related efflux (Baspinar *et al.,* 2018)
Inclusion complex formation molecules	Cyclodextrins	Forms complexes with drugs and increases the solubility and thus bioavailability of drugs (Kurkov & Loftsson, 2013; Stella *et al.,* 1999)
Fatty acids	Medium chain glycerides Long chain fatty acid esters (palmitoylcarnitine)	Dilation of tight junctions (paracellular) and epithelial cell damage or disruption of cell membranes (transcellular) causing reduction in the TEER value (Hamman *et al,* 2005; Juginger & Verhoef, 1998)
Anionic polymers	Poly (acrylic acid) derivatives	The combination of opening tight junctions by the depletion of extracellular calcium and enzymes (Hamman *et al.,* 2005)
Toxins and venom extracts	Zonula occludens toxin (ZOT) Melittin	Opens tight junctions by interacting with the zonulin surface receptor that induces actin polymerization (Salama *et al.,* 2006) A-helix ion channel formulation Bilayer micellization and fusion (Hamman *et al.,* 2005)
Bile salts	Sodium taurocholate Sodium taurodeoxycholate Sodium taurodihydrofusidate	Phospholipid solubilisation and cytolytic effects causing disruption of membrane integrity Mucus viscosity reduction (Hamman *et al.,* 2005)

Salicylates	Sodium salicylate Salicylate ion	Cell membrane fluidity increase Decrease in non-protein thiol concentration (Hamman *et al.,* 2005)
Chelating agents	Ethylenediaminetetraacetic acid (EDTA) Egtazic acid (EGTA)	Opening tight junctions by forming complexes with calcium and magnesium (Hamman *et al.,* 2005; Junginger & Verhoef, 1998)
Cationic polymers	Chitosan salts *N*-trimethyl chitosan chloride	Opening of tight junctions by combining mucoadhesion and ionic interactions with cell membranes (Kang *et al.,* 2009)

2.3.1.1 *Aloe vera* leaf materials

Aloe vera (L.) Burm. f. belongs to the Xanthorrhoeaceae family and is a xerophytic, perennial, succulent, pea-green coloured plant (IARC, 2017; López *et al.,* 2013). The pulp (innermost part of the leaves), also known as the gel, is made up of large, thin-walled parenchyma cells and is filled with the gel. The *A. vera* gel is colourless and consists primarily of water (>98%) and mono- and polysaccharides, such as pectins, hemicellulose, cellulose, glucomannan, acemannan and mannose derivatives. Acemannan is considered to be the main functional part of *A. vera*. The three most used parts of the plant include the *Aloe* latex, the *Aloe* gel and the extract of the whole leaves (Beneke *et al.,* 2012; Bozzi *et al.,* 2007; Chen *et al.,* 2009; Hamman, 2008). Whole leaf extracts are prepared by grinding the leaves and removing the antraquinones by means of filtration (Chen *et al.,* 2009).

Aloe vera leaves and *A. vera* gel have been used commercially and medically for many years. They possess beneficial medicinal, cosmetic and nutritional effects, although until recently, no scientific evidence was available to validate these uses (Muñoz *et al.,* 2015). Cosmetic industries use the gel mainly to treat scars, wounds and burns and is also the largest industry that uses *A. vera* gel. There is also an increase in the use of this gel in the food industry in drinks, ice creams and even beverages (Serrano *et al.,* 2006). Studies claim that *A. vera* whole leaf extract can be used for detoxification, treatment of constipation, improvement of digestion and for reducing illness risks (Boudreau *et al.,* 2013). Whole leaf extract can also be potentially used in the treatment of cancer and acquired immunodeficiency syndrome (Chen *et al.,* 2009).

An *in vivo* study in humans indicated an increase in the bioavailability of Vitamin C and E by *A. vera* juice preparations when compared to the control group, thus indicated an increase in the

absorption of these vitamins (Vinson *et al.,* 2005). A study conducted by Chen *et al.* (2009) indicated that *A. vera* gel and *A. vera* whole leaf extract can reduce the TEER of Caco-2 cell monolayers, thus indicating the enhancement of paracellular permeability of epithelial cell layers. It was concluded that the transport enhancement effect was possibly due to the opening of tight junctions, thus allowing paracellular transport of molecules. The TEER values returned to their original values at the end of the study, thus indicating that the effect of *A. vera* on the tight junctions is reversible. Confocal laser scanning microscopy images and TEER reductions confirmed the opening of tight junctions and paracellular transport enhancement of FITC-dextran by *A. vera* gel and whole leaf materials across Caco-2 cell monolayers (Haasbroek *et al*., 2019).

2.3.1.2 Piperine

Piperine is an alkaloid and bioactive compound extracted from *Piper nigrum* and *Piper longum*, commonly known as black pepper and long pepper, and is known as one of the main components of pepper (Di *et al.,* 2015; Wadhwa *et al.,* 2014; Zhao-Hui *et al*., 2018). Both peppers are more commonly used as food additives, where black pepper is the most frequently used spice worldwide. The components extracted from these peppers contribute to their value as food additives. The seeds are usually used to enhance taste and flavour of food, while the essential oils can prevent the spoilage of food because of its antioxidant and antimicrobial properties (Andrade *et al*., 2017).

Piperine, can be classified through its chemical structure as a cinnamamide, thus possessing sedative, hypnotic, antidepressant, anticonvulsant and skeletal muscle relaxing properties and can help to improve digestion (Atal & Bedi, 2010; Wadhawa *et al.,* 2014). Other important effects of piperine include anti-inflammatory, anti-angiogenic and anti-cancer activities (Baspinar *et al.,* 2018). The antioxidant and antimicrobial properties of piperine make this an excellent alternative and complementary therapy regime for various illnesses and disorders (Andrade *et al.,* 2017).

The bio-enhancing effects of piperine can be attributed to the formation of polar complexes with compounds, or by the inhibition of hepatic and gut metabolism (including UDP-glucuronyltransferase activities, arylhydrocarbon hydroxylase and ethylmorphine-*N*-demethylation). Piperine is one of the most potent bio-enhancers and inhibits the primary metabolizing enzyme, CYP3A4, as well as the P-gp related efflux, thus increasing drug absorption across the intestinal epithelium. It has also been proposed that piperine binds to the DNA receptor and can modulate cell signal transduction (Atal *et al.,* 2010; Di *et al.,* 2015; Zhao-Hui *et al.,* 2018). Figure 2.5 presents the chemical structure of piperine.

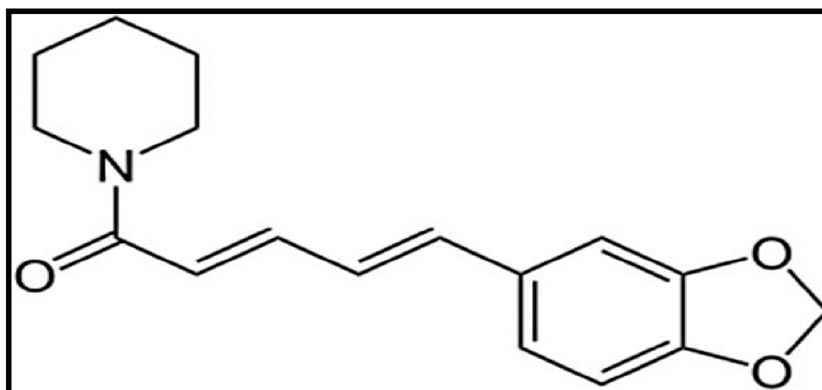


Figure 2-5: Chemical structure of piperine (Baspinar, *et al*., 2018)

The dose to be used for effective bio-enhancing effects varies, but studies have indicated that a dose of 10% (w/w) used could be regarded as a safe and effective dose. There are various advantages to using piperine as a bio-enhancer, including increased efficacy of the drug used due to an increase in bioavailability; reduced drug dosage and drug resistance risk due to the combination of a bio-enhancer; and minimized reactions or side effects and toxicity due to lower drug dosages (Wadhwa *et al.,* 2014).

2.4 Summary of literature review

The use of herbal or natural supplements are becoming more popular in the daily medicine regime of patients. The biggest problem concerning the use of these products is the lack of information regarding the products and the safety of these products when used with other medicines (Kaufman *et al.,* 2002). Most herbal medicine or supplements are not tested and reviewed appropriately for content of active ingredients, *in vitro* release, bioavailability and stability (Doogue & Thynne, 2013; Masupye *et al.,* 2015; Todorova *et al.,* 2016; Winterstein & Storrs, 2001).

Compounding pharmacies are compounding preparations that are not available in a certain strength, dosage form or that are not at all available for public use. These formulations, known as extemporaneously compounded medication, are not clinically tested and are sold or consumed without proper information regarding the safety and efficacy of the medication.

Beneficial pharmacokinetic interactions may be employed to the advantage of patients to improve the oral bioavailability of active compounds. Poor bioavailability may be overcome by including absorption enhancers such as; plant materials, complexation agents, surfactants, fatty acids and chelating agents in extemporaneously compounded medications (Baspinar *et al.,* 2018; Beneke *et al.,* 2012; Chen *et al.,* 2009, Desai *et al.,* 2012; Di *et al.,* 2015; Hamman *et al.,* 2005; Kurkov & Loftsson, 2013).

CHAPTER 3: MATERIALS AND METHODS

3.1 Introduction

The aim of this study was to prepare and evaluate different extemporaneously compounded astaxanthin formulations in the form of hard gelatine capsules. Additionally, selected bio-enhancers were added to the capsule formulations in varying concentrations to potentially improve the delivery of astaxanthin across the intestinal epithelium. Ten capsule formulations were prepared in total, each containing either astaxanthin alone or in combination with three bioenhancers namely; *Aloe vera* gel, *Aloe vera* whole leaf extract and piperine.

The formulations were first assayed for content and evaluated in terms of dissolution to determine the rate of astaxanthin release followed by astaxanthin delivery across excised pig intestinal tissues. The potential improvement of intestinal delivery of astaxanthin by selected bioenhancers was specifically evaluated using an *ex vivo* technique across excised pig intestinal tissues mounted between two half-cells of the Sweetana-Grass diffusion apparatus.

3.2 Materials

Table 3-1: Materials used to manufacture the various capsule formulations

Materials	Botch/Lot number	Supplier
Astaxanthin	Lot: 1-JLW-88-1	Toronto Research Chemicals
Aloe vera gel dehydrated powder	Batch: 700AQ11PK01	Donated by Improve USA
Aloe vera whole leaf decolorized spray dried powder	Batch: 715AQ11PK01	Donated by Improve USA
Piperine	Lot: MKCC0339	Sigma-Aldrich
Pharmacel®	-	-
Gelatine capsules	-	-

Table 3-2: Materials used to prepare Krebs-ringer bicarbonate buffer

Materials	Botch/Lot number	Supplier
D-Glucose	Batch: 8164	Ace chemicals
Magnesium Chloride hydrate	Batch: 26733	Ace chemicals
Potassium Chloride (KCl)	Batch: 34856	Ace chemicals
Sodium Chloride (NaCl)	Batch: 35310	Ace chemicals
Sodium Phosphate Dibasic	Batch: 34753	Ace chemicals
Sodium Phosphate Monobasic	Batch: 33442	Ace chemicals

Table 3-3: Materials used to prepare fed state simulated intestinal fluid (FeSSIF)

Materials	Botch/Lot number	Supplier
Sodium taurocholate hydrate	Lot # BCBW3135	Sigma-Aldrich
Lecithin	Lot E07Z037	Alta Aesar
Glacial acetic acid	-	-
Sodium Chloride (NaCl)	Batch: 35310	Ace chemicals
0.1 M Sodium hydroxide (NaOH) solution	-	Merck

Table 3-4: Materials used for method validation procedures and other miscellaneous materials

Materials	Botch/Lot number	Supplier
Lucifer Yellow	-	Sigma-Aldrich-
100% HPLC grade Methanol	Different batches used	Merck
HPLC grade Acetonitrile	Different batches used	Merck
DMSO	-	-

3.3 Validation of analytical methods

Validation is defined by Porter (2007) as establishing documented evidence that provide a high degree of assurance that a specific method will constantly provide results or products that meet the specifications and quality characteristics that were determined previously. Furthermore, method validation is performed to ensure that the methods used in a study are accurate, specific and reproducible over a specified range of specific analyte concentrations (Shabir, 2003).

3.3.1 Linearity

Linearity of an analytical method refers to the direct correlation between the concentration of an analyte (e.g. µg/ml) and the resultant instrument response (e.g. florescence value) within a given concentration range of the specific analyte (BP, 2018). For linearity to be considered acceptable, a correlation coefficient (R) value of ≥ 0.998 should be obtained for the line obtained when response is plotted as a function of concentration (Singh, 2013). The R value should be determined by a series of three to six evaluations/measurements over a minimum of five standard solutions (Singh, 2013; USP, 2017).

3.3.2 Accuracy

Accuracy can be defined as the closeness between test results obtained and true analyte concentration values (Shabir, 2003; USP, 2018). For an analytical method to be considered accurate, a mean recovery of 100 ± 2% should be obtained (Shabir, 2003), however. The % recovery was calculated with the following equation:

$$\% \text{ Recovery} = \frac{\text{Experimental concentration}}{\text{Theoretical concentration}} \times 100$$ **Equation 3.1**

3.3.3 Precision

Precision of an analytical method can be defined as the repeatability of the instrument response to analyse multiple samples of the same analyte solution at different times. Precision is usually expressed as the percentage relative standard deviation (%RSD) and can be divided into two sub-groups, namely intra- and inter-day precision (Shabir, 2003; Singh, 2013).

3.3.3.1 Intra-day precision

Intra-day precision is a measurement of precision under the same conditions over a short period of time (e.g. within the same day) (BP, 2018). The intra-day precision was determined using solutions of different concentrations taken on separate opportunities during the same day. For intra-day precision to be considered acceptable, a %RSD between the measurements of ≤ 2% should be obtained (BP, 2018; Shabir, 2003).

3.3.3.2 Inter-day precision

The inter-day precision of the analytical method was determined on three consecutive days using solutions with different concentrations. A %RSD between the measurements of ≤ 5% should be obtained for inter-day precision to be considered acceptable (BP, 2018).

3.3.4 Limit of quantification (LOQ)

The limit of quantification can be defined as the lowest concentration of an analyte in a sample that can be determined with acceptable linearity and precision under specified conditions (USP, 2018). The following equation was used to determine LOQ:

$$LOQ = 10 \times \frac{SD}{S} \qquad \textbf{Equation 3.2}$$

The SD represents the standard deviation of the blank samples (background noise) and S represents the slope of the standard curve that was obtained (Shabir, 2003; USP, 2018).

3.3.5 Limit of detection (LOD)

The limit of detection can be defined as the lowest amount of the analyte in a sample that can be detected, but not necessarily quantified, under specified experimental conditions (Shabir, 2003; USP, 2018). The following equation was used to determine the LOD:

$$LOD = 3.3 \times \frac{SD}{S} \qquad \textbf{Equation 3.3}$$

The SD represents the standard deviation of the blank samples (background noise) and S represents the slope of the standard curve that was obtained (Shabir, 2003; USP, 2018).

3.3.6 Specificity

Specificity can be defined as the ability to accurately detect and/or quantify the specific compound being investigated, while in the presence of other compounds that may also be present in the experimental samples (Singh, 2013).

3.4 Validation of the fluorometric analytical method for Lucifer Yellow

Prior to measuring the concentration of Lucifer Yellow (LY) in the transport samples, the fluorometric analytical method was validated with respect to linearity, accuracy, precision (inter- and intra-day precision), LOQ and LOD. A LY stock solution was prepared (50 µg/ml) and a SpectraMax® Paradigm® plate reader with the excitation wavelength set at 485 nm and the emission wavelength at 535 nm respectively was used for the validation of LY (Sigma-Aldrich, 2013).

3.4.1 Linearity

A 50 µg/ml stock solution of LY was prepared, after which a series of dilutions were prepared with a dilution factor of 2 to produce solutions with concentrations as shown in Table 3.5 below.

Table 3-5: Lucifer Yellow standard solution concentrations used to obtain a standard curve for linearity evaluation

Solution	Concentration LY (µg/ml)
1	50
2	25
3	12.5
4	6.25
5	3.125
6	1.563
7	0.781
8	0.391
9	0.195

3.4.2 Accuracy

Three different LY solutions with concentrations of 50, 25, and 12.5 µg/ml were prepared and ten samples from each solution were used in order to determine accuracy. The samples were analysed using the SpectraMax® Paradigm® plate reader. The percentage recovery in relation to the theoretical values were calculated using the concentration values of the samples determined with the fluorometer.

3.4.3 Precision

A 50 µg/ml stock solution of LY was used to obtain three different LY concentrations (e.g. 50, 25, and 12.5 µg/ml) to determine both the inter-day and intra-day precision of the analytical method.

3.4.3.1 Intra-day precision

To determine the inter-day precision of the analytical method data was obtained on three consecutive days and the %RSD values were calculated by dividing the standard deviation by the average obtained from the florescence values times 100%.

3.4.3.2 Inter-day precision

To determine the intra-day precision of the analytical method the samples were analysed on three separate occasions during the same day between 09:00 and 15:00, after which the %RSD values were calculated by dividing the standard deviation by the average obtained from the florescence values times 100%.

3.4.4 Limit of quantification (LOQ)

The limit of quantification of LY was determined using equation 3.2. The SD represents the standard deviation of the response of a blank solution (KRB) and S represents the slope of the standard curve that was obtained from LY data based on the analysis of serially diluted samples.

3.4.5 Limit of detection (LOD)

The limit of detection of LY was determined using equation 3.3. The SD represents the standard deviation of the response of a blank solution (KRB) and S represents the slope of the standard curve that was obtained from the LY data based on the analysis of serially diluted samples.

3.5 Validation of High-Performance Liquid Chromatography (HPLC) analytical methods for astaxanthin

The HPLC analytical methods were validated in terms of linearity, precision, LOQ, LOD and specificity. A stock solution consisting of 5.714 mg astaxanthin made up to 10 ml with methanol in a volumetric flask (571.429 µg/ml), serial dilutions were then prepared from the stock solution as needed for the relevant validations.

The initial assays of the capsule formulations were performed with the aid of a validated HPLC method (method one) but due to column failure and a lack of availability of a similar HPLC column a second HPLC method (method two) was also validated for analysis of astaxanthin. HPLC method one was adapted from Higuera-Ciapara, (2004), using a C_8 (5 µm), reversed-phase column, isocratic mobile phase consisting of 85% methanol, 10% acetonitrile and 5% HPLC grade water, with a flow rate of 1 ml/min. The temperature was set to 35 ^{0}C and the detection of astaxanthin was achieved at 480 nm, using UV detection and the run time was 10 min. A Hitachi® Chromaster HPLC system, equipped with a 5410 UV detector, 5260 auto sampler and 5160 pump was used for method one. Method one was used to determine the content astaxanthin present in each capsule formulation.

HPLC method two was adapted from Du *et al.,* (2016), using a C_{18} (5 µm) reversed-phase column, methanol and acetonitrile were used as the mobile phases (see table 3.6) with a flow rate of 1

ml/min. The detection of astaxanthin was achieved at 480 nm, using UV detection, and the run time was 10 min. An Agilent® 1100 Series HLPC system, equipped with a 1100 pump, diode array detector and auto sampler injection mechanism was used for method two. Method two was used for the analysis of samples obtained from the dissolution- and transport studies.

Table 3-6: Volumes of mobile phases and specific run time intervals

Time (min)	Mobile phase A: Acetonitrile	Mobile phase B: Methanol
0	92.0%	8.0%
5.50	92.0%	8.0%
6.00	100.0%	0.0%
8.00	100.0%	0.0%
8.10	92.0%	8.0%
10.00	92.0%	8.0%

3.5.1 Linearity

The linearity for both HPLC methods were determined by analysing a series of 5 astaxanthin concentrations, which were prepared with serial dilution from a stock solution as described below (Table 3.7). The results were then used to construct a standard curve followed by regression analysis to determine the resultant R-value.

Table 3-7: Standard dilutions used to create a standard curve for astaxanthin

Solution	Concentration (µg/ml)
1	571.429
2	114.285
3	28.571
4	9.524
5	4.762

3.5.2 Precision

3.5.2.1 Inter-day precision

To determine the inter-day precision for both HPLC methods, three solutions with different astaxanthin concentrations (e.g. 571.429, 114.286, and 9.524 µg/ml) were prepared and analysed at specific times on three consecutive days and the %RSD was calculated from the analytical results. Shabir (2003) stated that the accepted %RSD is ≤ 2%, previous studies reported that the

coefficient of variation (relative standard deviation) for natural supplements can be accepted at < 10% (Dwyer *et al.,* 2007; Roseland *et al.,* 2008).

3.5.2.2 Intra-day precision

To determine the intra-day precision for both HPLC methods, three solutions with different astaxanthin concentrations (e.g. 571.429; 114.286 and 9.524 µg/ml) were prepared and analysed during two separate occasions on the same day and the %RSD was calculated from the analytical results. An acceptable %RSD for intra-day precision is 5% (Shabir, 2003), however previous studies reported that the coefficient of variation (relative standard deviation) for natural supplements can be accepted at < 10% (Dwyer *et al.,* 2007; Roseland *et al.,* 2008).

3.5.3 Limit of quantification (LOQ)

For method 1 and 2, five astaxanthin solutions with different concentrations (e.g. 571.429, 114.285, 28.571, 9.524 and 4.762 µg/ml) were prepared by serial dilution and each were injected six times in order to obtain the necessary data to determine the LOQ, using equation 3.2 as stated above.

3.5.4 Limit of detection (LOD)

For method 1 and 2, five astaxanthin solutions with different concentrations (e.g. 571.429, 114.285, 28.571, 9.524 and 4.762 µg/ml), were prepared by serial dilution and each were injected six times in order to obtain the necessary data to determine the LOD, using equation 3.3 as stated above.

3.5.5 Specificity

To determine if any of the other compounds (e.g. *A.vera* gel; *A.vera* whole leaf extract; piperine and Pharmacel®) used in the capsule formulations and in the study would cause any interferences with the HPLC methods used, different solutions were made using methanol and above-mentioned compounds and injected into the HPLC using the two different methods. The solutions or media used for transport (FeSSIF and DMSO/KRB solution) were also analysed using the two methods (method 1 – FeSSIF and method 2 – both FeSSIF and the DMSO/KRB solution). The chromatograms were analysed in order to determine if there were any interactions with the chromatogram with the astaxanthin standard.

3.6 Capsule formulation

Ten capsule formulations were formulated as shown in Table 3.8. The individual components of each formulation were weighed accurately and added in small amounts to a mortar and pestle in order to obtain a homogeneous powder mixture. After mixing, 100 mg aliquots of the powder were weighed and transferred to size 1 capsule shells.

Table 3-8: Composition of the capsule formulations and the individual quantities required per ingredient for the formulation of a singe 100 mg capsule

Capsule formulation	Composition
Formulation 1	• 4 mg astaxanthin • Pharmacell® as filler (96 mg)
Formulation 2	• 4mg astaxanthin • 10 mg *Aloe vera* gel as bioenhancer • Pharmacell® as filler (86 mg)
Formulation 3	• 4 mg astaxanthin • 35 mg *Aloe vera* gel as bioenhancer • Pharmacell® as filler (61 mg)
Formulation 4	• 4 mg astaxanthin • 70 mg *Aloe vera* gel as bioenhancer • Pharmacell® as filler (26 mg)
Formulation 5	• 4 mg astaxanthin • 10 mg, *Aloe vera* whole leaf extract as the bioenhancer • Pharmacell® as filler (86 mg)
Formulation 6	• 4 mg astaxanthin • 35 mg *Aloe vera* whole leaf extract as bioenhancer • Pharmacell® as filler (26 mg)
Formulation 7	• 4 mg astaxanthin • 70 mg *Aloe vera* whole leaf extract as bioenhancer • Pharmacell® as filler (26 mg)
Formulation 8	• 4 mg astaxanthin • 0.299 mg piperine as bioenhancer • Pharmacell® as filler (95.701 mg)
Formulation 9	• 4 mg astaxanthin • 0.399 mg piperine as bioenhancer • Pharmacell® as filler (95.601 mg)
Formulation 10	• 4 mg astaxanthin • 0.499 mg piperine as bioenhancer • Pharmacell® as filler (95.501 mg)

3.7 Capsule evaluation

3.7.1 Assay

Assays were conducted to determine the quantity of astaxanthin in each of the capsule formulations. The ten capsule formulations were each assayed in triplicate and the average astaxanthin content was calculated for each formulation. Three capsules of each formulation were opened, and the contents were added to three individual 10 ml volumetric flasks and made up to volume with 100%, HPLC-grade methanol. The solutions were stirred for 15 min using a magnetic stirrer (MSH 10), after which the solutions were sonicated using an ultrasonic bath (Eumax®) for 15 min. The solutions were filtered, using a 0.45 µm syringe filter and analysed using the validated HPLC method 1.

The experimental concentration obtained for each formulation was compared to the intended (theoretical) astaxanthin concentration of each formulation. The % content was determined using the following equation:

$$\% \text{ Content} = \frac{\text{experimental concentration of astaxanthin}}{\text{theoretical concentration of astaxanthin}} \times 100 \qquad \textbf{Equation 3.4}$$

3.7.2 Preparation of fed state simulated intestinal fluid (FeSSIF)

FeSSIF was freshly prepared and used as medium in the dissolution and also in the transport studies. Due to the poor water solubility of astaxanthin, an alternative medium was needed for the dissolution and transport studies of astaxanthin, thus FeSSIF was used. Table 3.9 provides the quantities of the ingredients that were used to prepare the FeSSIF.

Table 3-9: Ingredients and quantities used to prepare 1 litre of fed state simulated intestinal fluid (FeSSIF)

Ingredient	Quantity
Sodium taurocholate hydrate	8.09 g
Lecithin	2.41 g
Glacial acetic acid	9.1 ml
Sodium Chloride (NaCl)	10.11 g

The ingredients in Table 3.9 were weighed and added to a 1000 ml volumetric flask, containing half the volume of deionised water needed. The contents were stirred with a magnetic stirrer, after which the glacial acetic acid was added with another 400 ml of deionised water. The mixture was heated to 40 °C while continuously stirring until all the ingredients were dissolved, and a clear solution was obtained. The pH of the solution was adjusted to 7.4 by adding 0.1 M NaOH solution

in small quantities until the desired pH was obtained. After a clear solution was obtained the magnetic stirrer was removed, and the solution was made up to volume using deionised water (Marques, 2004).

3.7.3 Dissolution studies

Dissolution studies were conducted on capsule formulation 1, 8, 9 and 10 in triplicate to determine the dissolution profile of astaxanthin from these formulations, which was necessary to understand and explain the membrane transport data of the formulations containing piperine better. Each selected capsule formulation was opened and the contents of one capsule was added to 50 ml test tubes and made up to volume using the FeSSIF solution. The test tubes were placed on a rotating shaft in a solubility bath that was kept at 37 ºC and 2 ml samples were withdrawn at 15, 30, 60, 90, 120, 180 and 240 min from each tube. After each sample withdrawal, fresh FeSSIF (2 ml) was added to each test tube to maintain a constant volume. The sample solutions were filtered into HPLC vials using 0.45 µm syringe filters and then analysed using the validated HPLC method 1 for capsule formulation 1, 4 and 10. HPLC method 2 was used for capsule formulations 1, 8, 9 and 10. The % dissolution was determined by using the astaxanthin concentration at each time point as well as the previous assay values of the capsule formulations (to calculate the theoretical concentration of astaxanthin if the complete dose was released) and by applying the following equation (Equation 3.5):

$$\% \text{ dissolution} = \frac{\text{experimental concentration of astaxanthin as per time interval}}{\text{theoretical concentration of astaxanthin}} \times 100 \qquad \textbf{Equation 3.5}$$

3.8 Evaluating membrane integrity using Lucifer Yellow

LY, a florescent marker, was used to determine the integrity of the excised pig intestinal tissue that was mounted between the two half cells of the Sweetana-Grass diffusion apparatus. A LY solution with a concentration of 50 µg/ml was prepared by dissolving 2.5 ml of previously prepared LY stock solution in 50 ml of KRB buffer solution; 7 ml of the 50 µg/ml solution was added to the apical side of all six chambers and 7 ml pre-heated KRB buffer solution was added to the basolateral side of all six chambers. Samples of 180 µl were withdrawn from the basolateral chamber every 20 min over a period of 2 h and replaced with 180 µl fresh KRB buffer solution. All the samples were analysed using a SpectraMax® Paradigm® plate reader with the excitation wavelength set at 485 nm and the emission wavelength a 535 nm respectively (Sigma-Aldrich, 2013).

The transport data were used to determine the percentage transport and calculate the P_{app} values. For membrane integrity to be considered acceptable, the percentage permeability or percentage

transport of LY should be <3%, and the P_{app} values in the range of 8.2 x 10^{-7} - 9.1 x 10^{-7} ($cm.s^{-1}$) (Bhushani *et al.,* 2016; Sigma-Aldrich, 2013).

3.9 *Ex vivo* transport of astaxanthin across excised pig intestinal tissues

3.9.1 Preparation of Krebs-ringer bicarbonate buffer

Table 3.10 provides the quantities of the ingredients that were used to prepare the Krebs-ringer bicarbonate (KRB) buffer. The intestinal tissue was rinsed with KRB buffer and then the tissue was submerged in ice cold KRB buffer and transported to the laboratory prior to the start of the experimental procedures.

Table 3-10: Ingredients and specific quantities used to prepare 1 litre of Krebs-ringer bicarbonate buffer

Component	Quantity
D-Glucose	1.8 g
Magnesium Chloride	0.1 g
Potassium Chloride	0.34 g
Sodium Chloride	7.0 g
Sodium Phosphate Dibasic	0.1 g
Sodium Phosphate Monobasic	0.18 g

The KRB was prepared according to the description of Sigma-Aldrich brochure, by measuring the required quantities powder and adding it to approximately 90 % of the final volume water required, while stirring gently. The original container was rinsed with a small amount of water to remove excess traces of powder and added to the solution. To the solution, 1.26 g sodium bicarbonate was added and stirred until dissolved. The solution was made up to volume by adding additional water. The solution was stored in the fridge for later use (June, 2007).

3.9.2 Preparation of 2% v/v dimethylsulfoxide (DMSO) solution in KRB buffer

A 2% v/v dimethylsulfoxide (DMSO) solution was prepared in KRB buffer, which was used as one of the diffusion media during the membrane transport studies to help with the solubility of astaxanthin and thereby creating a suitable environment for investigating its membrane permeation (Ingels & Augustijns, 2003). A 2% v/v DMSO solution in KRB buffer was made by adding 2 ml DMSO to KRB and making it up to 100 ml with KRB buffer. This solution was stored at 2°C in a fridge until the transport studies commenced.

3.9.3 Collection and preparation of pig intestinal tissue

The use of excised pig intestinal tissues obtained from animals slaughtered for meat production was approved by the North-West University's Ethics Committee (Animcare) and a certificate was issued with approval number NWU-00581-19-S5 (i.e. for a minimal risk, category 0 project where tissue is obtained from dead animals). The intestinal tissues were collected from the local abattoir in Potchefstroom immediately after pigs were slaughtered. A segment of +/- 40 cm of the proximal jejunum was cut and rinsed out with cold KRB buffer, after which the segment was placed in ice cold KRB buffer in a container and taken to the laboratory. On arrival at the laboratory, the tissue was pulled over a glass tube with the mesenteric border facing upwards. An incision was made along the mesenteric border and the serosa was removed using blunt dissection. The tissue was removed from the tube and placed on wet filter paper and cut into equal sized strips (tissue containing Peyer's patches was not used in the study). After completion of the surgical procedures, the tissue was mounted between the Sweetana-Grass diffusion chamber's half cells, placed in the diffusion apparatus, filled with pre-heated KRB buffer and connected to the carbogen supply as illustrated in Figure 3.1.

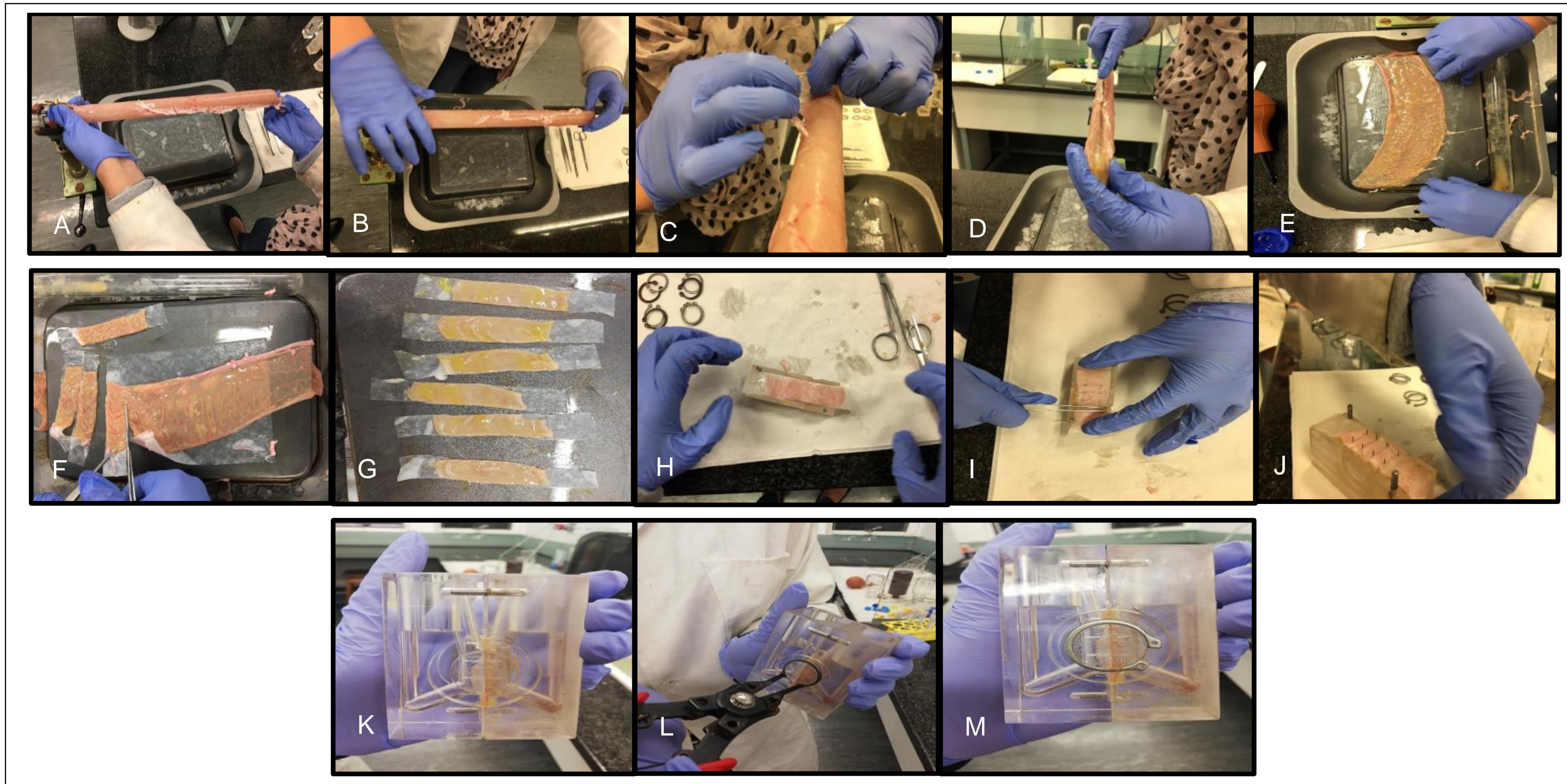


Figure 3-1: Photo's illustrating excised pig intestinal tissue being pulled over a glass tube (**A**); the mesenteric border being aligned (**B**); removing the serosa (**C**); tissue being cut along the mesenteric border (**D**); excised pig intestinal tissue being flattened out on filter paper after being cut (**E**); tissue being cut into segments (**F**); segments of tissue to be mounted (**G**); tissue segment being placed on chamber, filter paper on top (**H**); segment being placed on "spikes" on chamber (**I**); mounted segment, before removing filter paper (**J**); mounted tissue between the two chambers (**K**); clip being placed on chambers after tissue has been mounted to secure the chambers (**L**); mounted chambers secured by clip (**M**)

3.9.4 Transport studies

The transport of astaxanthin across pig intestinal tissue was investigated in both the apical-to-basolateral and basolateral-to-apical direction and all studies were performed in triplicate. After the tissue was mounted, both the chambers were filled with 7 ml pre-heated KRB buffer solution and a mixture of 95% O_2 and 5% CO_2 (carbogen supply) was allowed to bubble through the diffusion chambers and KRB buffer for 15 min in order to ensure that the tissue had acclimatised to the new environment (Grass and Sweetana, 1988).

The capsules were opened, and the powder content was added to 5 ml of the specific medium that was used in each chamber in a tube. For the apical-to-basolateral direction, the powder was added to 5 ml pre-heated FeSSIF, and for the basolateral-to-apical direction, the powder was added to 5 ml pre-heated 2% v/v DMSO/KRB solution. The tubes were vortexed in order to mix the powder with the medium and then sonicated for 10 min at 35 ^{o}C.

The KRB buffer was aspirated from both the chambers after 15 min, and the pre-mixed solutions were added to the appropriate chambers. For the apical-to-basolateral transport, the FeSSIF/capsule content mixture was added to each of the apical chambers and the tube washed out with 2 ml FeSSIF, making the final volume in the chamber 7 ml, while a volume of 7 ml fresh, pre-heated 2% v/v DMSO/KRB solution was added to each of the basolateral chambers. Samples of 100 µl were withdrawn from the basolateral (acceptor) chambers every 20 min over a period of 2 h.

For the basolateral-to-apical transport, the DMSO/capsule content mixture was added to each of the basolateral chambers and washed out with the 2 ml DMSO/KRB solution, making the final volume in the chamber 7 ml, while 7 ml of freshly prepared FeSSIF was added to the apical chambers. Samples of 100 µl were withdrawn from the apical (acceptor) chambers every 20 min over a period of 2 h.

The 100 µl samples that were withdrawn from the receiver chambers, at each of the specific time intervals, were mixed with 300 µl methanol and vortexed to be able to detect the astaxanthin that permeated across the membrane by means of HPLC analysis. The samples were placed in an ultra-sonic bath and sonicated for 5 min at 35 ^{o}C. Afterwards, the samples were placed in a centrifuge at 14 000 rpm for 10 min to separate the supernatant from the methanol mixture. Samples (200 µl) were withdrawn from the supernatant and analysed using HPLC method 2 and the percentage transport was calculated.

The percentage transport of astaxanthin was plotted on a graph as a function of time. Apparent permeability coefficient (P_{app}) values were calculated using equation 3.6:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A.Co.60}$$ **Equation 3.6**

Where P_{app} is the apparent permeability coefficient ($cm.s^{-1}$), $\frac{dQ}{dt}$ is the permeability rate (amount of measured astaxanthin in the receiver chamber over time), which was represented by the slope of the plotted percentage transport as a function of time. A is the surface area of the excised intestinal tissue between the apical and basolateral chambers (cm^2) and C_0 is the concentration ($\mu g.cm^{-3}$) on the apical side at the beginning of the study (Mahar Doan *et al.,* 2002; Yamashita *et al.*, 2000).

The transepithelial electrical resistance (TEER) was also measured every 20 min over the duration of all transport studies using a Warner Instruments® EC-825A epithelial voltage clamp, to give an indication of the tight junction integrity during the experimental procedures (Srinivasan *et al.,* 2015).

3.9.5 Astaxanthin retained in the intestinal tissue

Due to the lipophilic nature of astaxanthin, some of the molecules may not partition through the intestinal tissue into the aqueous medium of the acceptor chambers (basolateral or apical chambers) and a fraction may have been retained in the excised intestinal tissue. To determine if a fraction of astaxanthin was retained in the tissue, tissue samples were taken from the chambers after each transport study and rinsed with deionized water and transferred to 15 ml corning vials. A volume of 5 ml HPLC grade methanol was added to each vial and mixed using a Vortex® mixer. To aid lysis of the tissue, the vials were placed in an ultra-sonic bath for 10 min and then mixed thoroughly. After lysis of the tissue, the vials were placed in a centrifuge and spun at 3 000 rpm for 5 min in order to separate the solid particles from the supernatant; samples (200 µl) were withdrawn from each vial and analysed using the validated HPLC method 2. The percentage astaxanthin accumulated in the tissue was calculated using the following equation:

$$\% \text{ retention} = \frac{\text{Amount extracted from tissue}}{\text{Assay value from each formula}} \times 100$$ **Equation 3.7**

The amount of astaxanthin extracted from the lysis process was compared to the assayed value of each formula, this ratio was used to determine the percentage retention.

3.9.6 Statistical analysis of data

Statistical analysis of the transport data was performed by using Tukey HSD analysis and statistically significant differences were accepted when $p < 0.05$.

CHAPTER 4: RESULTS AND DISCUSSIONS

4.1 Introduction

The aim of this study was to formulate capsules containing astaxanthin in combination with the selected bioenhancers for improved oral delivery of astaxanthine. Three bioenhancers (including *Aloe vera* gel, *Aloe vera* whole leaf extract and piperine) were selected and each incorporated in three different concentrations in astaxanthin hard gelatine capsule formulations. The capsule formulations were evaluated in terms of uniformity of astaxanthin content, drug release profiles and the delivery of astaxanthin across excised pig intestinal tissues.

Ex vivo transport studies were conducted using excised pig intestinal tissues mounted on a Sweetana-Grass diffusion chamber apparatus in order to investigate the extent of astaxanthin transport across the intestinal tissues after application of the capsule formulations to the donor chamber. The percentage astaxanthin that permeated across the excised pig intestinal tissue was plotted as a function of time in both directions (apical-to-basolateral and basolateral-to-apical) and the apparent permeability coefficient (P_{app}) values were calculated. Retention studies were also performed on the excised pig intestinal tissues after each transport study to determine the quantity of astaxanthine that remained within the excised tissues. From the data obtained, the effectiveness of the bioenhancers in terms of the absorption enhancement potential of astaxanthin were calculated.

4.2 Validation of the fluorometric analytical method

Fluorescence spectrometry was used to measure the concentration of Lucifer yellow (LY) in transport samples, which were obtained from a transport study across excised pig intestinal tissue to confirm tissue integrity for the duration of the transport studies. The fluorometric analytical method was validated in terms of linearity, accuracy, precision (inter- and intra-day precision), limit of quantification (LOQ) and limit of detection (LOD).

4.2.1 Linearity

Figure 4.1 depicts the standard/regression curve of the mean fluorescence values plotted as a function of LY concentrations.

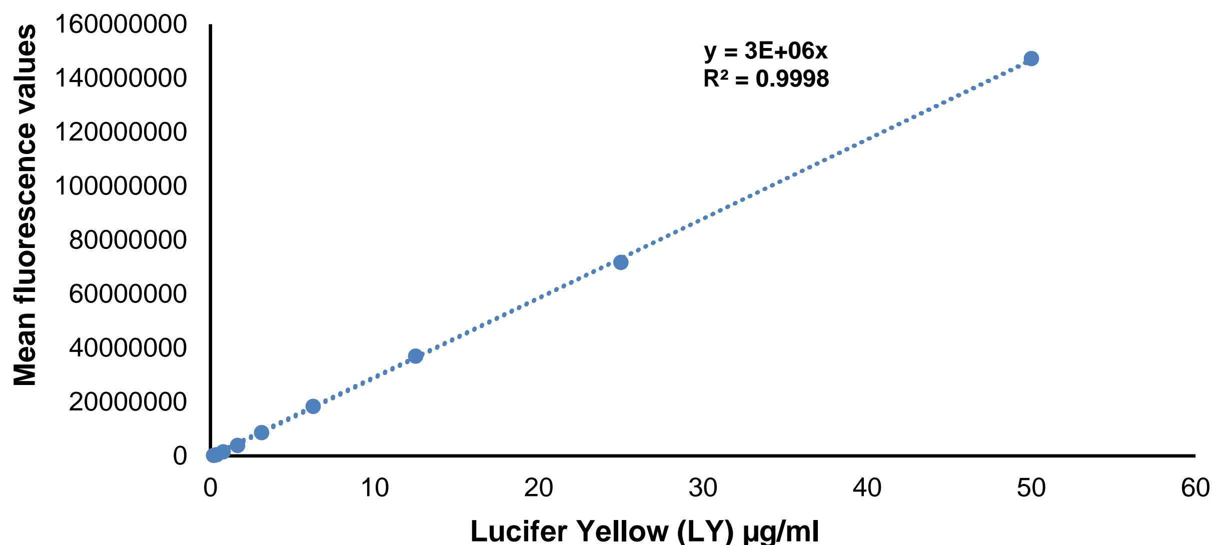


Figure 4-1: Linear regression curve of Lucifer Yellow with the representative straight line equation and the coefficient of determination (R^2) value

From Figure 4.1 it can be seen that the fluorometric analytical method did meet the specified requirements regarding linearity for LY, as is evident from the coefficient of determination (R^2) of the standard curve. The R^2 value of 0.9998 (> 0.998) indicate that there was a linear relationship between the concentrations and the recorded fluorescence values of LY (Shabir, 2003; Singh, 2013).

4.2.2 Accuracy

Accuracy was defined by Singh (2013) as the closeness between the results obtained and the true analyte concentration. For an analytical method to be considered as accurate, the percentage recovery of the analyte from the samples needs to be 100 ± 2% (Shabir, 2003). Table 4.1 presents the data obtained for the measurements of accuracy.

Table 4-1: Percentage recovery values of Lucifer Yellow to assess the accuracy of the fluorometric analytical method

Theoretical concentration (µg/ml)	Replicate	Actual concentration (µg/ml)	% Recovery	Average % Recovery
50	1	50.693	101.386	100.600 ± 0.946
	2	50.572	101.144	
	3	49.634	99.269	
25	1	25.054	100.217	100.401 ± 2.221
	2	25.802	103.208	
	3	24.444	97.778	
12.5	1	12.609	100.872	99.672 ± 2.026
	2	12.102	96.819	
	3	12.666	101.325	

The average percentage recovery for LY was within the required specified range (100 ± 2%) for an analytical method to be considered acceptable in terms of accuracy (Shabir, 2003).

4.2.3 Precision

4.2.3.1 Inter-day precision

The average standard deviation and percentage relative standard deviation (%RSD) was calculated using the average florescence values of three different LY concentrations (i.e. 50, 25 and 12.5 µg/ml). These values were obtained over the period of three consecutive days and is presented in Table 4.2.

Table 4-2: Inter-day precision measurements and the calculated standard deviation and percentage relative standard deviation (%RSD) of Lucifer Yellow

	Lucifer Yellow concentrations (µg/ml)		
	50	**25**	**12.5**
Day	**Mean fluorescence value**		
1	150 881 951.014	76 816 493.325	40 291 223.458
2	148 182 694.125	75 596 508.525	38 862 643.725
3	153 239 158.125	77 066 584.525	39 194 360.925
Average standard deviation	3 335 205.225	1 075 138.683	450 534.876
%RSD	2.210	1.404	1.145

The results presented in Table 4.2 confirmed that the fluorometric analytical method for LY did comply with the criteria regarding the inter-day precision with %RSD values of ≤ 5% (BP, 2018).

4.2.3.2 Intra-day precision

The intra-day precision of the fluorometric analytical method was determined by calculating the %RSD using the average florescence values and standard deviation values of three different LY concentrations (i.e. 50, 25 and 12.5 µg/ml). These values were obtained at three different time points during the same day and are presented in Table 4.3.

Table 4-3: Intra-day precision measurements and the calculated standard deviation and percentage relative standard deviation (%RSD) of Lucifer Yellow

	Lucifer Yellow concentrations (µg/ml)		
	50	**25**	**12.5**
Time point	**Mean fluorescence value**		
1	150 881 951.014	76 816 493.325	40 291 223.458
2	150 616 468.125	75 324 826.125	38 640 516.125
2	145 514 676.125	71 715 973.125	37 329 315.725
Average standard deviation	2 723 118.431	912 466.625	672 494.519
%RSD	1.826	1.211	1.757

Table 4.3 indicates that the average standard deviation and %RSD for LY did comply with the criteria regarding inter-day precision with a %RSD of ≤ 2% (BP, 2018; Shabir, 2003).

4.2.4 Limit of quantification (LOQ)

The standard deviation (2956.550) of the blank (KRB) fluorescence values represented the background noise and the slope of the regression/standard curve of LY (Figure 4.1) was used as the slope value in equation 3.1.

The LOQ for LY was calculated as 0.010089277 µg/ml. All the concentrations of LY in the experimental samples were higher than the calculated LOQ, thus the analytical method was able to accurately quantify all the experimental LY sample concentrations.

4.2.5 Limit of detection (LOD)

The standard deviation (2956.550) of the blank (KRB) fluorescence values represented the background noise and the slope of the regression/standard curve of LY (Figure 4.1) was used as the slope value in equation 3.2.

The LOD for LY was calculated as 0.003329461 µg/ml. All the concentrations for LY recovered from the experimental samples were higher than the calculated LOD, thus the analytical method was able to accurately detect all the experimental LY sample concentrations.

4.3 Validation of HPLC analytical methods

The quantity of astaxanthin in all samples acquired from assays, dissolution studies and transport studies were determined using validated HPLC analytical methods. The HPLC methods were validated in terms of linearity, precision (inter- and intra-day), LOQ, LOD and specificity.

4.3.1 Linearity

Figures 4.2 and 4.3 represent standard/regression curves obtained from average peak area values plotted as a function of astaxanthin concentrations.

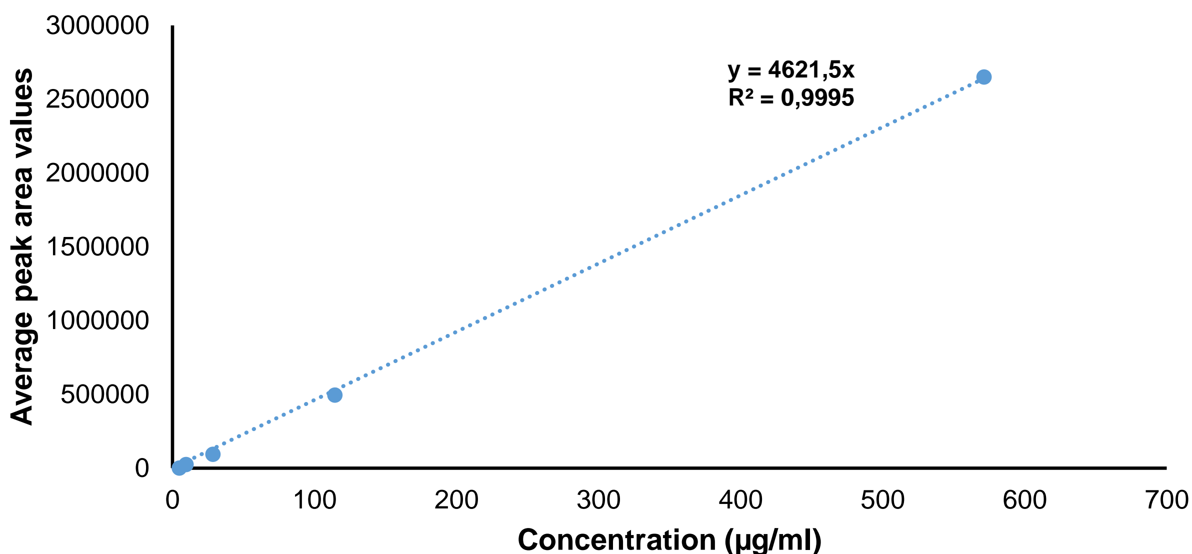


Figure 4-2: Linear regression curve of astaxanthin with the straight-line equation and the coefficient of determination (R^2) value for HPLC method one

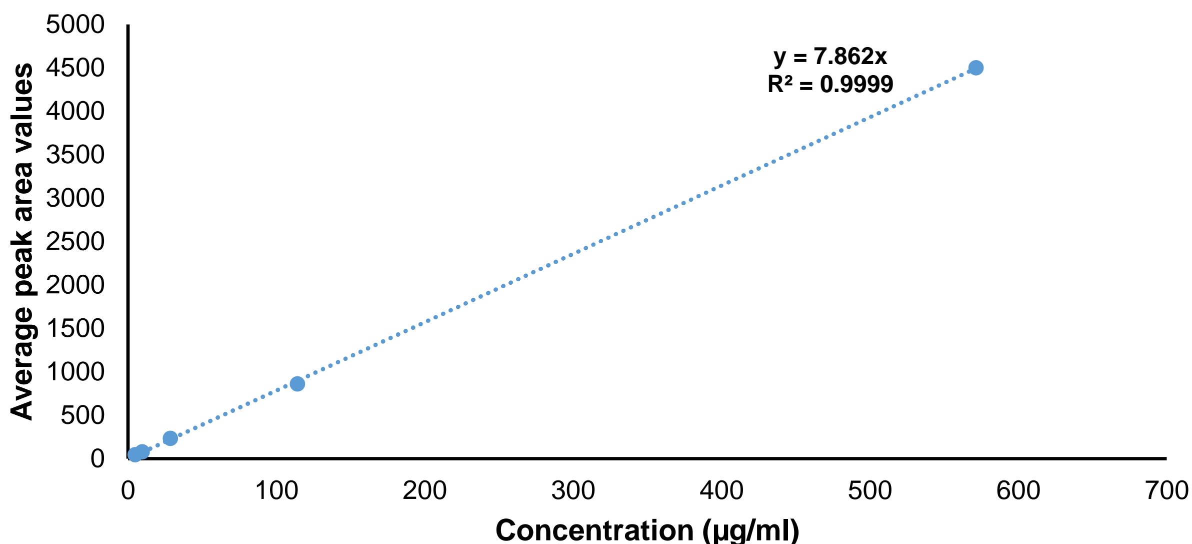


Figure 4-3: Linear regression curve of astaxanthin with the straight-line equation and the coefficient of determination (R^2) value for HPLC method two

Figures 4.2 and 4.3 show that the HPLC analytical methods did meet the specified requirements for linearity as determined from the R^2 value of the standard curve. The R^2 value of 0.9995 for method one and 0.9999 for method two indicate that there was a linear relationship between the concentrations and the resultant peak area values of astaxanthin (Shabir, 2003; Singh, 2013).

4.3.2 Precision

4.3.2.1 Inter-day precision

The standard deviation and %RSD values were calculated using the peak values of three different concentrations of astaxanthin (i.e. 571.429, 114.286 and 9.524 µg/ml). The values were obtained over a period of three consecutive days and are presented in Tables 4.4 and 4.5.

Table 4-4: Average peak area values used to calculate the standard deviation and percentage relative standard deviation (%RSD) obtained from the inter-day precision measurements of astaxanthin using HPLC method one

	Astaxanthin concentrations (µg/ml)		
	571.429	**114.286**	**9.524**
Day	**Mean peak area values**		
1	3925125.667	787553.500	28601.167
2	3800005.167	915167.500	49789.833
3	3745059.500	889945	49174.667
Standard deviation	72905.878	12230.480	1250.010
%RSD	1.896	1.415	2.8734

Table 4.4 indicates that this specific HPLC analytical method (method one) for astaxanthin did meet the requirements of the inter-day precision standards regarding analysis of supplements/compounds of natural origin with %RSD values of ≤ 10% (Dwyer *et al.,* 2007; Roseland *et al.,* 2008).

Table 4-5: Average peak area values used to calculate the standard deviation and percentage relative standard deviation (%RSD) obtained from the inter-day precision measurements of astaxanthin using HPLC method two

	Astaxanthin concentrations (µg/ml)		
	571.429	**114.286**	**9.524**
Day	**Mean peak area values**		
1	4500.067	858.883	77.067
2	4471.050	848.250	76.117
3	4423.600	843.933	70.867
Standard deviation	6.588	2.494	0.494
%RSD	0.147	0.293	0.669

Table 4.5 indicates that this specific HPLC analytical method (method two) for astaxanthin did meet the requirements for inter-day precision standards regarding analysis of supplements/compounds of natural origin with %RSD values of ≤ 10% (Dwyer *et al.,* 2007; Roseland *et al.,* 2008).

4.3.2.2 Intra-day precision

The standard deviation and %RSD values were calculated using the peak values of three different concentrations of astaxanthin (i.e. 571.429, 114.286 and 9.524 µg/ml). The values were obtained

from three measurements taken on the same day. Table 4.6 and 4.7 present the data obtained for HPLC method one and method two, respectively.

Table 4-6: Average peak area values and percentage relative standard deviation (%RSD) obtained from the intra-day precision measurements, using HPLC method one

	Astaxanthin concentrations (µg/ml)		
	571.429	**114.286**	**9.524**
Time point	**Mean peak area values**		
1	3861502.833	781513.667	28621.167
2	3745059.500	889945	49174.667
Standard deviation	75931.573	12231.988	1308.018
%RSD	1.972	1.468	3.442

Table 4.6 indicates that the intra-day precision for method one was acceptable and did meet the requirements for intra-day precision standards (%RSD ≤ 2) (Shabir, 2003), however, the lowest concentration presented a %RSD value above 2%. However, previous studies reported that the coefficient of variation (relative standard deviation) for natural supplements can be accepted at ≤ 10% (Dwyer *et al.,* 2007; Roseland *et al.,* 2008).

Table 4-7: Average peak area values and percentage relative standard deviation (%RSD) obtained from the intra-day precision measurements, using HPLC method two

	Astaxanthin concentrations (µg/ml)		
	571.429	**114.286**	**9.524**
Time point	**Mean peak area values**		
1	4500.067	858.883	77.06666667
2	44730400	851.567	76.43333333
Standard deviation	7.010	2.820	0.290059653
%RSD	0.156	0.330	0.378

Table 4.7 presents the data obtained for HPLC method two and confirms that the HPLC analytical method did comply with the required standards for intra-day precision (%RSD ≤ 10%) (Dwyer *et al.,* 2007; Roseland *et al.,* 2008).

4.3.3 Limit of quantification (LOQ)

The standard deviation and slope of the standard/regression curve of astaxanthin (Figure 4.2 and Figure 4.3) was used to calculate the LOQ.

The LOQ of method one was calculated as 0.11129969 µg/ml and the LOQ of method two was calculated as 0.045461725 µg/ml. All the experimental values were higher than the LOQ values. Both methods were thus acceptable for analysis of the experimental samples based on the calculated LOQ values.

4.3.4 Limit of detection (LOD)

The standard deviation and slope of the standard/regression curve of astaxanthin (Figure 4.2 and Figure 4.3) was used to calculate the LOD.

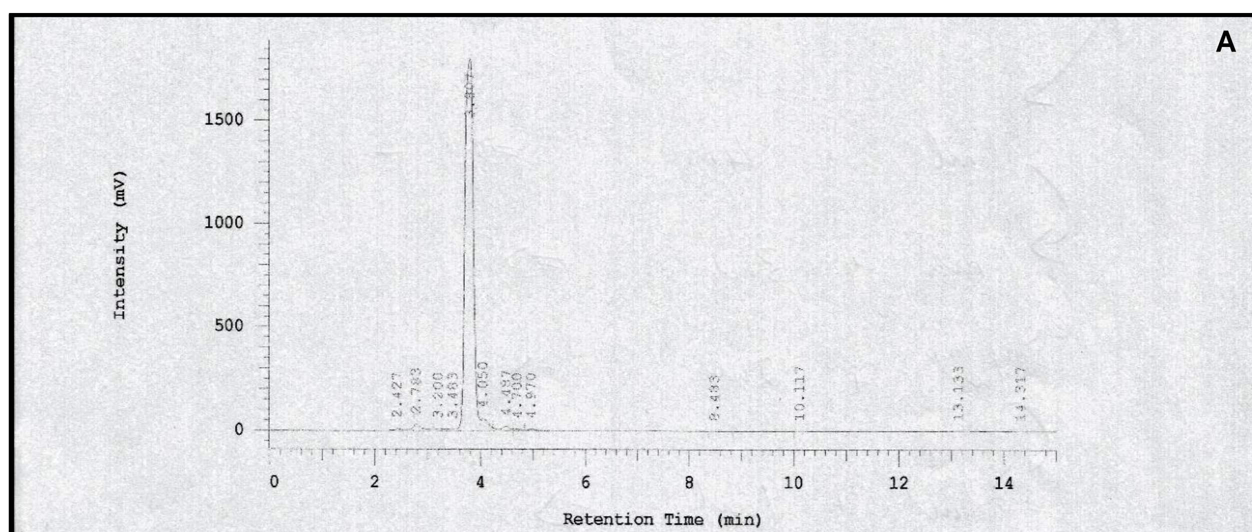
The LOD for method one was calculated as 0.036728898 µg/ml and the calculated LOD for method two was 0.015002369 µg/ml. The concentration values of the experimental samples were higher than the calculated LOD values for both methods, thus both methods were acceptable based on the LOD values.

4.3.5 Specificity

Specificity of an analytical method entails the capability to accurately detect the specific analyte in the presence of other components used in a study that may influence the detection of the specific compound by the instrument used in the analytical method. The different absorption enhancers, namely *A. vera* gel, *A. vera* whole leaf and piperine, as well as the FeSSIF ingredients, DMSO/KRB mixture, Pharmacel® and methanol used in the transport studies were analysed using the two different HPLC methods.

4.3.5.1 Astaxanthin

The chromatograms obtained from HPLC method one and two for an astaxanthine standard solution are presented in Figure 4.4.



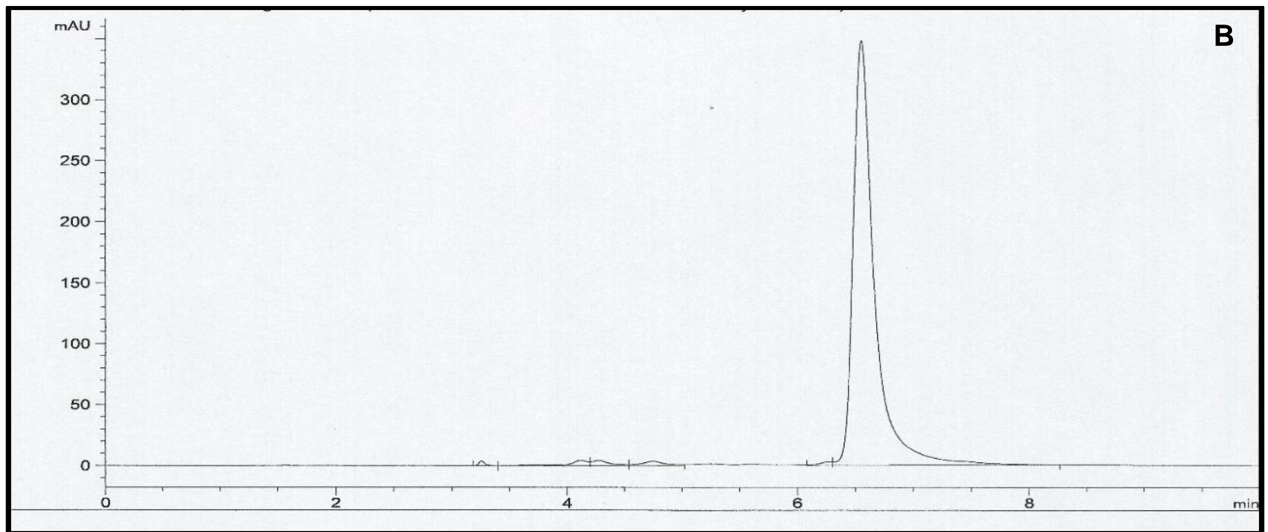
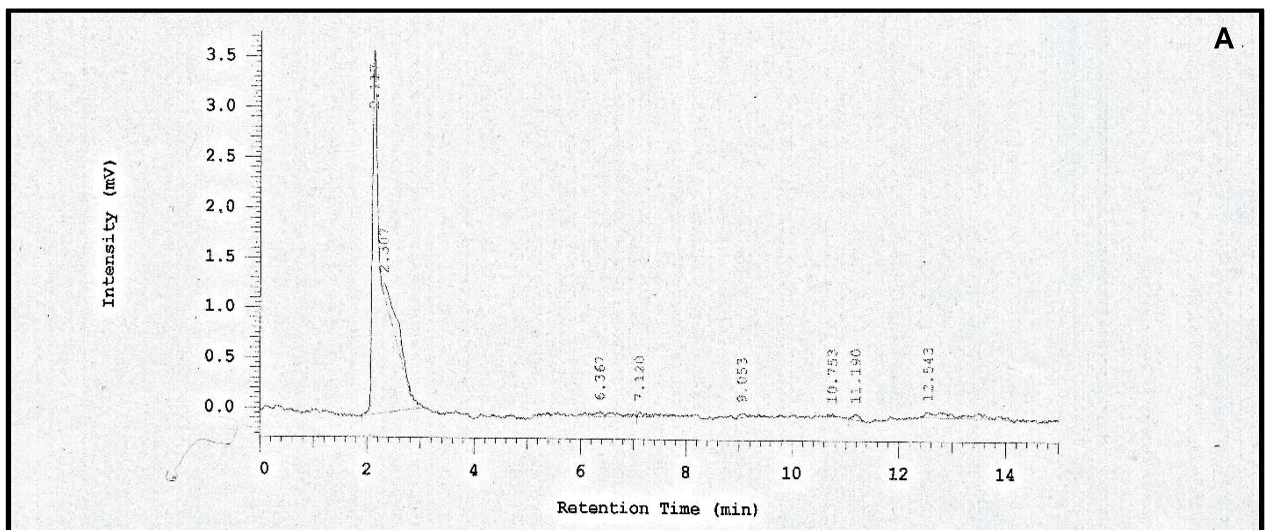


Figure 4-4: Chromatograms illustrating the peaks and retention times of an astaxanthin standard solution as obtained from HPLC method one (A) and method two (B)

As can be seen in Figure 4.4, the retention time for astaxanthin was 4 min for HPLC method one (A) and between 6.5 and 7 min for HPLC method two (B). No additional significant peaks were visible that might indicate interference of other compounds.

4.3.5.2 *Aloe vera* gel

The chromatograms obtained from HPLC method one and two for *A. vera* gel solution are presented in Figure 4.5.



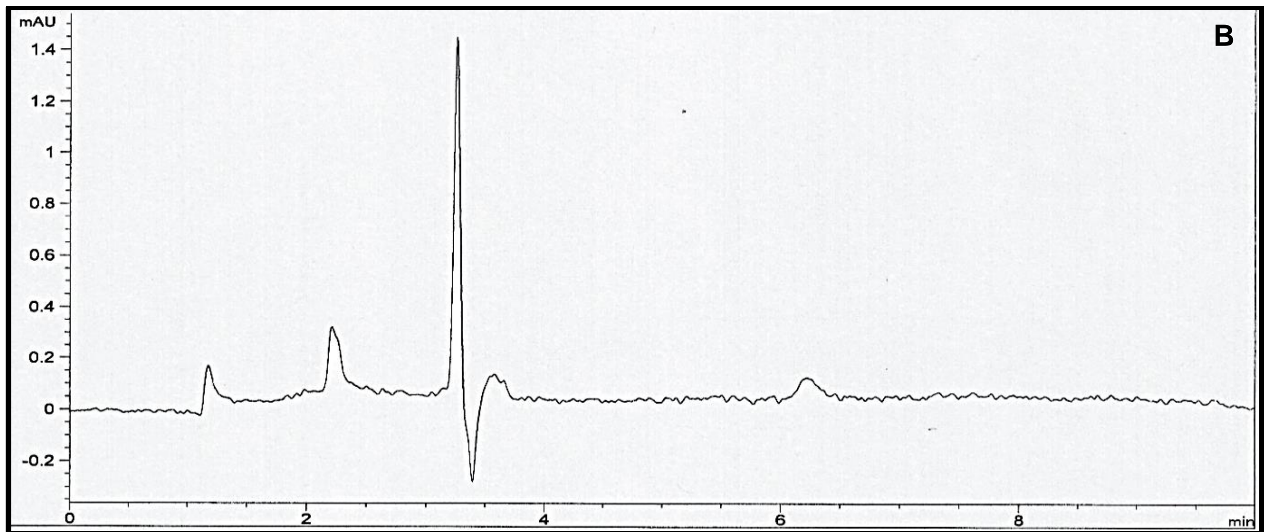


Figure 4-5: Chromatograms illustrating the peaks and retention times of *Aloe vera* gel solution when HPLC method one (A) and method two (B) were used

Comparing the chromatogram in Figure 4.5 (A) with the astaxanthin chromatogram in Figure 4.4 (A) for HPLC method one, it is evident that there was no overlap between the astaxanthin and *A. vera* gel peaks or retention times. *A. vera* gel only formed a low intensity peak after two minutes. Thus *A. vera* gel could be used as bioenhancer without interefering with HPLC method one.

Comparing Figure 4.5 (B) with the astaxanthin chromatogram in Figure 4.4 (B) for method two, no significant interactions between the peaks of *A. vera* gel and astaxanthin were found. Small peaks were formed with *A. vera* gel but did not interfere with the area or shape of the astaxanthin peak. *A. vera* gel could thus be used as a bioenhancer in the formulations and studies of astaxanthin without interfering with HPLC method two.

4.3.5.3 *Aloe vera* whole leaf extract

The chromatograms obtained from HPLC method one and two for *A. vera* whole leaf extract solution is presented in Figure 4.6.

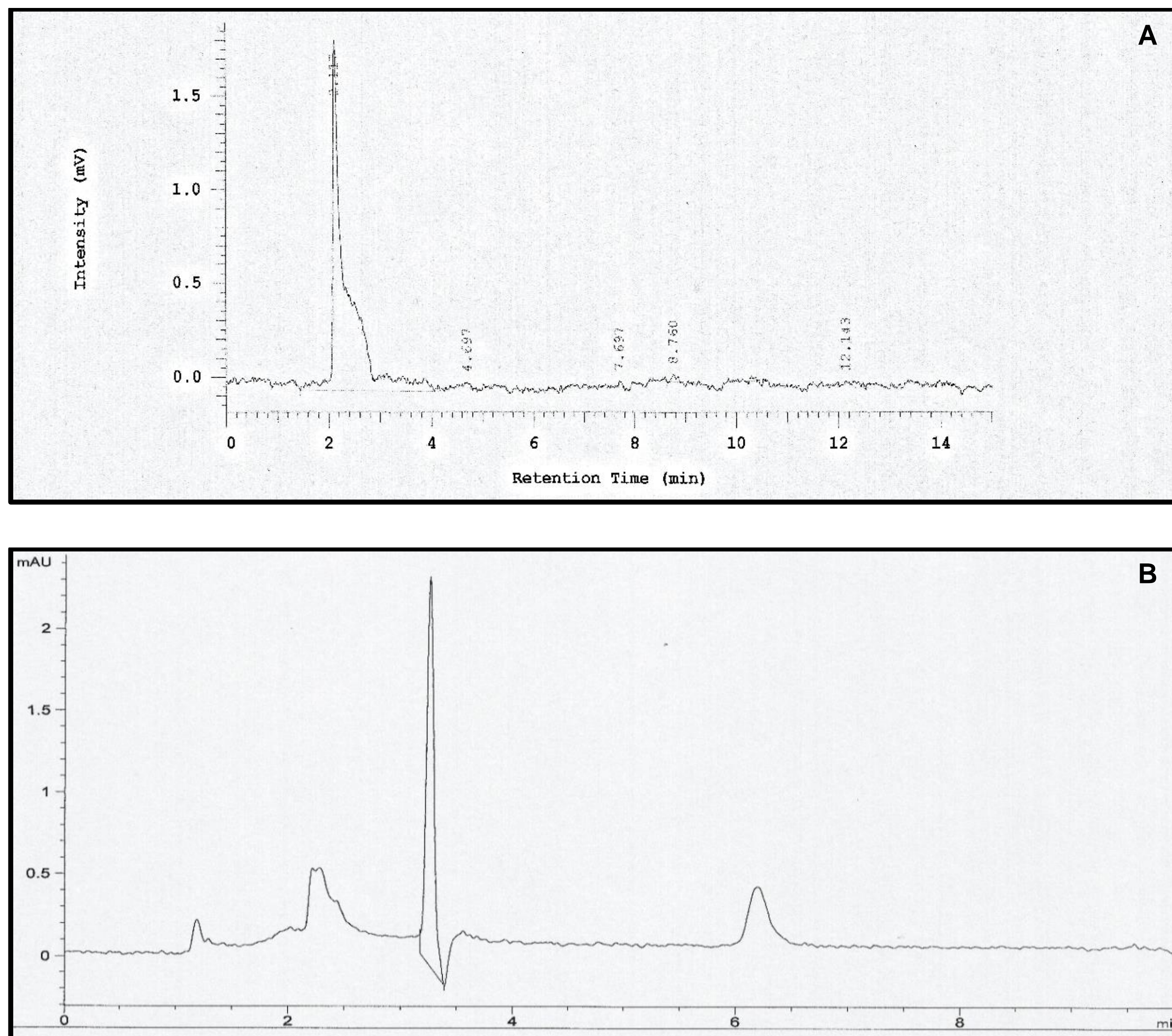


Figure 4-6: Chromatograms illustrating the peaks and retention times of *Aloe vera* whole leaf extract solution when HPLC method one (A) and method two (B) were used

Comparing Figure 4.6 (A) with the astaxanthin chromatogram in Figure 4.4 (A) for method one, it is evident that there was no overlap between the astaxanthin and *A. vera* whole leaf extract peaks and retention times. *A. vera* whole leaf extract formed a low intensity peak after two minutes for *A. vera* whole leaf extract. Thus *A. vera* whole leaf extract could be used as bioenhancer with method one.

Comparing the chromatogram in Figure 4.6 (B) with the astaxanthin chromatogram in Figure 4.4 (B) for HPLC method two, no overlap between the peaks of astaxanthin and *A. vera* whole leaf extract were found. A small, low intensity peak was formed between 6 and 6.5 min, which could possibly be explained by carry-over from the standard that was initially injected into the HPLC. The peak, however, was too low in intensity to be able to alter the shape or area of the astaxanthin peak. *A. vera* whole leaf extract could thus be used as a bioenhancer in the formulations and studies of astaxanthin without interfering with HPLC method two.

4.3.5.4 Piperine

The chromatograms obtained from HPLC method one and two for the piperine solution are presented in Figure 4.7.

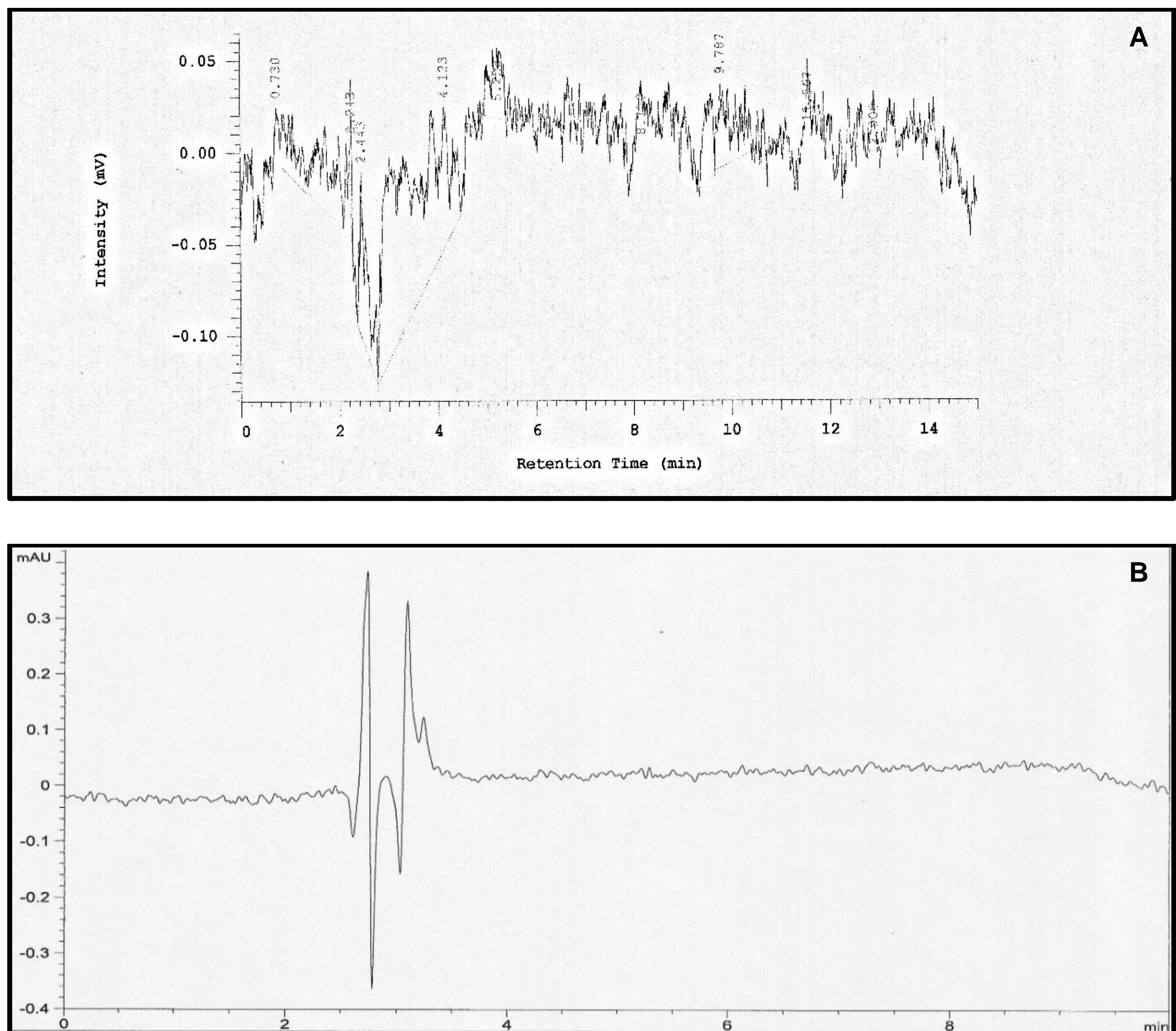


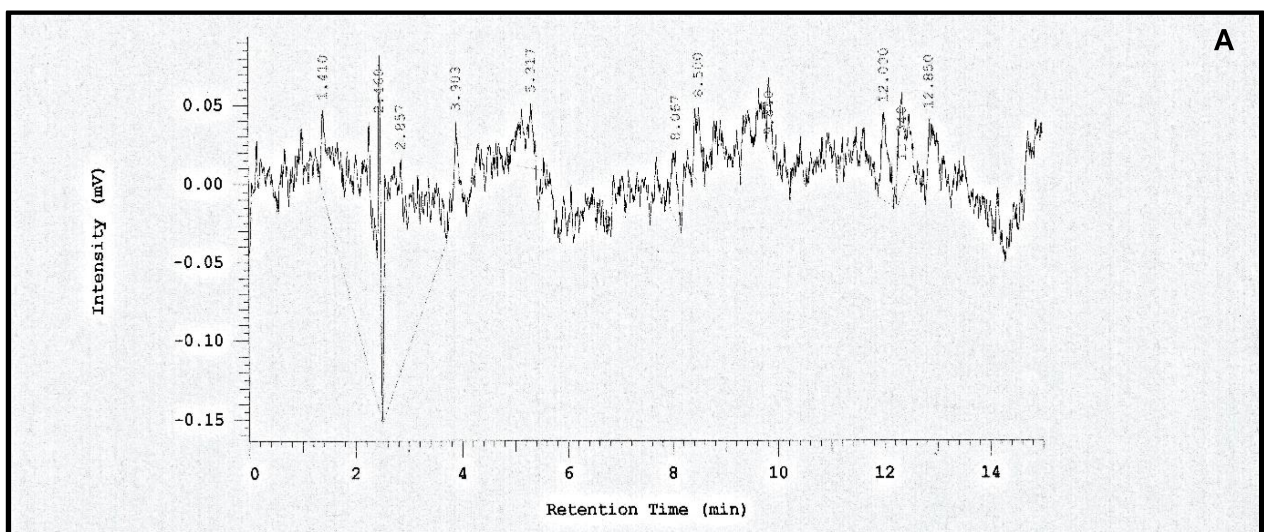
Figure 4-7: Chromatograms illustrating the peaks and retention time of piperine when HPLC method one (A) and method two (B) were used

Comparing the chromatogram in Figure 4.7 (A) with the astaxanthin chromatogram in Figure 4.4 (A) for HPLC method one, it is evident that there was no overlap between the astaxanthin and piperine peaks and retention times. Piperine only produced small peaks that can be considered as backround noise. Thus, piperine could be used as bioenhancer without interfering with HPLC method one.

Comparing the chromatogram in Figure 4.7 (B) with the astaxanthin chromatogram in Figure 4.4 (B) for HPLC method two, no interferences between the peaks of piperine and astaxanthin were found. Small peaks were formed with piperine between two and four minutes but did not interfere with the area or shape of the astaxanthin peak, piperine could thus be used as a bioenhancer in the formulations and studies of astaxanthin using HPLC method two.

4.3.5.5 Pharmacel®

The chromatograms obtained from HPLC method one and two for a Pharmacel® suspension are presented in Figure 4.8.



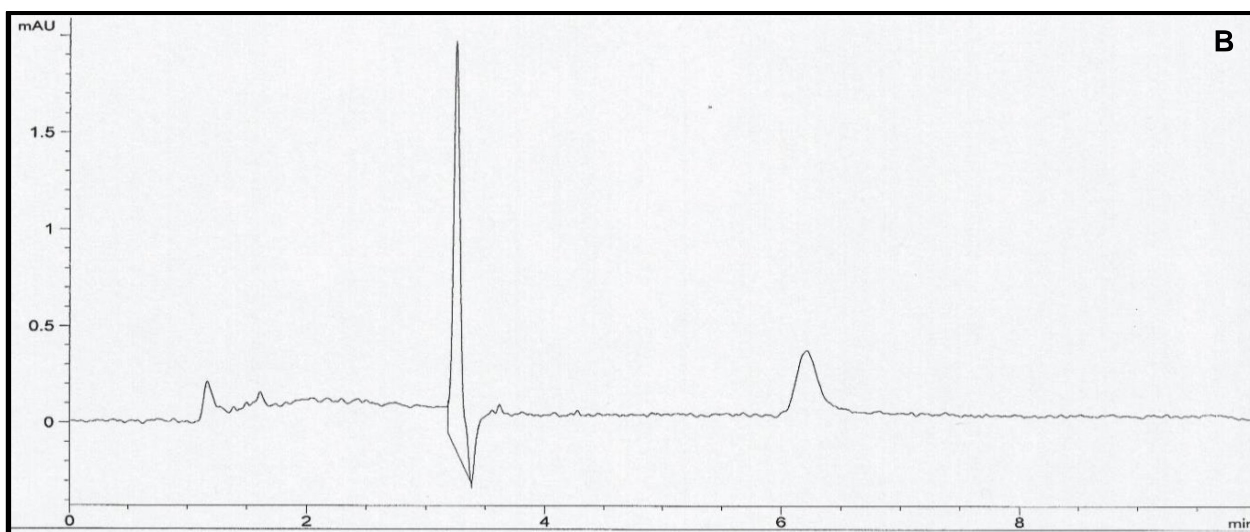


Figure 4-8: Chromatograms illustrating the peak and retention time of Pharmacel® suspension when HPLC method one (A) and method two (B) were used

Comparing the chromatogram in Figure 4.8 (A) with the astaxanthin chromatogram in Figure 4.4 (A) for HPLC method one, it is evident that there was no overlap between the astaxanthin and Pharmacel® peaks and retention times. Pharmacel® only formed small peaks that can be considered as backround noise. Thus Pharmacel® could be used as capsule filler without interfering with HPLC method one.

Comparing the chromatogram in Figure 4.8 (B) with the astaxanthin chromatogram in Figure 4.4 (B) for HPLC method two, no significant interactions between the peaks of Pharmacel® and astaxanthin were found. Small peaks were formed with Pharmacel® between three and four minutes and again after six minutes but did not interfere with the area or shape of the astaxanthin peak. Pharmacel® could thus be used as the capsule filler in the formulations and studies of astaxanthin without interfering with the HPLC method two.

4.3.5.6 FeSSIF

The chromatograms obtained from HPLC method one and two for FeSSIF are presented in Figure 4.9.

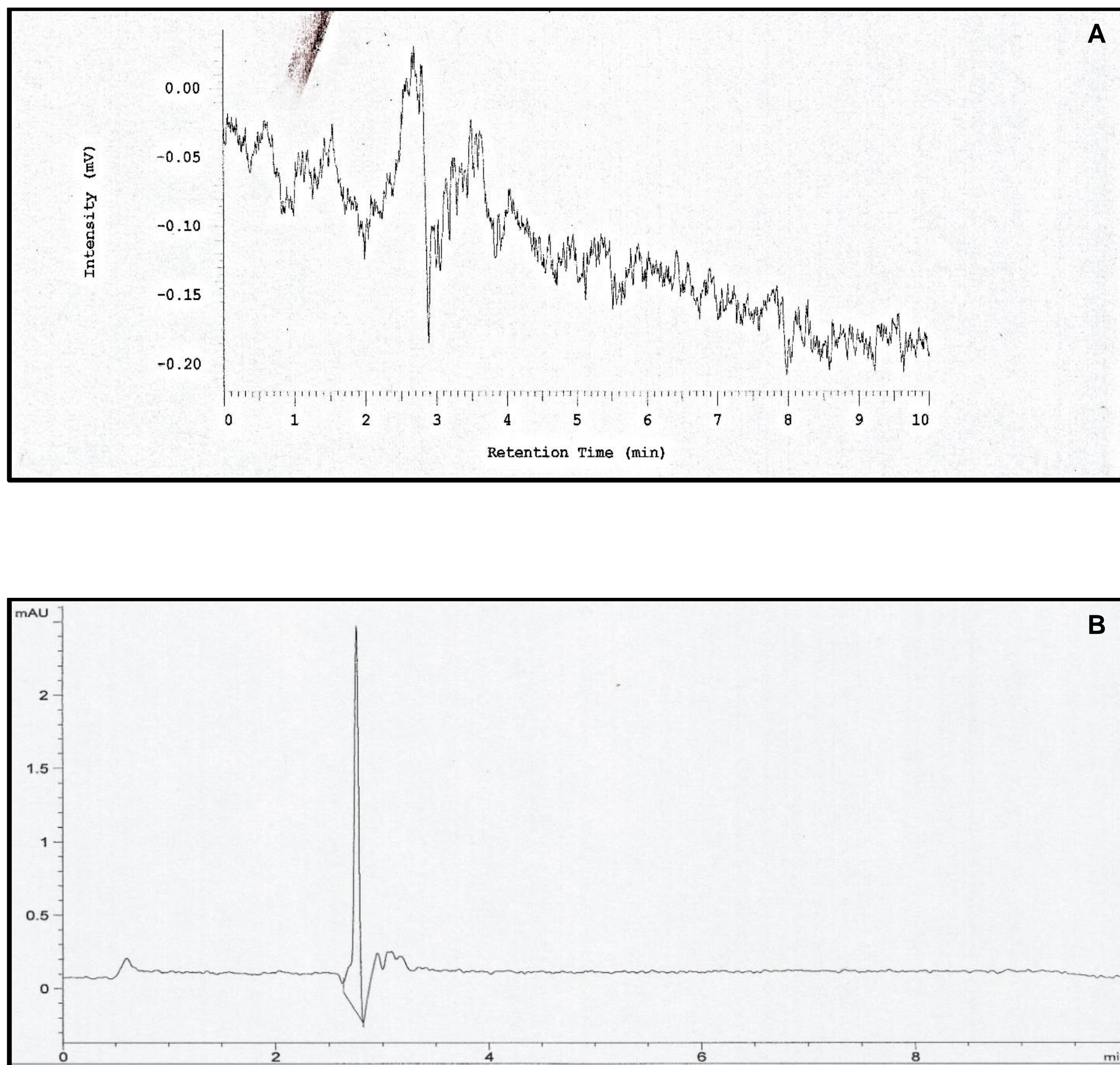


Figure 4-9: Chromatograms illustrating the peaks and retention time of FeSSIF ingredients when using HPLC method one (A) and method two (B)

Comparing the chromatogram in Figure 4.9 (A) with the astaxanthin chromatogram in Figure 4.4 (A) for HPLC method one, it is evident that there was no overlap between the astaxanthin and the FeSSIF solution peaks and retention times. The FeSSIF solution only formed small peaks that can be considered as backround noise. Thus, the FeSSIF solution could be used as a media without interefering with HPLC method one.

Comparing the chromatogram in Figure 4.9 (B) with the astaxanthin chromatogram in Figure 4.4 (B) for HPLC method two, no interefernce between the peaks of the FeSSIF solution and astaxanthin solution were found. Small peaks were formed with the FeSSIF solution between two and four minutes but did not interfere with the area or shape of the astaxanthin peak, FeSSIF could thus be used as a media in studies of astaxanthin using method two.

4.3.5.7 DMSO/KRB solution

The chromatogram obtained from HPLC method two for DMSO/KRB solution is presented in Figure 4.10.

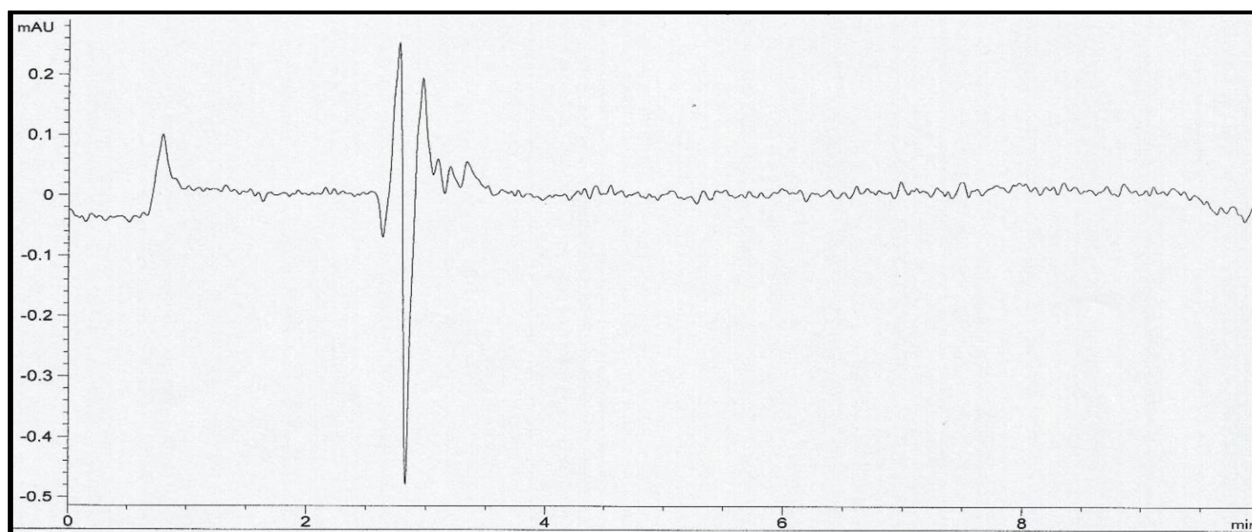


Figure 4-10: Chromatogram illustrating the peaks and retention time of DMSO/KRB solution when HPLC method two was used

Comparing the chromatogram in Figure 4.10 (B) with the astaxanthin chromatogram in Figure 4.4 (B) for HPLC method two, no interference between the peaks of the DMSO/KRB solution and astaxanthin were found. Small peaks were formed with the DMSO/KRB solution between two and four minutes but did not interfere with the area or shape of the astaxanthin peak, the DMSO/KRB solution could thus be used as a media in studies of astaxanthin using method two.

4.3.5.8 Methanol

The chromatogram obtained from HPLC method two for methanol is presented in Figure 4.11.

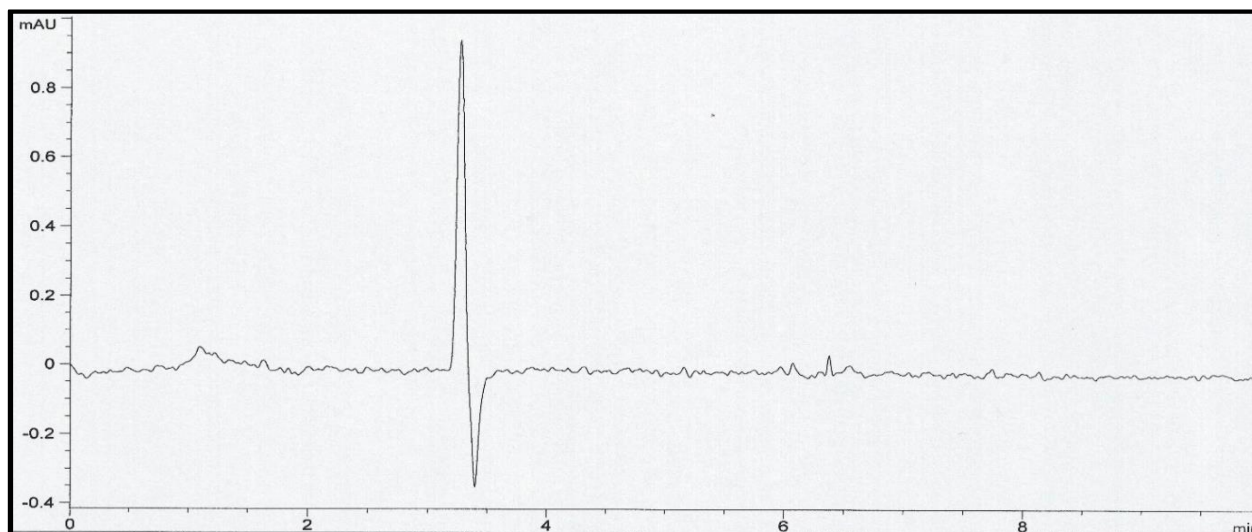


Figure 4-11: Chromatogram illustrating the peak and retention time of methanol when HPLC method two was used

When comparing the chromatogram in Figure 4.11 with the chromatogram of astaxanthin in Figure 4.4 (B), there were no interferences between the peaks and retention times of methanol and astaxanthin. Methanol formed a low intensity peak between 3 and 4 min and the peak could be clearly distinguished from the astaxanthin peak. Methanol could therefore be used in the transport studies of astaxanthin without interfering with the HPLC method used to quantify astaxanthin in the experimental samples.

4.4 Capsule evaluation

4.4.1 Assay

In order to determine the actual concentration of astaxanthin present in the capsule formulations, an assay was conducted on all ten formulations in triplicate and the average astaxanthin concentrations were calculated for each formulation. The results are presented in Table 4.8.

Table 4-8: Percentage astaxanthin content present in each capsule formulation

Astaxanthin formulation	% astaxanthin in sample 1	% astaxanthin in sample 2	% astaxanthin in sample 3	Average % of astaxanthin present in each formulation
1	116.721	114.383	116.879	115.994
2	115.914	125.355	109.699	116.989
3	113.836	119.986	113.643	115.823
4	113.505	113.026	105.760	110.764
5	111.933	111.531	109.171	110.878
6	101.758	110.269	105.940	105.989
7	96.812	96.134	95.608	96.185
8	118.769	120.544	112.093	117.135
9	112.172	117.676	115.895	115.248
10	109.806	114.594	117.346	113.915

4.4.2 Dissolution studies

Figure 4.12 indicates the percentage astaxanthin dissolution presented as a function of time for astaxanthin capsule formulations containing astaxanthin and piperine (formulation 1, 8, 9 and 10).

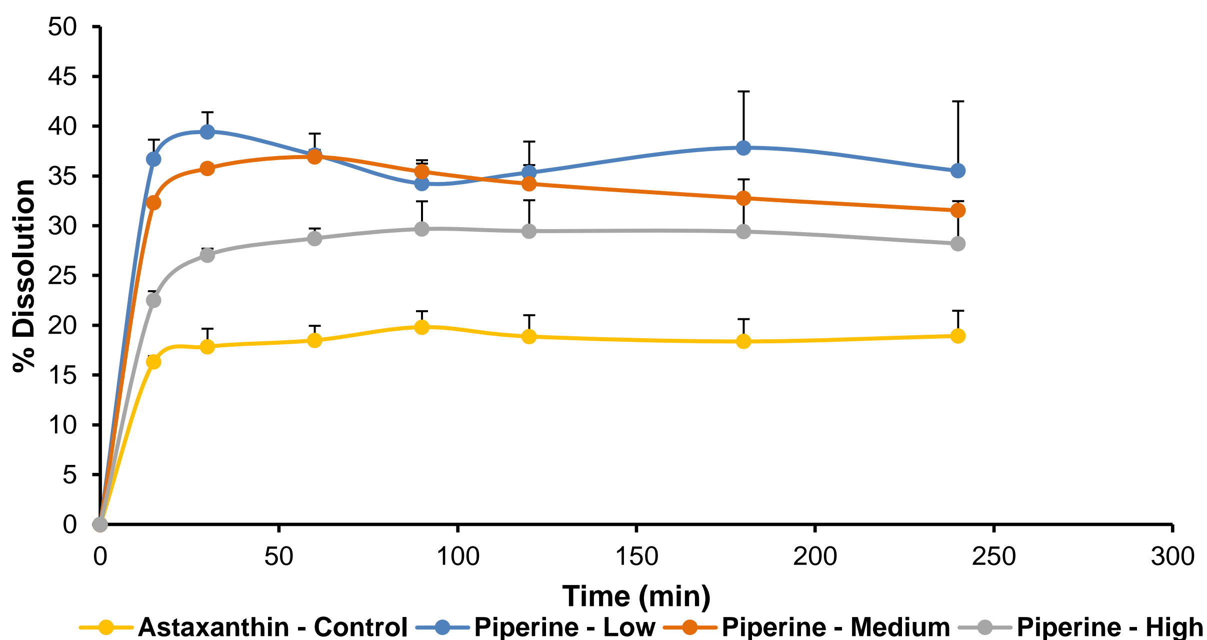


Figure 4-12: Percentage dissolution of astaxanthin from capsule formulations containing astaxanthin and piperine in three different concentrations

Figure 4.12 shows that when piperine was incorporated into the formula, the initial dissolution rate was higher in comparison to the control, which contained only astaxanthin and Pharmacel®. The release profiles of astaxanthin in combination with piperine (Formula 8 – 10) was investigated to help explain the inverse relationship (percentage transport versus piperine concentration) that were observed during the astaxanthin transport studies and it was evident that the addition of piperine to the experimental formulations did mediate an improvement in the dissolution of astaxanthin at all three test concentrations. However, the percentage dissolution of astaxanthin for the high, medium and low concentrations of piperine were 28.21%, 31.53% and 35.52%, respectively, after 240 min. The percentage dissolution showed a concentration dependent decrease in astaxanthin release with increased piperine concentrations.

4.5 Evaluating membrane integrity using Lucifer Yellow

The percentage transport of Lucifer Yellow (LY), an exclusion marker, across excised pig intestinal tissue is illustrated in Figure 4.13. The average transport of LY was 0.73%. This is well below the recommended transport of 3% for intact membranes, as stated by Sigma Aldrich (2013). This is an indication that the membrane integrity of the tissue was maintained over the course of the transport study period of 120 min. The P_{app} values obtained from the transport data is illustrated in Figure 4.14. The average P_{app} was calculated as 5 x 10^{-7} cm/s, which is well below the maximum range of 8.2 – 9.1 x 10^{-7} cm/s (Bhushani *et al.,* 2016).

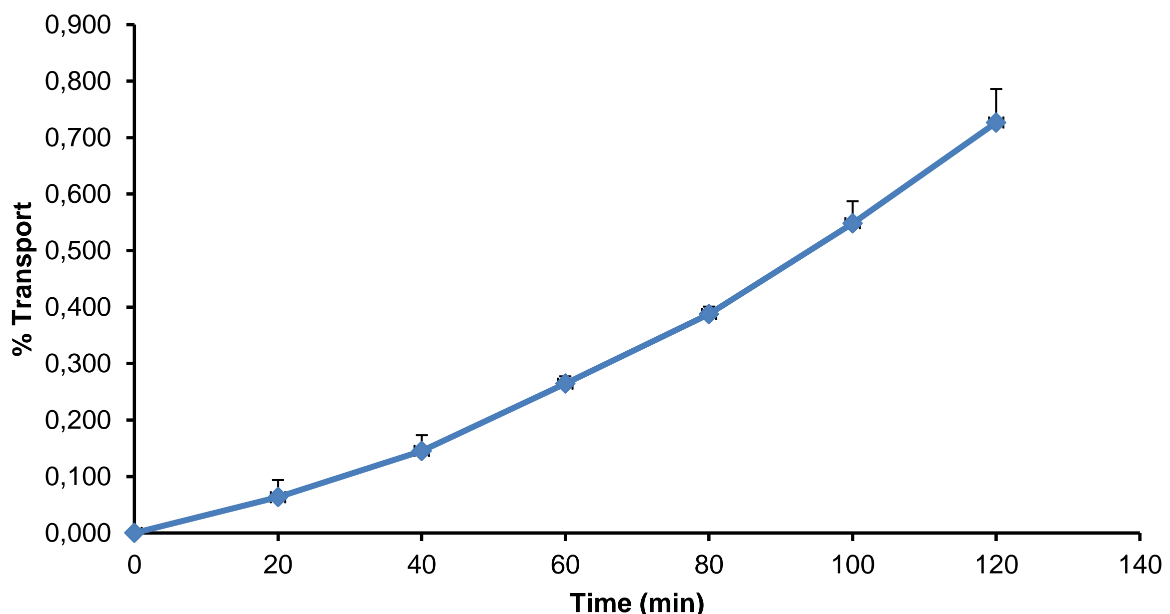


Figure 4-13: Graph illustrating the % transport of Lucifer Yellow over a period of 120 min

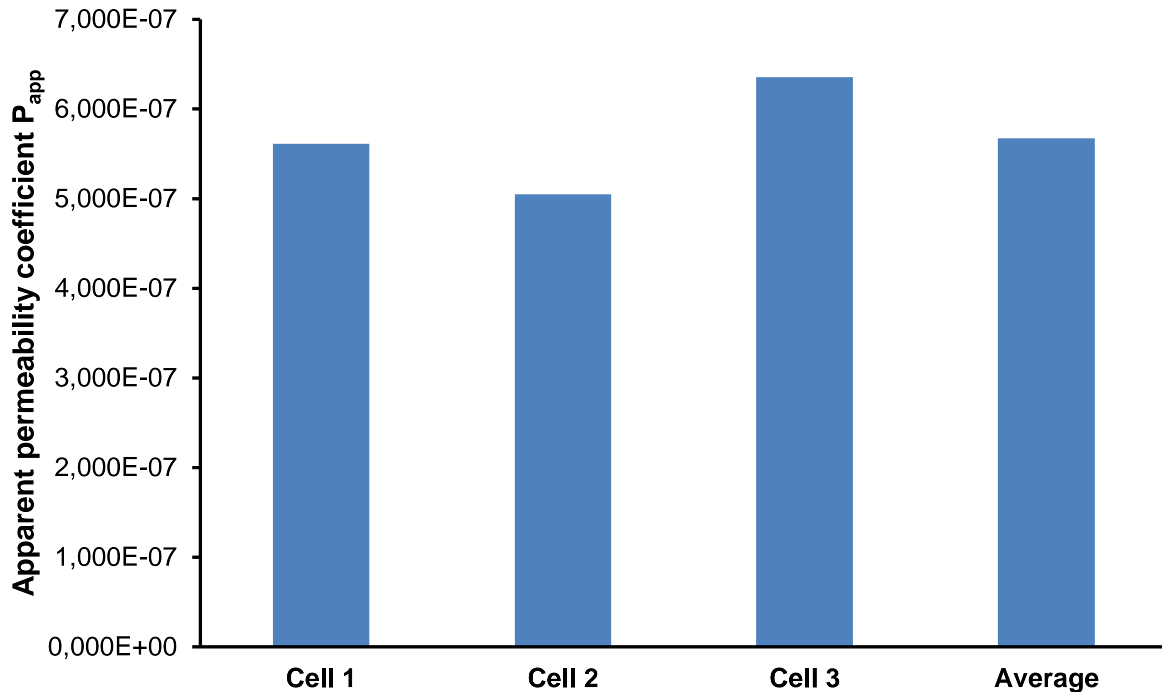


Figure 4-14: Graph illustrating the apparent permeability coefficient values of Lucifer Yellow after the duration of the transport study (120 min)

4.6 Bi-directional transport studies of astaxanthin

4.6.1 Astaxanthin

4.6.1.1 Apical-to-basolateral transport

Figure 4.15 illustrates the percentage astaxanthin transport across excised pig intestinal tissue after application of the capsule formulation consisting of only astaxanthin in combination with Pharmacel® to the apical chamber. This formulation was used as the control formulation since it did not contain any bioenhancing agents. The transport was conducted in the apical-to-basolateral direction across the excised pig intestinal tissue to measure the absorptive transport of astaxanthin alone (without any absorption enhancing agent).

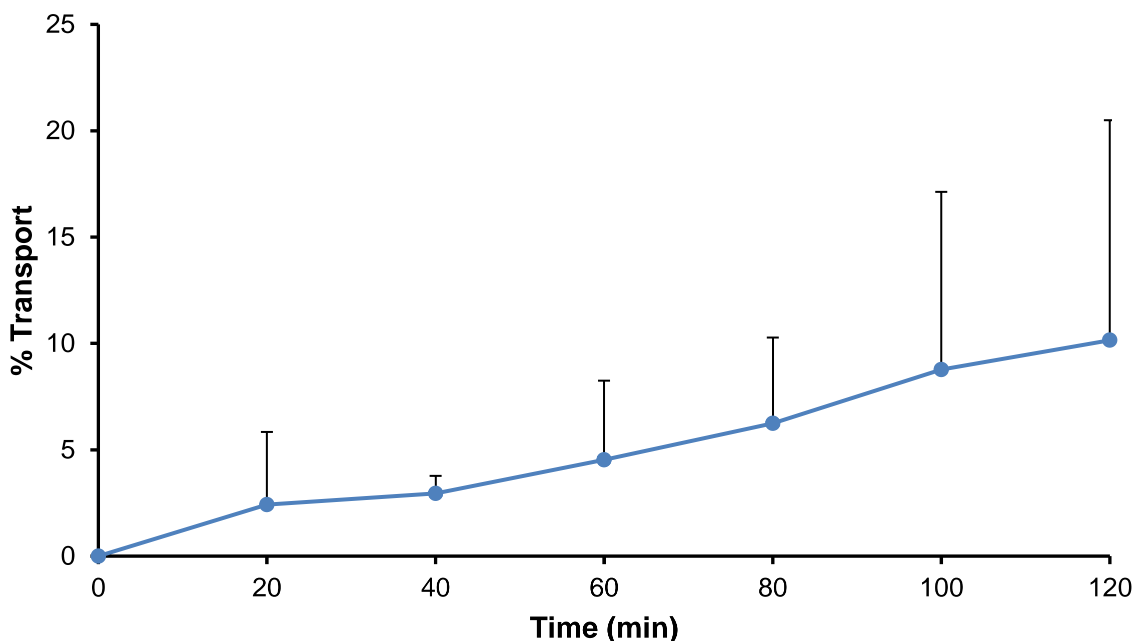


Figure 4-15: Graph illustrating the percentage transport of astaxanthin from the control formulation in the apical-to-basolateral direction

Figure 4.15 shows that the percentage transport of astaxanthin across excised pig intestinal tissue, in the apical-to-basolateral direction (absorptive transport), was 10.149% of the applied dose, over a period of 120 min. The extremely poor water solubility of astaxanthin may have contributed to the relatively limited permeability of astaxanthin, while its lipophilic nature has contributed to good membrane permeability (as opposed to the comparatively extremely low membrane permeability of the hydrophilic compound Lucifer Yellow of less than 1%) (Krishna *et al.*, 2001).

4.6.1.2 Basolateral-to-apical transport

Figure 4.16 illustrates the percentage basolateral-to-apical transport of astaxanthin across excised pig intestinal tissue after the control formulation was added to the basolateral side of the Sweetana-Grass diffusion chamber. This was used to determine the basolateral-to-apical transport (or secretory transport) of astaxanthin.

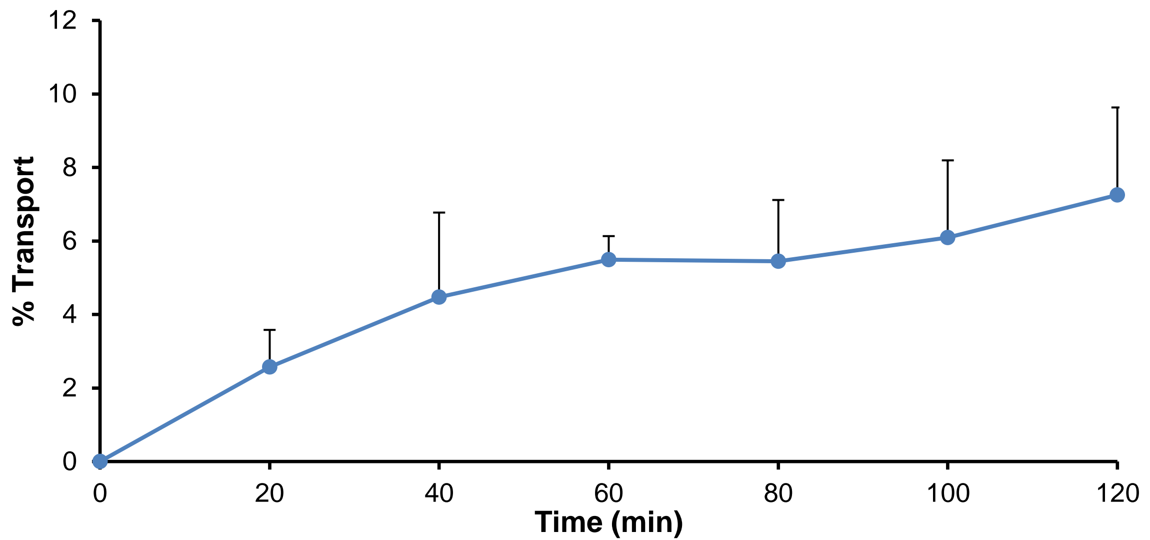


Figure 4-16: Graph illustrating the percentage transport of astaxanthin from the control formulation in the basolateral-to-apical direction

Figure 4.16 shows that when the control formulation was added to the basolateral side of the excised tissue in the Sweetana-Grass diffusion apparatus, an accumulative amount of 7.252% of the applied dose of astaxanthin was present in the acceptor (i.e. apical) chamber after 120 min.

In general, it has been found with the control formulation that the transport of astaxanthin was lower in the secretory direction than in the absorptive direction, which means that no active efflux of asataxanthin occurred.

4.6.2 *Aloe vera* gel

4.6.2.1 Apical-to-basolateral transport

Figure 4.17 illustrates the percentage transport of astaxanthin across excised pig intestinal tissue of three different capsule formulations of astaxanthin in combination with *A. vera* gel in three different concentrations, which was used as bioenhancer in combination with the filler (Pharmacel®).

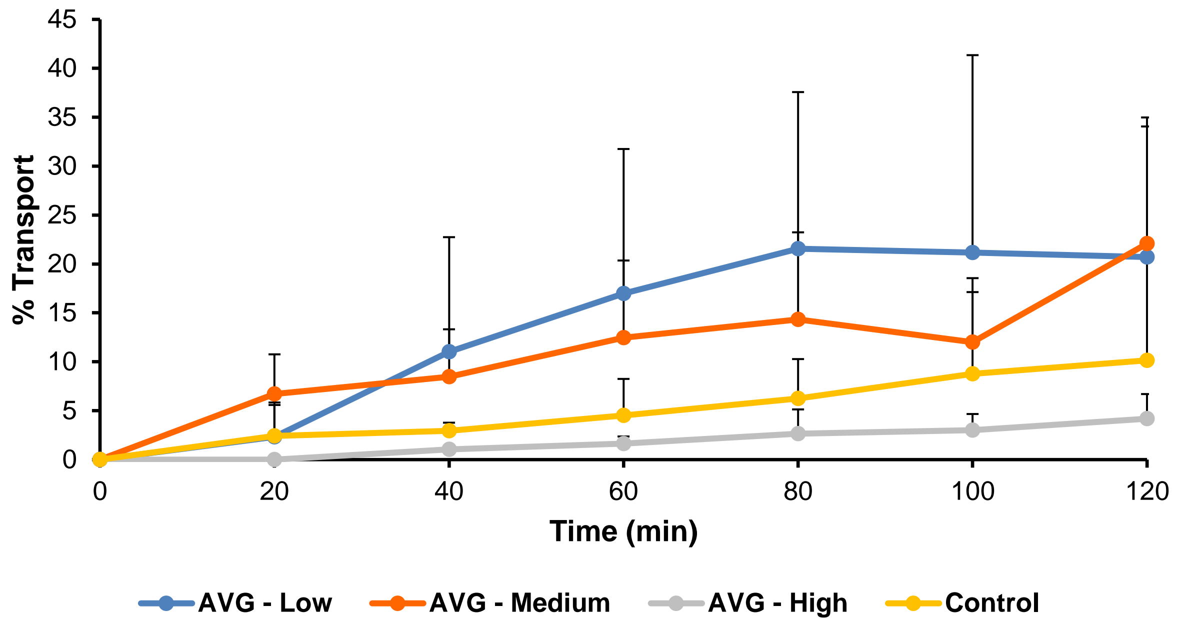


Figure 4-17: Graph illustrating the percentage transport of astaxanthin in the apical-to-basolateral direction across excised pig intestinal tissue in combination with *Aloe vera* gel in comparison to the control formulation. AVG low = *A. vera* gel in lowest dose (10 mg), AVG medium = *A. vera* gel in medium dose (35 mg) and AVG high = *A. vera* gel in highest dose (70 mg)

From Figure 4.17, it can be seen that when astaxanthin was formulated in combination with *A. vera* gel, the rate and extent of astaxanthin transport was improved (low and medium *A. vera* gel concentration) when compared to the control formulation. Cumulative percentage transport values of astaxanthin of 20.710 and 22.080% were obtained with the formulations containing *A. vera* gel in low and medium concentrations, respectively. In comparison, the percentage cumulative astaxanthin transport of the control formulation was 10.149%. The improved transport (in the presence of low and medium *A. vera* gel concentrations) might be explained by the enhanced paracellular transport of astaxanthin due to the ability of *A. vera* gel to open tight junctions between epithelial cells (Beneke *et al.,* 2012; Chen *et al.,* 2009).

However, the highest concentration of *A. vera* gel mediated a decrease in astaxanthin transport (cumulative transport of 4.181%), in comparison with the control formulation (10.1 % cumulative transport). This can possibly be explained by the high viscosity of *A. vera* gel that delayed the release of astaxanthin and also hampered diffusion of astaxanthin molecules to the surface of the membrane as explained by a previously published transport study (Gerber *et al.,* 2019).

The results of the study showed that the addition of *A. vera* gel (at 0.1; 0.5 and 1.0%) can help to improve the membrane transport of astaxanthin. A study conducted by Vinson *et al.* (2005) also

showed that *A. vera* gel increased the absorption of Vitamin E (a fat-soluble vitamin). However, the amount of *A. vera* gel added to formulations should be taken into account, as the high viscosity factor of the *A. vera* gel at higher concentrations can have a negative impact on astaxanthin transport at higher concentrations (1.0%).

4.6.2.2 Basolateral-to-apical transport

Figure 4.18 illustrates the percentage transport of astaxanthin across excised pig intestinal tissues of three different formulations of astaxanthin in combination with *A. vera* gel in three different concentrations, used as bioenhancer in combination with Pharmacel®.

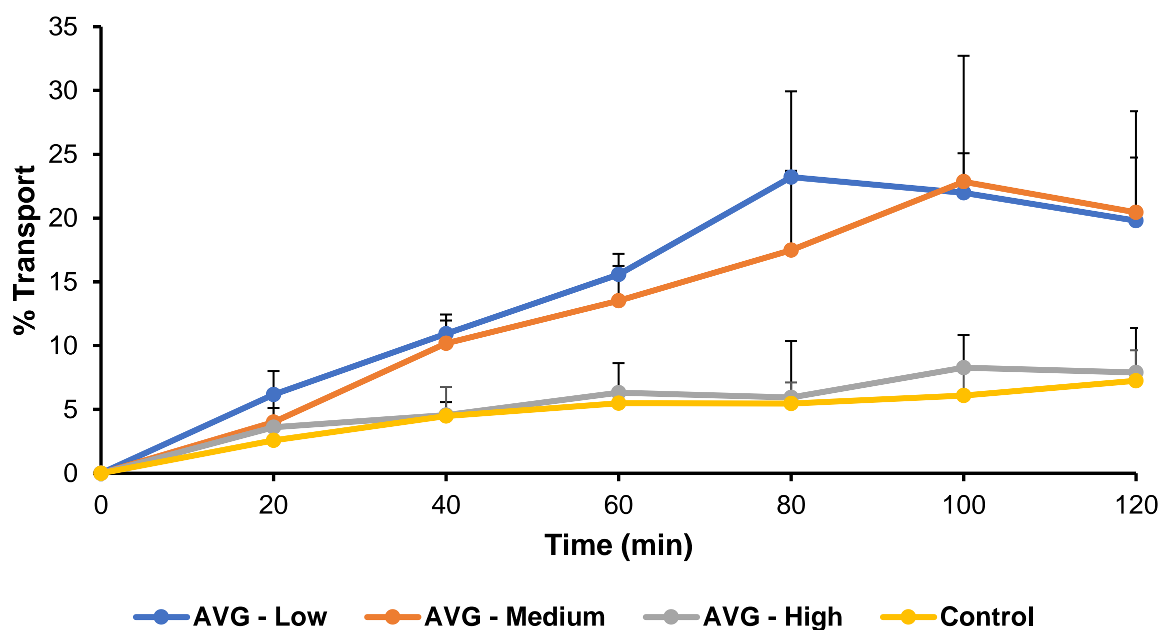


Figure 4-18: Graph illustrating the percentage transport of astaxanthin in the basolateral-to-apical direction across excised pig intestinal tissue in combination with *Aloe vera* gel in comparison to the control formulation. AVG low = *A. vera* gel in lowest dose (10 mg), AVG medium = *A. vera* gel in medium dose (35 mg) and AVG high = *A. vera* gel in highest dose (70 mg)

Figure 4.18 shows that the addition of astaxanthin in combination with *A. vera* gel in varying concentrations (0.1; 0.5 and 1.0%) altered the rate and extent of astaxanthin transport in the secretory direction. The low *A. vera* gel concentration mediated a cumulative astaxanthin transport of 19.786%, while the medium concentration exhibited a cumulative transport value of 20.452%. The high concentration of *A. vera* gel mediated a marginal increase in the extent of astaxanthin transport with a cumulative transport value of 7.904%.

This increased transport of astaxanthin by *A. vera* gel in the basolateral-to-apical direction can also be described by the opening of tight junctions with increased transport of astaxanthin molecules via the paracellular pathway.

4.6.3 *Aloe vera* whole leaf extract

4.6.3.1 Apical-to-basolateral transport

Figure 4.19 illustrates the percentage transport of astaxanthin across excised pig intestinal tissues in the apical–to-basolateral direction after application of three different formulations of astaxanthin in combination with *A. vera* whole leaf extract, in three different concentrations, to the apical chamber.

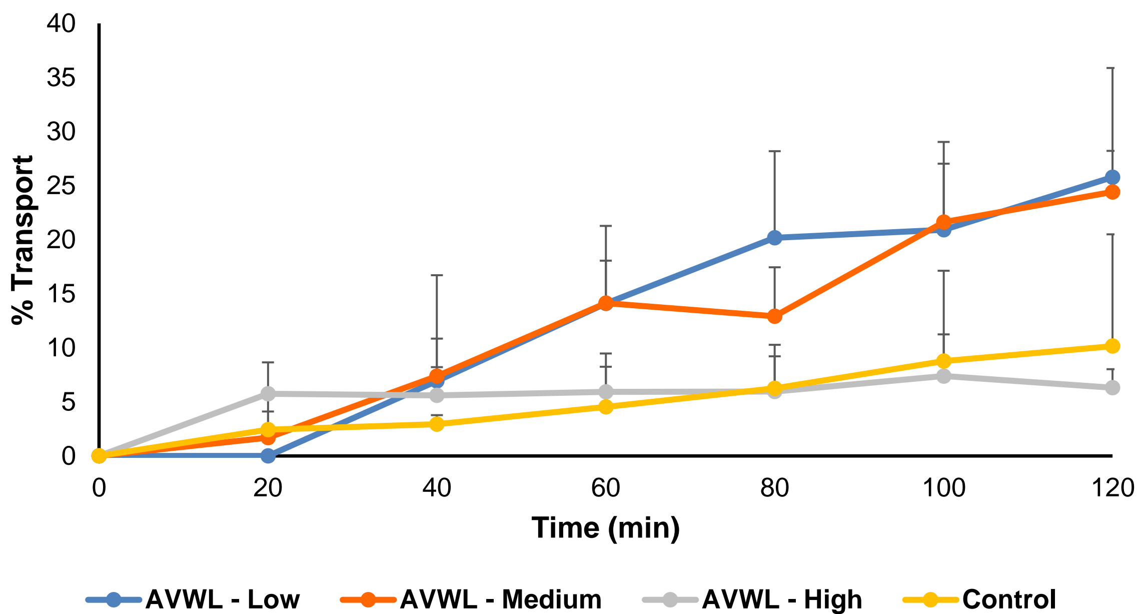


Figure 4-19: Graph illustrating the percentage transport of astaxanthin across excised pig intestinal tissue in the apical-to-basolateral direction in combination with *Aloe vera* whole leaf extract in comparison to the control formulation. AVWL low = *A. vera* whole leaf extract in lowest dose (10 mg), AVWL medium = *A. vera* whole leaf extract in medium dose (35 mg) and AVWL high = *A. vera* whole leaf extract in highest dose (70 mg)

Figure 4.19 shows that the addition of *A. vera* whole leaf extract to the capsule formulations mediated an increase in the rate and extent of astaxanthin transport across the excised pig intestinal tissues. The control formulation exhibited a cumulative transport value of 10.149%, while the low and medium concentrations of *A. vera* whole leaf extract mediated an increase in

astaxanthin transport and exhibited cumulative transport values of 25.780% and 24.417%, respectively. The increase in transport may be explained by the ability of *A. vera* whole leaf extract to open the tight junctions between adjacent epithelial cells to allow for paracellular transport (Beneke *et al.,* 2012; Chen *et al.,* 2009).

The high concentration of *A. vera* whole leaf extract mediated a decrease in astaxanthin transport (6.306% cumulative transport) when compared to the control group (10.149%). This occurrence may possibly be explained by the increase in viscosity of the experimental solution due to the addition of the *A. vera* whole leaf extract, which hampered the diffusion of the astaxanthin molecules (Gerber *et al.,* 2019).

It is evident that the cumulative transport of astaxanthin was increased to a greater extent by *A. vera* whole leaf extract than by *A. vera* gel.

4.6.3.2 Basolateral-to-apical transport

Figure 4.20 illustrates the percentage transport of astaxanthin across excised pig intestinal tissues in the basolateral-to-apical direction after application of three different capsule formulations of astaxanthin in combination with *A. vera* whole leaf extract in three different concentrations.

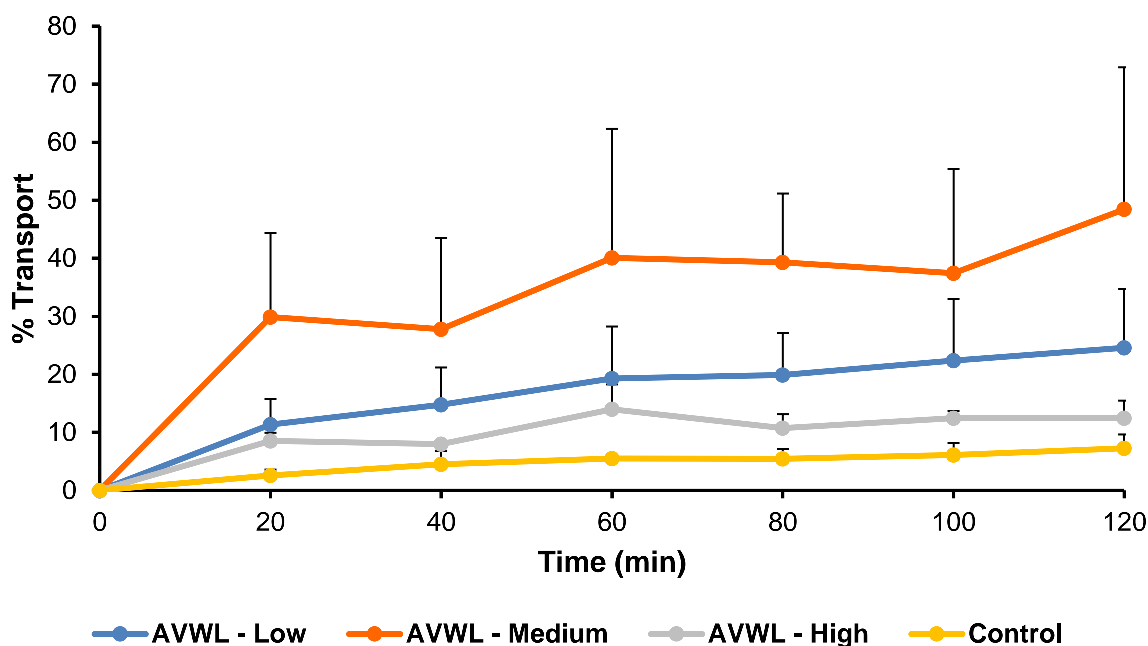


Figure 4-20: Graph illustrating the percentage transport of astaxanthin across excised pig intestinal tissue in the basolateral-to-apical direction in combination with *Aloe vera* whole leaf extract in comparison to the control formulation. AVWL low = *A. vera* whole leaf extract in lowest dose (10 mg), AVWL medium = *A. vera* whole leaf extract in medium dose (35 mg) and AVWL high = *A. vera* whole leaf extract in highest dose (70 mg)

Figure 4.20 depicts the percentage transport of astaxanthin in the secretory direction when combined with *A. vera* whole leaf extract. The results show that the low and medium *A. vera* whole leaf extract concentrations had mediated increased transport of astaxanthin (24.572% and 48.424% cumulative transport, respectively) to a greater extent than the high *A. vera* whole leaf extract concentration (12.447%). The extent of astaxanthin transport was very similar for both the control formulation and the high *A. vera* whole leaf extract concentration.

4.6.4 Piperine

4.6.4.1 Apical-to-basolateral transport

Figure 4.21 illustrates the percentage transport of astaxanthin across excised pig intestinal tissues in the apical-to-basolateral direction after application of three different capsule formulations of astaxanthin in combination with piperine, in three different concentrations.

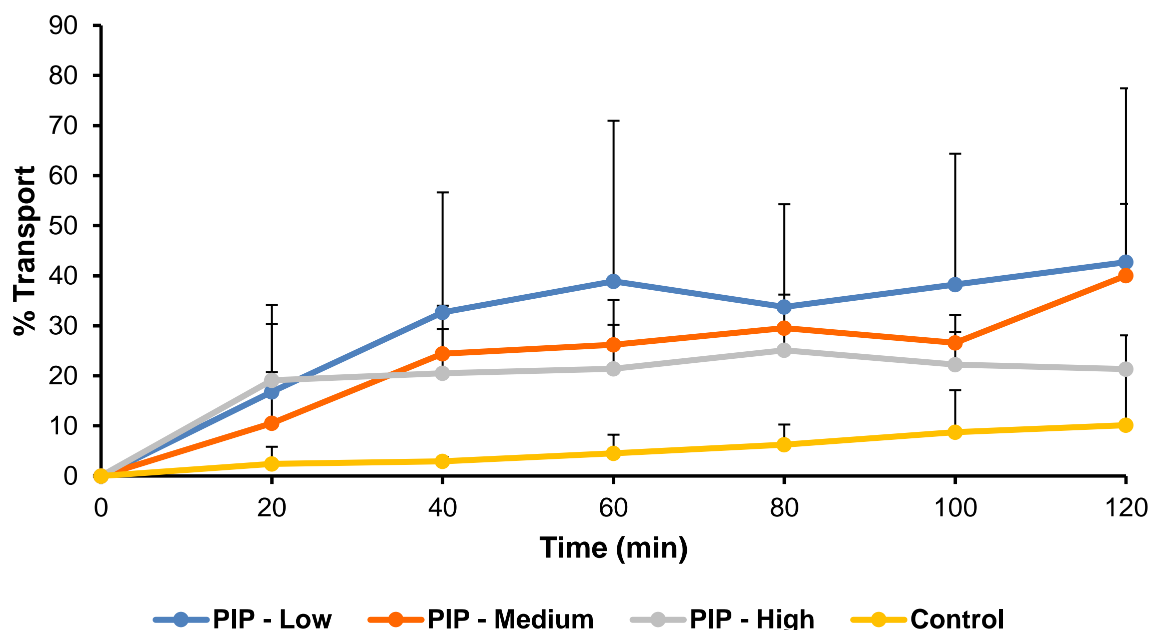


Figure 4-21: Graph illustrating the percentage transport of astaxanthin across excised pig intestinal tissue in the apical-to-basolateral direction in combination with piperine in comparison to the control formulation. PIP low = piperine in lowest dose (0.299 mg), PIP medium = piperine in medium dose (0.399 mg) and PIP high = piperine in highest dose (0.499 mg)

Figure 4.21 illustrates the the effect of piperine, as a bioenchancer, on the percentage transport of astaxanthin across excised pig intestinal tissue. When compared to the control formulation (10.15% cumulative transport), the lowest concentration of piperine showed the largest increase in astaxanthin transport (42.71% cumulative transport). The percentage transport of astaxanthin was similar at the medium piperine concentration, which yielded a cumulative transport value of 40.02%, while the highest piperine concentration yielded a cumulative transport value of 21.33%. The increase in astaxanthin transport can be explained by the absorption enhancing mechanisms that have been identified for piperine. Piperine has been shown to alter membrane dynamics (Khajuria *et al.,* 2002).

However, the percentage astaxanthin transported was inversely proportional to the piperine concentration included in the capsule formulations. This phenomenon was also reported by Gerber *et al.*, (2019) when piperine was investigated as drug absorption enhancer across nasal epithelial membranes.

4.6.4.2 Basolateral-to-apical transport

Figure 4.22 illustrates the percentage transport of astaxanthin across excised pig intestinal tissues in the basolateral-to-apical direction after application of three different capsule formulations of astaxanthin in combination with piperine in three different concentrations.

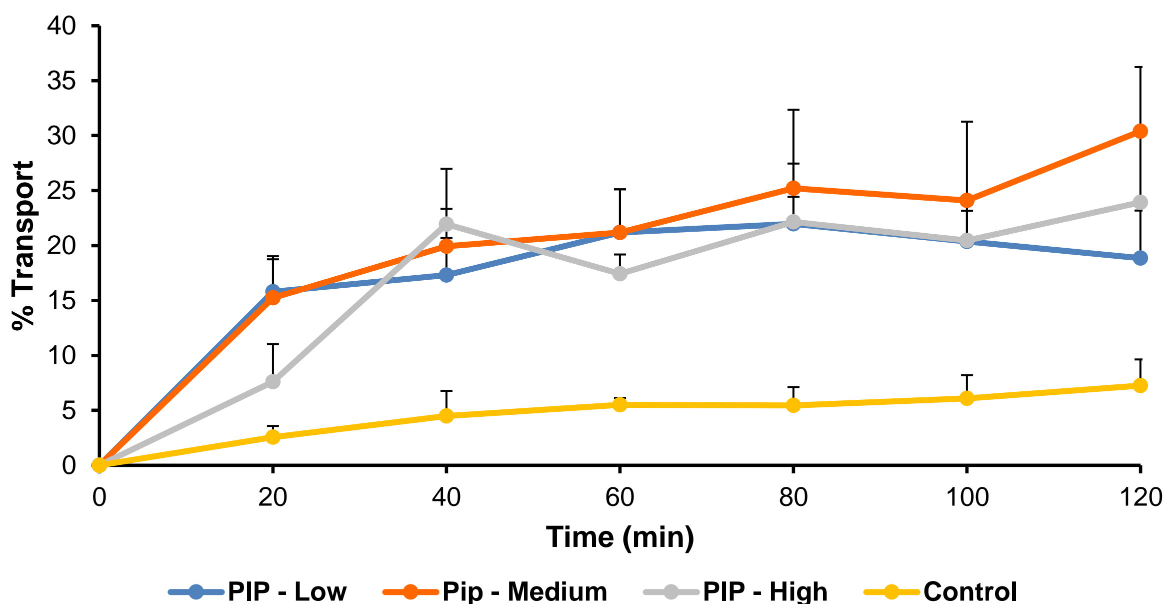


Figure 4-22: Graph illustrating the percentage transport of astaxanthin across excised pig intestinal tissue in combination with piperine formulations in comparison to the control formulation. PIP low = piperine in lowest dose (0.299 mg), PIP medium = piperine in medium dose (0.399 mg) and PIP high = piperine in highest dose (0.499 mg)

The percentage transport of astaxanthin with the medium and low concentrations of piperine was 30.399 and 18.847%, respectively, showing an increase in the transport compared to the control group. The highest piperine concentration used in the formulations with astaxanthin presented an increase in the percentage transport of astaxanthin with a cumulative transport value of 23.928%.

4.6.5 Comparison of astaxanthin permeability across excised pig intestinal tissue

The apparent permeability coefficient (P_{app}) values for astaxanthin were calculated for each formulation and type of bioenhancer investigated. The following figures (Figure 4.23 - 4.25) show the P_{app} values for astaxanthin from all ten capsule formulations in two directions.

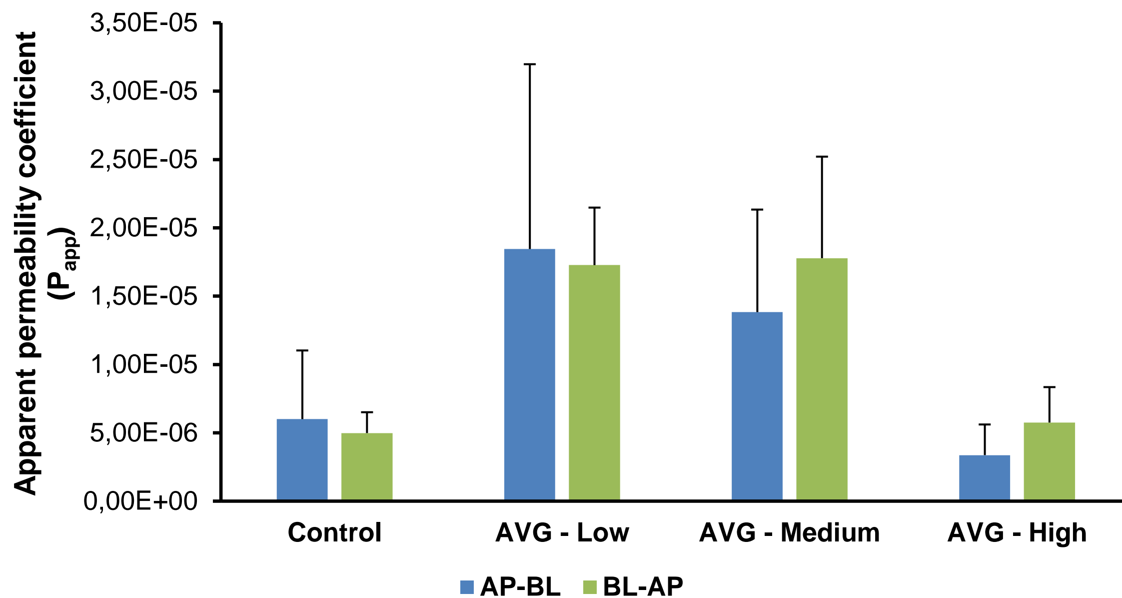


Figure 4-23: Graph comparing the apparent permeability coefficient values of astaxanthin when formulated with *Aloe vera* gel in comparison with the control group in both transport directions, apical-to-basolateral and basolateral-to-apical. AVG low = *A. vera* gel in lowest dose (10 mg), AVG medium = *A. vera* gel in medium dose (35 mg) and AVG high = *A. vera* gel in highest dose (70 mg)

When comparing the apical-to-basolateral and basolateral-to-apical transport of astaxanthin in the control group, the basolateral-to-apical transport was slightly lower than the apical-to-basolateral transport, showing that there was no efflux of astaxanthin. Both the apical-to-basolateral and basolateral-to-apical transport of astaxanthin increased in the presence of the low and medium concentration of *A. vera* gel. The high concentration of *A. vera* gel showed the lowest P_{app} values for astaxanthin in both the apical-to-basolateral and basolateral-to-apical direction. The potential for *A. vera* gel to enhance the permeability of astaxanthin can be explained by the tight junction opening mechanicm of *A. vera* gel thus, enhanced pracellular transport. There was a reduction in the TEER values (data not shown), that is an indicator that the tight junctions between adjacent epithelial cells were opened and the paracaellular transport did increase (Beneke *et al.,* 2012; Chen *et al.,* 2009). A study done by Gerber *et al.,* (2019) explained that higher concentrations of *A. vera* gel have presented higher viscosity values, thus,

possibly explaining the decrese in transport of the higher concentration of *A. vera* gel due to reduction in diffusion rate of the astaxanthin molecules. According to results from the ANOVA statistical analysis, there were no significant statistical differences between the astaxanthin P_{app} values in the control group and the astaxanthin P_{app} values in the *A. vera* gel formulations.

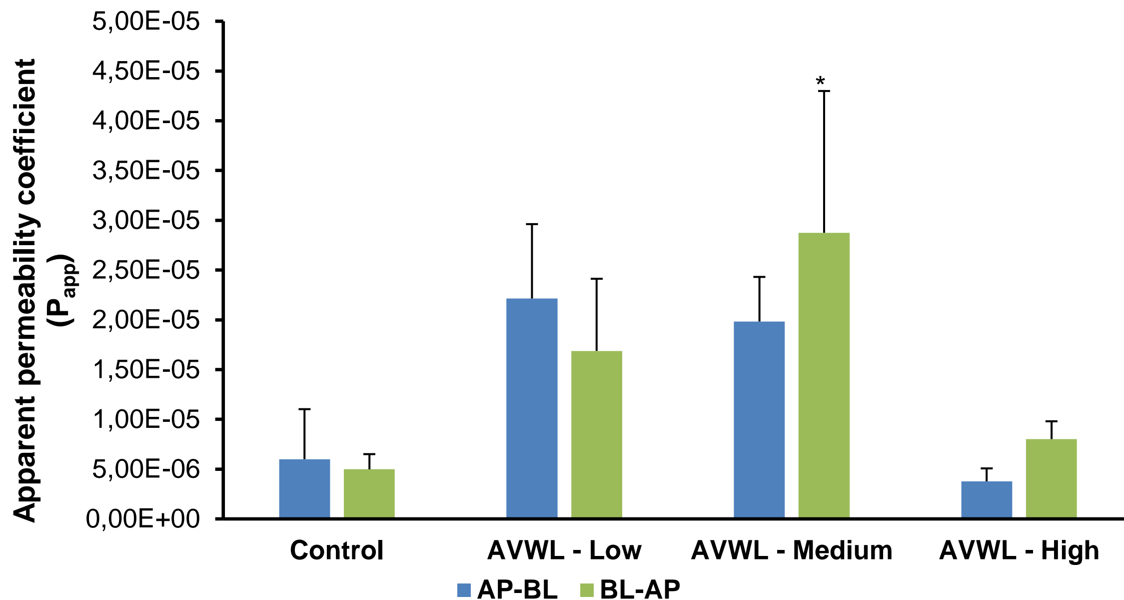


Figure 4-24: Graph comparing the apparent permeability coefficient values of astaxanthin when mixed with *Aloe vera* whole leaf extract, in comparison with the control group in both the transport directions. Apical-to-basolateral and basolateral-to-apical. AVWL low = *A. vera* whole leaf extract in lowest dose (10 mg), AVWL medium = *A. vera* whole leaf extract in medium dose (35 mg) and AVWL high = *A. vera* whole leaf extract in highest dose (70 mg)

Both the apical-to-basolateral and basolateral-to-apical direction presented an increase in the transport of astaxanthin with the low concentration of *A.vera* whole leaf extract, the medium concentration also presented an increase in transport in both directions, although the basolateral-to-apical direction presented an even higher degree of transport than in the apical-to-basolateral direction. The highest concentration presented a decrease in transport in the apical-to-basolateral direction and in the basolateral-to-apical direction it was also lower than the other concentrations that were tested but slightly higher than the control group. *A. vera* whole leaf extract has the ability to open the tight juctions between adjacent epithelial cells and increase the paracellular transport of astaxanthin, which might possibly explain the increase in astaxanthin permeation (Beneke *et al.,* 2012; Chen *et al.,* 2009). According to results from the ANOVA statistical analysis, there were no statistically significant differences between the control group and the *A. vera* whole leaf extract formulations. However, there was a statistically significant

difference between the control group and the formulation containing the medium concentration *A. vera* whole leaf extract in the basolateral-to-apical direction.

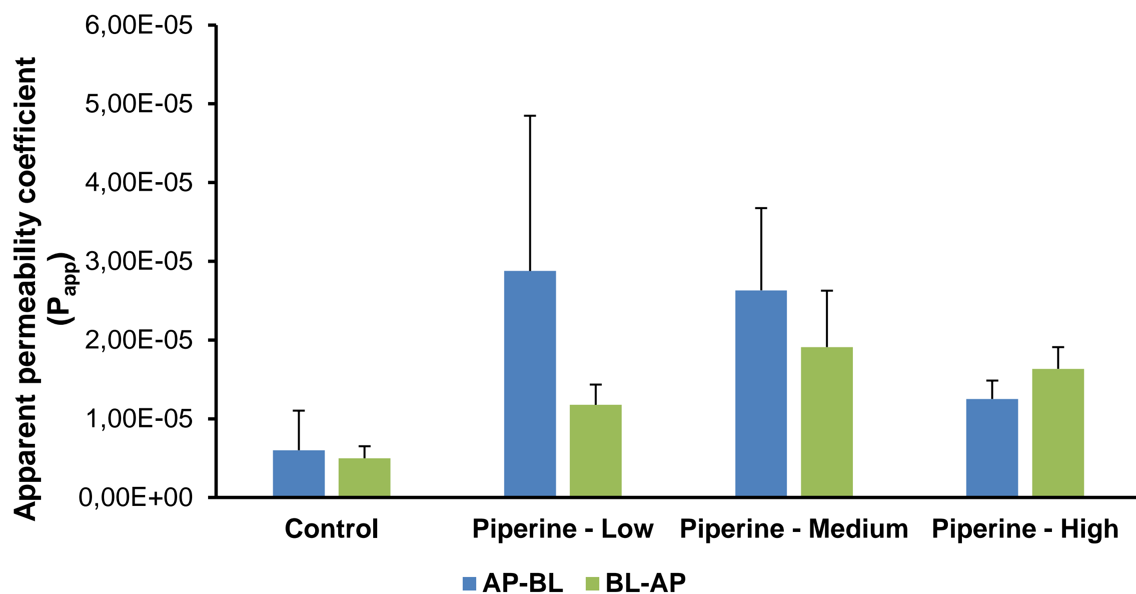


Figure 4-25: Graph comparing the apparent permeability coefficient values of astaxanthin when mixed with piperine in comparison with the control group in both transport directions. Apical-to-basolateral and basolateral-to-apical. PIP low = piperine in lowest dose (0.299 mg), PIP medium = piperine in medium dose (0.399 mg) and PIP high = piperine in highest dose (0.499 mg)

The apical-to-basolateral transport of astaxanthin decreased as the concentration of piperine increased. This phenomenon was also observed during a study by Gerber *et al.* (2019), where piperine was investigated as a bioenhancer to increase the permeation of compounds across nasal epithelial membranes. The dissolution data in this study indicated that the highest concentration piperine presented the lowest amount of astaxanthin release, which may explain the inversely proportional transport behaviour of astaxanthin as a function of piperine concentration.

Both the apical-to-basolateral and basolateral-to-apical directions presented an increase in astaxanthin transport. However, as the concentration of the bioenhancer increased, the opposite effect was seen with the transport. The increase in astaxanthin transport can be explained by the mechanisms of action that has been identified for piperine's membrane permeation enhancement effects. Piperine has membrane altering dynamics and may also open the tight junctions between adjacent epithelial cells (Khajuria *et al.,* 2002). Additionally, it is possible that Piperine had formed complexes with the drugs or compounds to help the added compound to reach the target site (Wadhwa *et al.,* 2014). According to results from the ANOVA statistical analysis, there were no

statistically significant differences between the transport of astaxanthin in the control group when compared to the piperine formulations.

4.6.6 Retention studies

Retention studies were performed in order to determine how much astaxanthin was taken up and retained in the excised pig intestinal tissues after the transport studies were conducted.

4.6.6.1 Percentage astaxanthin retained in the excised pig intestinal tissue when formulated in combination with *Aloe vera* gel

The percentage of astaxanthin that remained in the excised pig intestinal tissues after exposure to the formulations containing *A. vera* gel is shown in Figure 4.26.

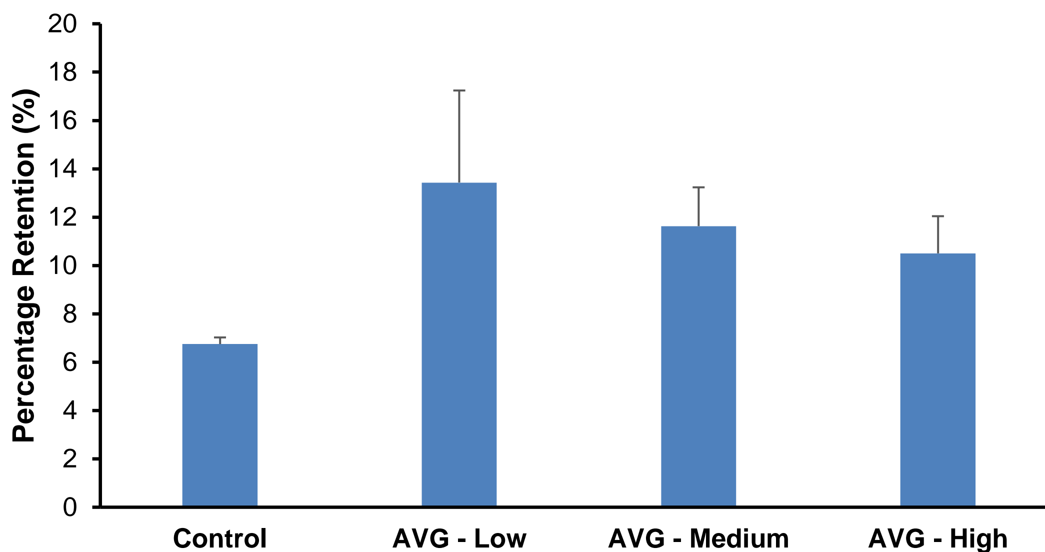


Figure 4-26: Graph illustrating the percentage retention of astaxanthin in combination with *Aloe vera* gel when compared to the control group. AVG low = *A. vera* gel in lowest dose (10 mg), AVG medium = *A. vera* gel in medium dose (35 mg) and AVG high = *A. vera* gel in highest dose (70 mg)

Figure 4.26 shows that the retention of astaxanthin in the control group, was 6.75%, while the formulation containing the low concentration A. *vera* gel presented the highest percentage retention of 13.43%. The percentage retention decreased as the concentration of the *A. vera* gel increased (medium – 11.63% and high – 10.50%). The *A. vera* gel thus increased the amount of astaxanthin retained in the membrane compared to the control, although the extent of retention had decreased with an increase in the concentration of *A. vera* gel. This data correlates with the

apical-to-basolateral transport data of astaxanthin combined with *A. vera* gel. This can most likely be explained by an increase in viscosity as the concentration increases (Gerber *et al.,* 2019).

4.6.6.2 Percentage astaxanthin retained in the excised pig intestinal tissue when formulated in combination with *Aloe vera* whole leaf extract

The percentage of astaxanthin that remained in the excised pig intestinal tissues after exposure to the formulations containing *A. vera* whole leaf extract is shown in Figure 4.27.

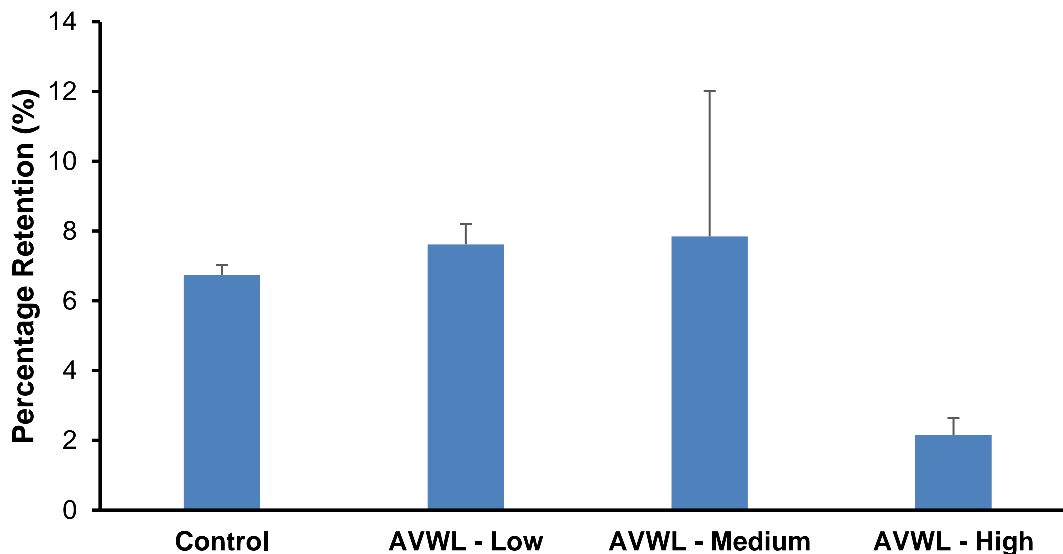


Figure 4-27: Graph illustrating the percentage retention of astaxanthin in combination with *Aloe vera* whole leaf extract when compared to the control group. AVWL low = *A. vera* whole leaf extract in lowest dose (10 mg), AVWL medium = *A. vera* whole leaf extract in medium dose (35 mg) and AVWL high = *A. vera* whole leaf extract in highest dose (70 mg)

When looking at the different concentrations of *A. vera* whole leaf extract added to the different formulations, the medium concentration of *A.vera* whole leaf extract presented the highest percentage retention of astaxanthin (7.84%), whereas the highest concentration of *A.vera* whole leaf extract presented the lowest percentage retention (2.15%), which was even lower than the control group. The lowest concentration of *A. vera* whole leaf extract presented a retention percentage of 7.62%, which was slightly lower than the percentage astaxanthin retained when combined with a medium concentration of *A. vera* whole leaf extract.

4.6.6.3 Percentage astaxanthin retained in the excised pig intestinal tissue when formulated in combination with piperine

The percentage of astaxanthin that remained in the excised pig intestinal tissues after exposure to the formulations containing piperine is shown in Figure 4.28.

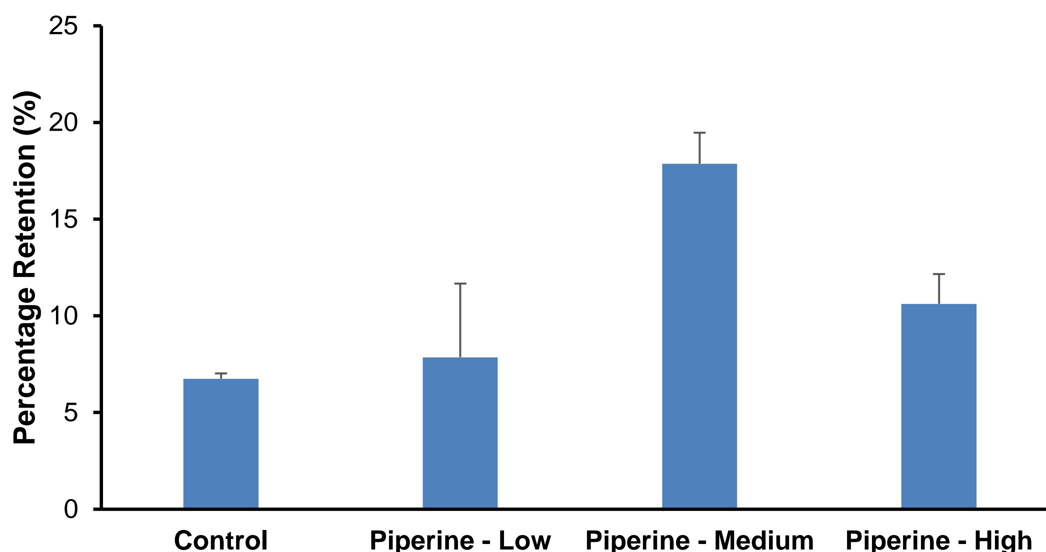


Figure 4-28: Graph illustrating the percentage retention of astaxanthin in combination with piperine when compared to the control group. PIP low = piperine in lowest dose (0.299 mg), PIP medium = piperine in medium dose (0.399 mg) and PIP high = piperine in highest dose (0.499 mg)

The control group presented a retention percentage of 6.75% for astaxanthin, whereas the low, medium, and high concentrations of piperine yielded astaxanthin retention of 7.86%, 17.86%, and 10.62%, respectively. The medium concentration of piperine therefore presented the highest percentage retention of astaxanthin and the lowest concentration of piperine the lowest percentage retention. All three concentrations of piperine presented a higher percentage retention of astaxanthin, when compared to the control group.

4.7 Conclusion

The analytical methods used to measure the concentration of astaxanthin in the different studies complied with the validation specifications of linearity, precision, limit of detection, limit of quantification and specificity. Different formulations were made and assayed to determine the amount of astaxanthin present in each formulation. The dissolution studies of the formulations showed that the percentage of astaxanthin released was inversely proportional to the piperine concentration. The membrane integrity was evaluated using Lucifer Yellow, and was maintained

through the experimental studies. All the formulations, except the formulation containing the highest concentration of *A. vera* gel, showed an increase in the transport of astaxanthin across the excised pig intestinal tissues, albeit not statistically significant. Previous studies have shown that *A.vera* gel, *A.vera* whole leaf extract and piperine can be used to enhance the permeability of drugs across the epithelia of intestinal tissue (Beneke *et al.,* 2012; Chen *et al.,* 2009; Vinson *et al.,* 2005) and that piperine can be used to inhibit efflux of drugs and open tight junctions (Gerber *et al.,* 2019; Wadhwa *et al.,* 2014). The results obtained from this study confirmed the permeability enhancement properties of the three selected bioenhancers. However, the concentrations used for permeability enhancement of astaxanthin should be further evaluated, as differing results may be obtained with some of the bioenhancers. For this study, piperine proved to have the largest effect on the transport enhancement of astaxanthin. The highest concentration of piperine had the lowest effect on astaxanthin transport and the lowest concentration of piperine had the largest effect, corresponding with the findings of the dissolution studies. This study has made a positive contribution in finding a safe and effective way to enhance the transport of astaxanthin, although more studies are recommended to determine the effectiveness in the bio-enhancement of astaxanthin.

CHAPTER 5: FINAL CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE STUDIES

5.1 Final conclusion

The main aim of this study was to compound capsules containing astaxanthin in combination with selected bioenhancers to potentially improve the extent of membrane permeability of astaxanthin. The selected bioenhancers (i.e. *Aloe vera* gel, *Aloe vera* whole leaf extract and piperine) were each added to the experimental formulations in three different concentrations, high, medium and low, and made up to weight using Pharmacel® as a filler. The powder ingredients were mixed and added to empty capsule shells. The astaxanthin content of each capsule formulation was determined by means of high-performance liquid chromatography (HPLC). Additionally, the dissolution/drug release of astaxanthin from the piperine containing formulations were also evaluated to aid in the elucidation of the transport results.

The dissolution studies regarding the piperine containing formulations revealed that the addition of piperine had mediated an increase in the percentage dissolution of astaxanthin, in comparison to the control formulation, at all three piperine concentrations that were evaluated. However, an inverse relationship was evident in terms of the pipirine concentration versus the extent of dissolution improvement, which means the low piperine concentration exhibited the most pronounced increase in astaxanthin release/dissolution.

The results of the *ex vivo* transport studies showed that the formulations containing the selected bioenhancers increased the transport of astaxanthin across the excised pig intestinal tissues at specific concentrations in both the apical-to-basolateral and basolateral-to-apical directions. However, the addition of a high concentration of either *A. vera* gel or *A. vera* whole leaf extract to the experimental formulations had an impeding effect on the extent of astaxanthin transport in the apical-to-basolateral direction. Both *A. vera* gel and *A. vera* whole leaf extract presented an inverse relationship with the transport of astaxanthin, showing a concentration dependant decrease in transport with an increase in the concentration of these bioenhancers. The basolateral-to-apical transport of astaxanthin in combination with *A. vera* gel presented an increase in the transport of astaxanthin with all three concentrations used, however, the highest concentration presented the lowest percentage transport. Similar results were obtained with the basolateral-to-apical transport of astaxanthin in combination with *A. vera* whole leaf extract. In this case, the highest concentration *A. vera* whole leaf extract also presented the lowest percentage astaxanthin transport. The results also showed that the addition of piperine to the experimental formulations exerted an inverse relationship between the extent of astaxanthin

transport and the piperine concentration included in the formulations. This means that the lowest piperine concentration exhibited the most pronounced improvement on astaxanthin transport in the apical-to-basolateral transport. The inverse relationship between the piperine concentration versus the extent of transport improvement correlates well with the results acquired from the dissolution evaluation of the piperine containing formulations. The extent of astaxanthin transport was higher than that of the control formulation at all three pipirine concentrations that were tested in the apical-to-basolateral direction. The basolateral-to-apical direction showed the transport of astaxanthin improved with all three concentrations of piperine. The transport data of the formulations containing the bioenhancers did not prove statistically significant when compared to the control group, except for the formulation containing the medium concentration *A. vera* whole leaf extract in the basolateral-to-apical direction. However, the results revealed that the selected bioenhancers have pronounced effects on the transport of astaxanthin across excised pig intestinal tissues.

5.2 Future recommendations

The results from this study showed that intestinal absorption of astaxanthin may be improved with the addition of selected bioenhancers to experimental capsule formulations, however, additional future studies are recommended. Possible future studies include:

- Toxicity studies to investigate the effect of prolonged exposure of biological tissue to various bioenhancers;
- To investigate the possibility of incompatibility and/or chemical interactions between astaxanthin and selected bioenhancers;
- Refinement of compounding techniques;
- Additional *in vitro* studies should be performed with the focus on region specific effects of bioenhancers on the extent of membrane permeation of astaxanthin;
- The use of biological membranes in transport studies are known to produce variable results and future *In vitro* studies should be performed in more replicates to circumvent this scenario and to obtain more data for accurate statistical evaluation;
- *In vivo* studies should be conducted to evaluate the possibility of bioavailability improvement of astaxanthin following oral administration when combined with bioenhancers.

REFERENCES

Ajazuddin, Alexander, A., Qureshi, A., Kumari, L., Vaishnav, P., Sharma, M., Saraf, S. & Saraf, S. 2014. Role of herbal bioactives as a potential bioavailability enhancer for Active Pharmaceutical Ingredients. *Fitoterapia,* 97:1 – 14.

Alderborn, G. & Frenning, G. 2018. Tablets and compaction. (*In*, Aulton, M.E., *ed.* Aulton's Pharmaceutics. The design and manufacturing of medicines. New York: Elsivier. 517 – 549.).

Allen, L.V. 2008. Dosage form design and development. *Clinical Therapeutics,* 30(11):2102 – 2111.

Ambati, R.R., Phang, S.M., Ravi, S. & Aswathanarayana, R.G. 2014. Astaxanthin: Sources, extraction, stability, biological activities and its commercial applications – A review. *Marine drugs,* 12:128 – 152.

American Cancer Society. 2015. Risks and side effects of dietary supplements. https://www.cancer.org/treatment/treatments-and-side-effects/complementary-and-alternative-medicine/dietary-supplements/risks-and-side-effects Date accessed: 5Nov. 2019.

Amidon, G.L., Lennernäs, H., Shah, V.P. & Crison, J.R. 1995. A theoretical basis for a biopharmaceutic drug classification: the correlation of in vitro drug product dissolution and in vivo bioavailability. *Pharmaceutical Research,* 12(3):413 – 420.

Anand, O., Yu, LX., Conner, D.P. & Davit, B.M. 2011. Dissolution testing for generic drugs: An FDA perspective. *The AAPS Journal*, 13(3), 328 – 335.

Andrade, K.S., Trivellin, G. & Ferreira, S.R.S. 2017. Piperine-rich extracts obtained by high pressure methods. *The Journal of Supercritical Fluids,* 128:370 – 377.

Aquilina, A., 2013. The extemporaneous compounding of paediatric medicines at Mater Dei Hospital. *Journal of the Malta College of Pharmacy Practice,* 19:28 – 30.

Ashford, M. 2018. Gastrointestinal tract – physiology and drug absorption. (*In*, Aulton, M.E., *ed.* Aulton's Pharmaceutics. The design and manufacturing of medicines. New York: Elsivier. 300 – 318.).

Atal. N. & Bedi, K.L. 2010. Bioenhancers: Revolutionary concept to market. *Journal of Ayurveda & Integrative Medicine,* 1(2):96 – 99.

Aungst, B.J. 2012. Absorption enhancers: Applications and advances. *The AAPS Journal,* 14(1):10 – 18.

Balimane, P.V., Chong, S. & Morrison, R. 2000. Current methodologies used for evaluation of intestinal permeability and absorption. *Journal of Pharmacological and Toxicological Methods,* 44:301 – 312.

Barth, L., Woodley, J. & Houin, G. 1999. Gastrointestinal absorption of drugs: methods and studies. *Fundamental Clinical Pharmacology,* 13:154 – 168

Baspinar, Y., Üstündas, M., Bayraktar, O. & Sezgin, C. 2018. Curcumin and piperine loaded zein-chitosan nanoparticles: Development and in-vitro characterisation. *Saudi Pharmaceutical Journal.*

Beneke, C., Viljoen, A. & Hamman, J. 2012. *In vitro* drug absorption enhancement effects of *Aloe vera* and *Aloe ferox. Scientia Pharmaceutica,* 80:475 – 486.

Berben, P., Bauer-Brand, A., Brand, M., Faller, B., Flaten, G.E., Jacobsen, A., Brouwers, J. & Augustiins, P. 2018. Drug permeability profiling using cell-free permeation tools: Overview and applications. *European Journal of Pharmaceutical Sciences,* 119:219 – 233.

Berman, B.M., Singh, K., Lao, L., Singh, B.B., Ferentz, K.S. & Hartnoll, S.M. 1995. Physicians' attitudes toward complementary or alternative medicine: A regional survey. *Alternative Medicine,* 8(5):361 – 366.

Bhushani, J.A., Karthik, P. & Anandharamakrishnan, C. 2016. Nanoemulsion based delivery system for improved bioaccessibility and Caco-2 cell monolayer permeability of green tea catechins. *Food Hydrocolloids,* 56:372 – 382.

Boodoo, J.M. Compounding problems and compounding confusion: Federal regulation of compounded drug products and the FDAMA circuit split. *American Journal of Law & Medicine,* 36:221 – 248.

Boudreau, M.D., Mellick, P.W., Olson, G.R., Felton, R.P., Thorn, B.T & Beland, F.A. 2013. Clear Evidence of carcinogenic activity by a whole-leaf extract of *Aloe barbadensis* Miller (Aloe vera) in F344/N rats. *Toxicological Sciences,* 131(1):26 – 39.

Bozzi, A., Perrin, C., Austin, S. & Arce Vera, F. 2007. Quality and authenticity of commercial aloe vera gel powders. *Food Chemistry,* 103:22 – 30.

BP (British Pharmacopoeia). 2018. London: HMSO.

Brion, F., Nunn, A.J. & Rieutord, A. 2003. Extemporaneous (magistral) preparation of oral medicines for children in European hospitals. *Acta Paediatrica,* 92:486 – 490.

Capelli, B., Bagchi, D. Cysewski, G.R. 2013. Synthetic astaxanthin is significantly inferior to algal-based astaxanthin as an antioxidant and may not be suitable as a human nutraceutical supplement. *Nutrafoods,* 12:145 – 152.

Chen, W., Lu, Z., Viljoen, A. & Hamman, J. 2009. Intestinal drug transport enhancement by *Aloe vera*. *Journal of Medicinal Plant and Natural Product Research,* 75:587 – 595.

Cook, G.K., Ling, J.W.H. & Lee, R. 2007. Extemporaneous compounding in Queensland hospitals. *Journal of Pharmacy Practice and Research,* 37(3):204 – 209.

Crater, J.S. & Carrier, R.L. 2010. Barrier properties of gastrointestinal mucus to nanoparticle transport. *Macromolecular Bioscience,* 10:1473 – 1483.

da Cunha, M.G., Franco, G.C.N., Franchin, M., Beutler, J.A., de Alencar, S.M., Ikegaki, M. and Rosalen, P.L. 2016. Prediction of pharmacokinetic and toxicological parameters of a 4-phenylcoumarin isolated from geopropolis: In silico and in vitro approaches. *Toxicology Letters*, 263:6 – 10.

Daugherty, A.L. & Mrsny, R.J. 1999. Transcellular uptake mechanisms of intestinal epithelial barrier Part one. *PSTT,* 2(4):144 – 151.

Desai, P.P., Date, A.A. & Patravale, V.B. 2012. Overcoming poor oral bioavailability using nanoparticle formulations – opportunities and limitations. *Drug Discovery Today: Technologies,* 9(2):e87 – e95.

DeSesso, J., Jacobson, C.F & Williams, A.L. 2012. Anatomical and physiological parameters that influence gastrointestinal absorption. *Encyclopedia of Drug Metabolism and Interactions,* 2:43 – 78.

Di, X., Wang, X., Di, X. & Liu, Y. 2015. Effect of piperine on the bioavailability and pharmacokinetics of emodin in rats. *Journal of Pharmaceutical and Biomedical Analysis*, 115:144 – 149.

Dodou, K. 2018. Rectal and vaginal drug delivery. (*In*, Aulton, M.E., *ed.* Aulton's Pharmaceutics. The design and manufacturing of medicines. New York: Elsivier. 739 – 756.).

Doogue, M.P. & Thynne, T.R.J. 2013. What is quality medicine. 2013. *Internal Medicine Journal,* 43:1057 – 1058.

Dresser, G.K., Spence, J.D. & Bailey D.G. 2000. Pharmacokinetic-pharmacodynamic consequences and clinical relevance of cytochrome P450 3A4 inhibition. *Clinical Pharmacokinetics,* 38(1):41 – 57.

Du, P., Jin, M., Yang, L., Chen, G., Zhang, C., Jin, F., Shao, H., Yang, M., Yang, X., She, Y,, Wang, S., Zheng, L. & Wang, J. 2016. Determination of astaxanthin in feeds using high performance liquid chromatography and an efficient extraction method. *Journal of Liquid Chromatography & Related Technologies,* 39(1):35 – 43.

Dwyer, J.T., Picciano, M.F., Betz, J.M., Fishera, K.D., Saldanha, L.G., Yetley, E.A., Coates, P.M., Milner, J.A., Whitted, J., Burt, V., Radimer, K., Wilger, J., Sharpless, K.E., Holden, J.M., Andrews, K., Roseland, J., Zhao, C., Schweitzer, A., Harnly, J., Wolf, W.R. & Perry, C.R. 2008. Progress in developing analytical and label-based dietary supplement databases at the NIH Office of Dietary Supplements. *Journal of Food Composition and Analysis,* 21:S83 – S93.

Edwards, D.J. 2012. Beneficial pharmacokinetic drug interactions. *Advances in Pharmacoepidemiology & Drug Safety,* 10(4172):2167 – 1052.

Ensign, L.M., Cone, R. & Hanes, J. 2012. Oral drug delivery with polymeric nanoparticles: The gastrointestinal mucus barriers. *Advanced Drug Delivery Reviews,* 64:557 – 570.

Fagerholm, U. & Lennernäs, H. 1995. Experimental estimation of the effective unstirred water layer thickness in the human jejunum, and its importance in oral drug absorption. *European Journal of Pharmaceutical Sciences,* 3:249 – 253.

Feldschuh, M. 2008. Compounding in community pharmacy. *Australian Prescriber,* 31:30 – 31.

Freire, A.C., Basit, A.W. 2018. Dissolution testing of solid dosage form. (*In*, Aulton, M.E., *ed.* Aulton's Pharmaceutics. The design and manufacturing of medicines. New York: Elsivier. 626 – 637.).

Gerber, W., Steyn, J.D., Kotzé, A.F. & Hamman, J.H. 2018. Beneficial pharmacokinetic drug interactions: A tool to improve the bioavailability of poorly permeable drugs. *Pharmaceutics,* 10(106):1 – 15.

Gerber, W., Steyn, D., Kotzé, A., Svitina, H., Wheldon, C. & Hamman, J. 2019. Capsaicin and Piperine as functional excipients for improved drug delivery across nasal epithelial models. *Planta Medica,* 85:1114 – 1123.

Giam, J.A., McLachlan, A.J. & Krass, I. 2011. Community pharmacy compounding-impact on professional status. *International Journal of Clinical Pharmacy,* 33:177 – 182.

Grass, G.M. 1997. Simulation models to predict oral drug absorption from in vitro data. *Advanced Drug Delivery Reviews,* 23:199 – 219.

Grass, G.M. & Sweetana, S.A. 1988. *In vitro* measurement of gastrointestinal tissue permeability using a new diffusion cell. *Pharmaceutical Research,* 5(6):372 – 376.

Gudeman, J., Jozwiakowski, M., Chollet, J. & Randell, M. 2013. Potential risk of pharmacy compounding. *Drugs R&D,* 13:1 – 8.

Guerra, A., Etienne-Mesmin, L.E., Livrelli, V., Denis, S., Blanquet-Diot, S. & Alric, M. 2012. Relevance and challenges in modeling human gastric and small intestinal digestion. *Trends in Biotechnology*, 30(11):591 – 600.

Haasbroek, A., Willers, C., Glyn, M., du Plessis, L. & Hamman, J. 2019. Intestinal drug absorption enhancement by *Aloe vera* gel and whole leaf extract: *In vitro* investigations into the mechanisms of action. *Pharmaceutics,* 11(36):1 – 17.

Harrison, A.P., Erlwanger, K.H., Elbrønd, V.S., Andersen, N.K. & Unmack, M.A. 2004. Gastrointestinal-tract models and techniques for use in safety pharmacology. *Journal of Pharmacological and Toxicological Methods,* 49:187 – 199.

Hamman, J.H. 2008. Composition and applications of *Aloe vera* leaf gel. *Molecules,* 13:1599 – 1616.

Hamman, J.H., Enslin, G.M. & Kotze, A.F. 2005. Oral delivery of peptide drugs: Barriers and development. *Biodrugs,* 19(3):165 – 177.

Hayashi, M., Sakai, T., Hasegawa, Y., Nishikawahara, T., Tomioka, H., Iida, A., Shimizu, N., Tomita, M. & Awazu, S. 1999. Physiological mechanism for enhancement of paracellular drug transport. *Journal of Controlled Releases,* 62:141 – 148.

Hayeshi, R., Masimirembwa, C., Mukanganyama, S. & Ungell, A.B. 2006. The potential inhibitory effect of antiparasitic drugs and natural products on P-glycoprotein mediated efflux. *European journal of pharmaceutical sciences,* 29:70 – 81.

Haywood, A. & Glass, B.D. 2013. Liquid dosage forms extemporaneously prepared from commercially available products – Considering new evidence on stability. *Journal of Pharmacy and Pharmaceutical Sciences,* 16(3):441 – 455.

He, L., Yin, Y., Li, T., Huang, R., Xie, M., Wu, Z. & Wu, G. 2013. Use of the Ussing chamber technique to study nutrient transport by epithelial tissue. *Frontiers in Bioscience,* 18:1266 – 1274.

Hidalgo, I.J. 2001. Assessing the absorption of new pharmaceuticals. *Current Topics in Medicinal Chemistry*, 1(5):385 – 401.

Higuera-Ciapara, I., Felix-Valenzuela, L., Goycoolea, F.M. & Argüelles-Monal. W. 2004. Microencapsulation of astaxanthin in a chitosan matrix. *Carbohydrate Plymers,* 56:41 – 45.

Holmstock, N., Annaert, P. & Augustijns, P. 2012. Boosting of HIV Protease inhibitors by Ritonavir in the intestine: The relative role of cytochrome P450 and P-Glycoprotein inhibition based on Caco-2 monolayers versus *in situ* intestinal perfusion in mice. *Drug Metabolism and Disposition*, 40(8):1473 – 1477.

IARC Monographs: 108. *Aloe vera.* 37 – 72.

Ingels, F.M. & Augustijns, P.F. 2003. Biological, pharmaceutical, and analytical considerations with respect to the transport media used in the absorption screening system, Caco-2. *Journal of Pharmaceutical Sciences,* 92(8):1545 – 1558.

Jaksic, I., Lukic, M., Malenovic, A., Reichl, S., Hoffman, C., Müller-Goymann, C., Daniels, R. & Savic, S. Compounding of a topical drug with prospective natural surfactant-stabilized pharmaceutical bases: Physiochemical and *in vitro in vivo* characterization – A ketoprofen case study. *European Journal of Pharmaceutics and Biopharmaceutics,* 80:164 – 175.

Jivraj, M., Martini, L.G, & Thomson, C.M. 2000. An overview of the different excipients useful for the direct compression of tablets. *Pharmaceutical Science & Technology Today*, 3(2):58 – 63.

Jones, B.E. 2018. Hard capsules. (*In*, Aulton, M.E., *ed.* Aulton's Pharmaceutics. The design and manufacturing of medicines. New York: Elsivier. 597 – 11.).

Juan, P., WenZhou, X., QuanMing, T., Ni, S., Feng, C. & JianPing, Y. 2008. Comparative analysis of astaxanthin and its esters in the mutant E1 of *Haematococcus pluvialis* and other green algae by HPLC with a C30 column. *Science in China Series C: Life Sciences,* 51(12):1108 – 1115.

Junginger, H.E. & Verhoef, J.C. 1998. Macromolecules as safe penetration enhancers for hydrophilic drugs – a fiction?. *PSTT,* 1(9):370 – 376.

Kang, M.J., Cho, J.Y., Shim, B.H., Kim, D.K. & Lee, J. 2009. Bioavailability enhancing activities of natural compounds from medicinal plants. *Journal of Medicinal Plants Research,* 3(13):1204 – 1211.

Khajuria, A., Thusu, N. & Zutshi, U. 2002. Piperine modulates permeability characteristics of intestine by inducing alterations in membrane dynamics: Influence on brush border membrane fluidity, ultrastructure and enzyme kinetics. *Phytomedicine,* 9:224 – 231.

Kidd, P. 2011. Astaxanthin, cell membrane nutrient with diverse clinical benefits and anti-aging potential. *Alternative Medicine Review,* 16(4):355 – 364.

Kobayashi, M., Kakizono, T., Nishio, N., Nagai, S., Kurimura, Y. & Tsuji, Y. 1997. Antioxidant role of astaxanthin in the green alga *Haematococcus pluvialis*. *Applied Microbiology and Biotechnology.* 48:351 – 356.

Kohli, K., Chopra, S., Dhar. D., Arora, S. & Khar, R.K. 2010. Self-emulsifying drug delivery systems: an approach to enhance oral bioavailability. *Drug Discovery Today,* 15(21/22):958 – 965.

Koziolek, M., Grimm, M., Becker, D., Iordanov, V., Zou, H., Shimizu, J., Wanke, C., Garbacz, G., Weitschies, W. 2015. Investigation of pH and temperature profiles in the GI tract of fasted human subjects using the Intellicap® system. *Journal of Pharmaceutical Science*, 104:2855 – 2863.

Krishna, G., Chen, K., Lin, A. & Nomeir, A.A. 2001. Permeability of lipophilic compounds in drug discoveryusing in-vitro human absorption model, Caco-2. *International Journal of Pharmaceutics,* 222:77 – 89.

Kurkov, S.V. & Loftsson, T. 2013. Cyclodextrins. *International Journal of Pharmaceutics,* 453:167 – 180.

Le Ferrec, E., Chesne, C., Artusson, P., Brayden, D., Fabre, G., Gires, P., Guillou, F., Rousset, M., Rubas, W. & Scarino, M. 2001. *In Vitro* models of the intestinal barrier. *Alternatives to Laboratory Animals,* 29:649 – 668.

Liang, J., Tian, Y., Yang, F., Zhang, J. & Skibsted, L.H. 2009. Antioxidant synergism between carotenoids in membranes. Astaxanthin as a radical transfer bridge. *Food Chemistry,* 115:1437 – 1442.

Lieberman, H.R., Marriott, B.P., Williams, C., Judelson, D.A., Glickman, E.L., Geiselman, P, J., Dotson, L. & Mahoney, C.R. 2015. Patterns of dietary supplement use among college students. *Clinical Nutrition,* 34:976 – 985.

Linert, W., Bridge, M.H., Huber, M., Bjugstad, K.B., Grossman, S. & Arendash, G.W. 1999. In vitro and in vivo studies investigating possible antioxidant actions of nicotine: relevance to Parkinson's and Alzheimer's diseases. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, 1454(2):143 – 152.

Liu, X., Luo, Q., Rakariyatham, K., Cao, Y., Goulette, T., Liu, X. & Xiao, H. 2016. Antioxidant and anti-ageing activities of different stereoisomeric astaxanthin in vitro and in vivo. *Journal of Functional Foods,* 25:50 – 61.

Liu, Z., Wang, S. & Hu. M. 2009. Oral absorption basics: Pathways, physico-chemical and biological factors affecting absorption. *Developing Solid Oral Dosage Forms: Pharmaceutical Theory and Practise,* II:265 – 288.

López, A., Suárez de Tangil, M., Vega-Orellana, O., Ramírez, A.S. & Rico, M. 2013. Phenolic constituents, antioxidant and preliminary antimycoplasmic activities of leaf skin and flowers of *Aloe vera* (L.) Burm.f. (syn. *A. barbadensis* Mill). From the Canary Islands (Spain). *Molecules,* 18:4942 – 4954.

Lowe, R. 2018. Parenteral drug delivery. (*In*, Aulton, M.E., *ed.* Aulton's Pharmaceutics. The design and manufacturing of medicines. New York: Elsivier. 638 – 652.).

Lowey, A. & Jackson, M. 2008. How to ensure the quality and safety of unlicensed oral medicines. *The Pharmaceutical Journal,* 1 – 11.

Mahar-Doan, K.M., Humphreys, J.E., Webster, L.O. Wring, S.A., Shampine, L.J., Serabjit-Sing, C.J., Adkison, K.K. & Polli, J.W. 2002. Passive permeability and P-glycoprotein-mediated efflux differentiate Central Nervous System (CNS) and non-CNS marketed drugs. *Journal of Pharmacology and Experimental Therapeutics,* 303(3):1029 – 1037.

Marques, M. 2004. Dissolution Media Simulating Fasted and Fed States. *Dissolution Technology.*

Martini, F.H., Nath, J.L. & Bartholomew, E.F. 2014. The digestive system. (*In*, Martini, F.H., *ed.* Fundamentals of Anatomy & Physiology. Edinburgh: Pearson Education Limited. 899 – 949.).

Maske, T.T., Kuper, N.K., Hollanders, A.C.C., Bronkhorst, E.M., Cenci, M.S. & Huysmans, M.C.D.N.J.M. 2018. Secondary caries development and the role of a matrix metalloproteinase inhibitor: A clinical in situ study. *Journal of Dentistry*, 1 – 5.

Masupye, E.M., Suleman, F. & Govender, T. 2015. Investigating extemporaneous compounding practises in Polokwane tertiary hospital pharmacies in South Africa – a pilot study. *African Journal of Pharmacy and Pharmacology,* 9(48):1099 – 1105.

Merchant, H.A., McConnell, E.L., Liu, F., Ramaswamy, C., Kulkarni, R.P., Basit, A.W. & Murdan, S. 2011. Assessment of gastrointestinal pH, fluid and lymphoid tissue in the guinea pig, rabbit and pig, and implications for their use in drug development. *European Journal of Pharmaceutical Sciences,* 42:3 – 10.

Meurens, F., Summerfield, A., Nauwynck, H., Saif, L. & Gerdts, V. 2012. The pig: a model for human infectious diseases. *Trends in microbiology,* 20(1):50 – 57.

Muñoz, O.M., Leal, X., Quitral, V. & Cardemil, L. 2015. Extraction, characterization and properties of the gel of *Aloe vera* (*Aloe barbadensis* Miller) cultivated in Chile. *Medicinal & Aromatic Plants,* 4(3):1 – 7.

Murdan,S. 2018. Solutions. (*In*, Aulton, M.E., *ed*. Aulton's Pharmaceutics. The design and manufacturing of medicines. New York: Elsivier. 407 – 416.).

Naguib, Y.M.A. 2000. Antioxidant activities of Astaxanthin and related carotenoids. *Journal of Agricultural and Food Chemistry,* 48:1150 – 1154.

Nahata, M.C. & Allen, L.V. 2008. Extemporaneous drug formulations. *Clinical Therapeutics,* 30(11):2112 – 2119.

Nakagawa, K., Kiko, T., Miyazawa, T., Burdeos, G.C., Kimura, F., Satoh, A. & Miyazawa, T. 2011. Antioxidant effect of astaxanthin on phospholipid peroxidation in human erythrocytes. *British Journal of Nutrition,* 105:1563 – 1571.

National Institute of Health. 2011. Dietary supplements: What you need to know. https://ods.od.nih.gov/pubs/DS_WhatYouNeedToKnow.pdf. Date of access: 5Nov. 2019.

Nunes, R., Silva, C. & Chaves, L. 2016. Tissue-based *in vitro* and *ex vivo* models for intestinal permeability studies. *Concepts and Models for Drug Permeability Studies,* 203 – 233

O'Dea, J.A. 2003. Consumption of nutritional supplements among adolescents: usage and perceived benefits. *Heath Education Research,* 15(1):98 – 107.

Odeberg, J.M., Lignell, A., Pettersson, A. & Höglund, P. 2003. Oral bioavailability of the antioxidant astaxanthin in humans is enhanced by incorporation of lipid based formulations. *European Journal of Pharmaceutical Sciences,* 19:299 – 304.

Ohnishi, N., Kusuhara, M., Yoshioka, M., Kuroda, K., Soga, A., Nishikawa, F., Koishi, T., Nakagawa, M., Hori, S., Matsumoto, T., Yamashita, M., Ohta, S., Takara, K. & Yokoyama, T. 2003. Studies on interactions between functional foods or dietary supplements and medicines. Effects of *Ginkgo biloba* leaf extract in the pharmacokinetics of diltiazem in rats. *Biological and Pharmaceutical Bulletin,* 26(9):1315 – 1320.

Onel, S. 2005. Dietary Supplements: A Definition that is black, white and grey. *American Journal of Law & Medicines,* 31:341 – 348.

Orosa, M., Franqueira, D., Cid, A. & Abalde, J. 2005. Analysis and enhancement of astaxanthin accumulation in *Haematococcus pluvialis*. *Bioresource Technology,* 96:373 – 378.

Otton, R., Marin, D.P., Bolin, A.P., dos Santos, R.dC.M., Polotow, T.G., Sampaio, S.C. & de Barros, M.P. 2010. Astaxanthin ameliorates the redox imbalance in lymphocytes of experimental diabetic rats. *Chemo-Biological Interactions,* 186:306 – 315.

Palmer, M.E., Haller, C., McKinney, P.E., Klein-Schwartz, W., Tschirgi, A., Smolinske, S.C., Woolf, A., Sprague, B.M., Ko, B., Everson, G., Nelson, L.S., Dodd-Butera, T., Bartlett, W.D. & Landzberg, B.R. 2003. Adverse events associated with dietary supplements: an observational study. *The Lancet,* 361:101 – 106.

Parry, D.A., Oeppen, R.S., Amin, M. &Brennan, P.A. 2017. Can dietary supplements improve a clinician's well-being and health?. *British Journal of Oral and Maxillofacial Surgery.*

Patterson, J.K., Lei, X.G. & Miller, D.D. 2008. The pig as an experimental model for elucidating the mechanisms governing dietary influence on mineral absorption. *Experimental Biology and Medicine,* 233:651 – 664.

Pauletti, G.M., Gangwar, S., Siahaan, T.J., Aubé, J. & Borchardt, R.T. 1997. Improvement of oral peptide bioavailability: Peptidomimetics and prodrug strategies. *Advanced Drug Delivery Reviews,* 27:235 – 256.

Persson, E.M., Gustafsson, A.S., Carlsson, A.S., Nilsson, R.G., Knutson, L., Forsell, P., Hanisch, G., Lennernäs, H. & Abrahamsson, B. 2005. The effects of food on the dissolution of poorly soluble drugs in human and in model small intestinal fluids. *Pharmaceutical research*, 22:2141 – 2151.

Perzborn, E., Strassburger, J., Wilmen, A., Pohlmann, J., Roehrig, S., Schlemmer, K.H. & Straub, A. 2005. In vitro and in vivo studies of the novel antithrombotic agent BAY 59-7939-an oral, direct Factor Xa inhibitor. *Journal of Thrombosis and Haemostasis*, 3(3):514 – 521.

Peterson, B., Weyers, M., Steenekamp, H.H., Steyn, J.D., Gouws, C. & Hamman, J.H. 2019. Drug bioavailability enhancing agents of natural origin (Bioenhancers) that modulate drug membrane permeation and pre-systemic metabolism. *Pharmaceutics*, 11(33):1 – 46.

Porter, D. 2007. Qualification, validation and verification. *PharmTech*.

Pund, S., Rasve, G. & Borade, G. 2013. *Ex vivo* permeation characteristics of venlafaxine through sheep nasal mucosa. *European Journal of Pharmaceutical Science,* 48:195 – 201.

Radimer, K.L. 2000. Nonvitamin, nonmineral dietary supplements: Issues and findings from NHANES III. *Journal of the American Dietetic Assosiation,* 100(4):447 – 454.

Rees, D.A. & Alcolado, J.C. 2005. Animal models of diabetes mellitus. *Diabetes UK. Diabetic Medicine,* 22:359 – 370.

Reis, J.M., Dezani, A.B., Pereira, T.M., Avdeef, A. & Serra, C.H.R. 2013. Lamivudine permeability study: A comparison between PAMPA, *ex vivo* and *in situ* Single-Pass Intestinal Perfusion (SPIP) in rat jejunum. *European Journal of Pharmaceutical Science,* 48:781 – 789.

Roseland, J.M., Holden, J.M., Andrews, K.W., Zhao, C., Schweitzer, A., Harnly, J., Wolf, W.R., Perry, C.R., Dwyer, J.T., Picciano, M.F., Betz, J.M., Saldanha, L.G., Yetley, E.A., Fisher, K.D. & Sharpless, K.E., 2008. Dietary supplement ingredient database (DSID): Preliminary USDA studies on the composition of adult multivitamin/mineral supplements. *Journal of Food Composition & Analysis,* 21(1):S69 — S77.

Rouge, N., Buri, P. Doelker, E. 1996. Drug absorption sites in the gastrointestinal tract and dosage forms for site-specific delivery. *International Journal of Pharmaceutics,* 136:117 – 139.

Salama, N.N., Eddington, N.D., Fasano, A. 2006. Tight junction modulation and its relationship to drug delivery. *Advances Drug Delivery Reviews*, 58:15 – 28.

Schneeberger, E.E. & Lynch, R.D. 2004. The tight junction: a multifunctional complex. *AJP-Cell Physiol*, 286:C1213 – C1228.

Serrano, M., Valverde, J.M., Guillén, F., Castillo, S., Martínez-Romero, D. & Valero, D. 2006. Use of *Aloe vera* gel coating preserves the functional properties of grapes. *Journal of Agricultural and Food Chemistry*, 54:3882 – 3886.

Shabir, G.A. 2003. Validation of high-performance liquid chromatography methods for pharmaceutical analysis. Understanding the differences and similarities between validation requirements of the US Food and Drug Administration, the US Pharmacopeia and the International Conference on Harmonization. 2003. *Journal of Chromatography A,* 987:57 – 66.

Shanmugam, S. 2015. Natural bioenhancers: Current Outlook. *Clinical Pharmacology & Biopharmaceutics,* 4(2):1 – 3.

Shikanga, E.A., Hamman, J.H., Chen, W., Combrinck, S., Gericke, N. & Viljoen, A.M. 2012. *In Vitro* permeation of mesembrine alkaloids from *Sceletium tortuosum* across porcine buccal, sublingual and intestinal mucosa. *Planta Medica,*78:260 – 268.

Shargel, L., Wu-Pong, S. & Yu, A.B.C. 2012. Physiologic factors related to drug absorption. (*In*, Shargel, L., *ed.* Applied Biopharmaceutics & Pharmacokinetics. Great Britain. 321 – 359.).

Sigma-Aldrich. 2013. Lucifer Yellow: Product information. https://www.sidmaaldrich.com/content/dam/sigmaaldrich/docs/Sigma/Bulletin/1/mtox1000p24bul.pdf. Date of access: 25Oct. 2019.

Sigma-Aldrich. KREBS-Ringer Bicarbonate Buffer. 2007. https://www.sigmaaldrich.com/content/dam/sigmaaldrich/docs/Sigma/Product_Information_Sheet/1/k4002pis.pdf. Date of access: 27Oct. 2019.

Singh, R. 2013. HPLC method development and validation – an overview. *Journal of Pharmaceutical Education and Research,* 4(1):26 – 33.

Sjögren, E., Abrahamsson, B., Augustijns, P., Becker, D., Bolger, M.B., Brewster, M., Brouwers, J., Flanagan, T., Harwood, M., Heinen, C., Holm, R., Juretschke, H.P., Kubbinga, M., Lindahl, A., Lukacova, V., Münster, U., Neuhoff, S., Nguyen, M.A., van Peer, A., Reppas, C., Hodjegan, A.R., Tannergren, C., Weitschies, W., Wilson, C., Zane, P., Lennernäs, H., Langguth, P. 2014. *In vivo* methods for drug absorption – Comparative physiologies, model selection, correlations with in vitro methods (IVIVC), and applications for formulation/API/excipient characterization including food effects. *European Journal of Pharmaceutical Sciences,* 57:99 – 151.

Sonaje, K., Chuang, E., Lin, K., Yen, T., Su, F., Tseng, M.T. & Sung, H. 2012. Opening of epithelial tight junctions and enhancement of paracellular permeation by chitosan: Microscopic, ultrastructural, and computed-tomographic observations. *Molecular Pharmaceutics,* 9:1271 – 1279.

Song, N., Zhang, S. & Liu, C. 2004. Overview of factors affecting oral drug absorption. *Asian Journal of Drug Metabolism and Pharmacokinetics,* 4(3):167 – 176.

Srinivasan, B., Kolli, A.R., Esch, M.B., Abaci, H.E., Shuler, M.L. & Hickman, J.J. 2015. TEER measurement techniques for *In vitro* barrier model systems. *Journal of Laboratory Automation,* 20(2):107 – 126.

Stella, V.J., Rao, V.M., Zannou, E.A. & Zia, V. 1999. Mechanisms of drug release from cyclodextrin complexes. *Advanced Drug Delivery Reviews,* 36:3 – 16.

Sullivan, T.P., Eaglstein, W.H., Davis, S.C. & Mertz, P. 2001. The pig as a model for human woand healing. *Wound Repair and Regeneration,* 9(2):66 – 76.

Todorova, A., Gugleva, V. & Georgieva, L. 2016. Doctors' point of view on Pharmacy Compounding – Advantages and disadvantages. *IAIM,* 3(2):45 – 50.

Troutman, M.D. & Takker, D.R. 2003. Efflux ratio cannot access P-glycoprotein-mediated attenuation of absorptive transport: Asymmetric effect of P-glycoprotein on absorptive and secretory transport across Caco-2 cell monolayers. *Pharmaceutical Research,* 20(8):1200 –1209.

van der Horst, K. & Siegrist, M. 2011. Vitamin and mineral supplement users. Do they have healthy or unhealthy dietary behaviours?. *Appetite,* 57:758 – 764.

Varma, K. 2016. Excipients used in the formulation of tablets. *Research and Reviews; Journal of Chemistry,* 5(2):143 – 154.

Versantvoort, C.H.M., Rompelberg, C.J.M. & Sips, A.J.A.M. 2000. Methodologies to study human intestinal absorption. *Research for man and environment,* 630030 001:4 – 55.

Vinson, J.A., Al Kharrat, H. & Andreoli, L. 2005. Effect of *Aloe vera* preparations on the human bioavailability of vitamins C and E. *Phytomedicine,* 12:760 – 765.

Wadhwa, S., Singhal. S. & Rawat, S. 2014. Bioavailability enhancement by piperine: A review. *Asian Journal of Biomedical and Pharmaceutical Sciences,* 4(36):1 – 8.

Watkins, P.B. 1997. The barrier function of CYP3A4 and P-glycoprotein in the small bowel. *Advanced Drug Delivery Reviews,* 27:161 – 170.

Winterstein, A.P. & Storrs, C.M. 2001. Herbal supplements: Considerations for the athletic trainer. *Journal of Athletic Trainer,* 36(4):425 – 432.

Yamashita, S., Furubayashi, T., Kataoka, M., Sakane, T., Sezaki, H. & Tokuda, H. 2000. Optimized conditions for prediction of intestinal drug permeability using Caco-2 cells. *European Journal of Pharmaceutical Sciences,* 10(3):195 – 204.

Zhang. D., Luo, G., Ding, Z. & Lu, C. 2012. Preclinical experimental models of drug metabolism and disposition in drug discovery and development. *Acta Pharmaceutica Sinica B,* 2(6):549 – 561.

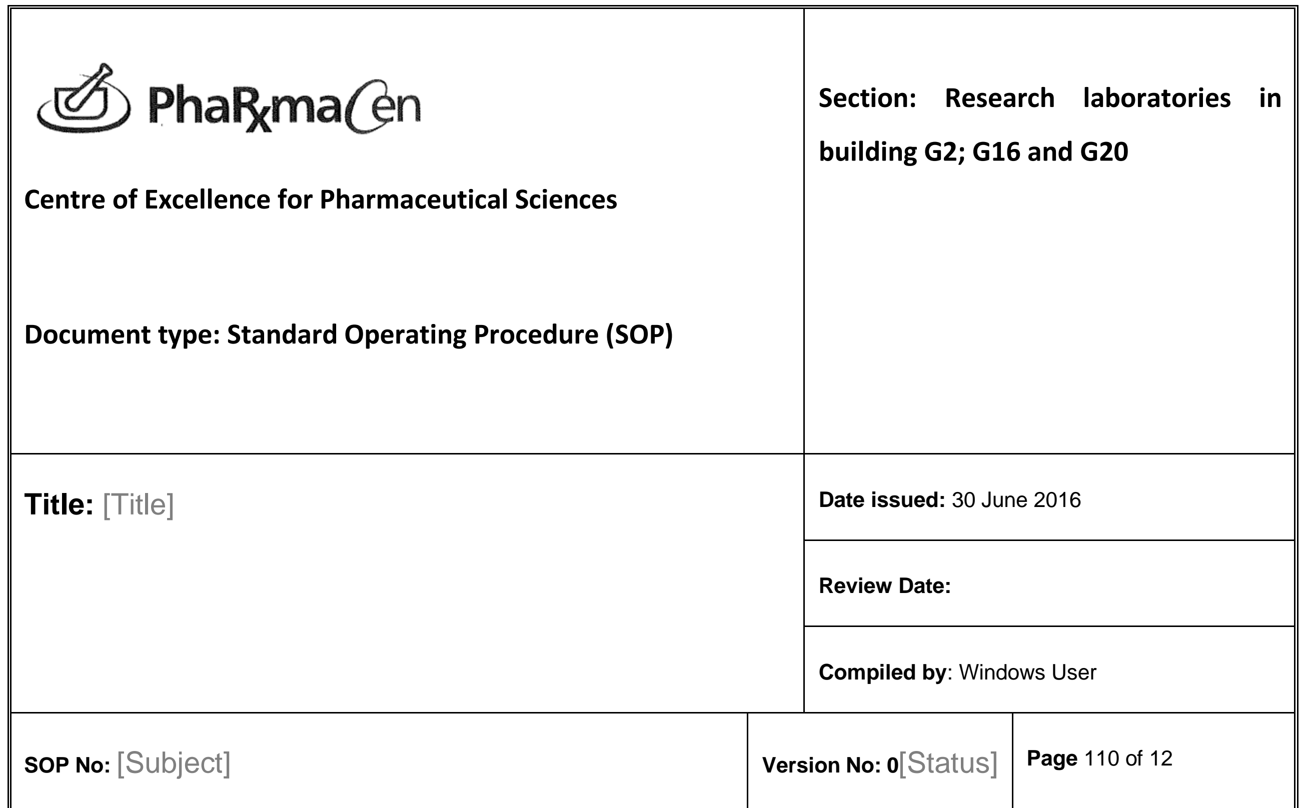
Zhang, Y. & Benet, L.Z. 2001. The gut as a barrier to drug absorption combined role of cytochrome P450 and P-glycoprotein. *Clinical Pharmacokinetics,* 40(3):159 – 168.

Zhao-Hui, J., Wen, Q., Hui, L., Xue-Hua, J. & Ling, W. 2018. Enhancement of oral bioavailability and immune response of Ginsenoside Rh2 by co-administration with piperine. *Chinese Journal of Natural Medicines,* 16(2):0143 – 0149.

Ziegler, A., Gonzalez, L. & Blikslager, A. 2016. Large Animal Models: The Key to Translational Discovery in Digestive Disease Research. *Cellular and Molecular Gastrointestinal and Hepatology,* 2(6):716 – 724.

ADDENDUM A

STANDARD OPERATING PROCEDURES: BIOLOGICAL WASTE MANAGEMENT



PhaRxmaCen Centre of Excellence for Pharmaceutical Sciences Document type: Standard Operating Procedure (SOP)		Section: Research laboratories in building G2; G16 and G20
Title: [Title]		**Date issued:** 30 June 2016
		Review Date:
		Compiled by: Windows User
SOP No: [Subject]	**Version No: 0**[Status]	**Page** 110 of 12

1. Definitions

The following definitions are applicable to this SOP:

Waste (according to the Waste Amendment Act, 2014 (Act No 26 of 2014)): “Any substance, material or object, that is unwanted, rejected, abandoned, discarded or disposed of, by the holder of the substance, material or object, whethter or not such substance, material or object can be re-used, recycled or recovered and includes all wastes as defined in Schedule 3 to this Act”.

Hazardous waste (Act No 26 of 2014): “Any waste that contains organic or inorganic elements of compounds that may, owing to the inherent physical, chemical or toxicological characteristics of that waste, have a detrimental impact on health and the environment”.

Biological waste: Waste containing mostly natural organic materials such as cell cultures, animal/human tissues or blood, animal excrements, microbiological cultures etc.

2. Purpose

To manage radioactive waste in the research laboratory of Building G20 (Room G14) on the Potchefstroom campus of the North-West University to ensure the safety and health of the researchers (both staff and students) as well as to ensure that the environment is not contaminated by waste materials of a potential hazardous nature. This ensures compliance with the policy regarding the use of unsealed radioactive nuclides, which is subject to regulatory control in terms of the Hazardous Substance Act, 1973 (Act 15 of 1973).

3. Objective

To ensure safe handling, storage and removal of liquid and/or solid biological waste materials as well as needles and sharp waste generated in the research laboratories of Buildings G2, G16 and G20.

4. Scope

This SOP is applicable to the all research laboratories in buildings G2, G11 and G20 generating potentially hazardous liquid and/or solid biological and sharp waste waste materials.

5. Responsibilities

All staff and students working inside the research laboratories of buildings G2, G16 and G20 are responsible to follow the procedures outlined in this SOP. The safety officers are responsible for completion of documentation such as disposal request form, treatment request form, communication with the medical waste company (currently Oricol), as well as reporting any incidents to the Occupational Health and Safety Committee.

6. Apparatus and equipment

Apparatus/equipment	Location (Room No.)	Check points	Criteria for approval/rejection
Autoclave	LAMB (G14)	Preheat until jacket pressure and temperature reach minimum level to start an autoclave cycle	Autoclave must be serviced once a year, and Sterikon+ bio-indicator for check on autoclaving quality must be done quarterly
Hazardous waste containers	All research laboratories	Appropriately labelled	Correct label and container for waste type

7. List of other SOPs relevant to this SOP

None.

8. Safety measures

The following general safety measures should always be followed:

- All staff and students working in the research laboratories of buildings G2, G16 and G20 should familiarise themselves with the potential hazardousness and other safety aspects such as incompatibilities between chemicals or potential presence of pathogens in biological material before handling them.
- All staff and students must be appropriately trained in handling hazardous waste materials.
- Adherence to dress code: always wear a laboratory coat, gloves and, if the need arise, eye protective goggles or a face mask during handling of biological materials.
- All biological materials should be regarded as potentially hazardous and therefore be handled with care.

- Emergency post for eye washing should be available in all laboratories.
- Disinfect all needles, blades, loops and slides by autoclaving before discarding.
- Keep large enough bins for broken glass in the laboratory (store room).

9. Procedures

9.1 Waste containers

9.1.1 Only use waste containers supplied by the removal company that is compatible with the type of waste material.

9.1.2 Different types of waste containers shall be available for different types of waste as prescribed by the waste company which may include: biological waste – sharps/needles (plastic bucket with lid), biological waste – solids (box with red liner), biological waste – liquid (plastic drum with screw lid), medical waste – solids, liquids and sludge (plastic drum with screw lid) (Please refer to Addendum A).

9.1.3 Containers shall be labeled correctly with all appropriate information: Site information include campus name, building number, room number and date, Waste information include full names and class of biological material as prescribed by the waste removal company before removal.

9.1.4 Containers must remain closed at all times (except when waste is added) and be sealed appropriately.

9.1.5 Containers must be kept clean and dry.

9.1.6 Containers for liquids must never be filled to the top to allow space for expansion (only fill to about 90% of container volume). Incompatible liquids are not allowed to be mixed in any waste container.

9.2 Contaminated sharps and needles

9.2.1 Place the sharps and needles in the designated waste bins provided by the waste removal company (yellow buckets).

9.2.2 No waste other than the sharps and needles shall be placed in these containers.

9.2.3 When the need arises for removal of the waste (e.g. container is full), the safety officer should be informed to complete a disposal request form and to notify the medical waste company (currently Oricol) according to the procedure described in Addendum A.

9.3 Biological materials and contaminated disposable items

9.3.1 Biological materials (e.g. tissue and blood) and contaminated disposable items (other than sharps and needles) are placed in the disposable red plastic bag that is placed inside the appropriate waste container and stored in the refrigerator in Room G14, Building G20.

9.3.2 When the need arises for removal of the waste (e.g. container is full), the safety officer should be informed to complete a disposal request form and to notify the medical waste company (currently Oricol) according to the procedure described in Addendum A.

9.4 Contaminated broken glassware

9.4.1 Place the broken glassware (after it has been decontaminated by autoclaving) in the designated bins for broken glass.
9.4.2 No other waste should be placed in this container.

10. Records and data sheets

There should be material safety data sheets (MSDS) available on all chemicals used in the specified laboratories.

Record the type and volume of waste disposed on record books placed next to the waste container.

All records (including copies of disposal request forms and treatment request forms) must be kept for 5 years at the North-West University by the safety officer.

11. Scheme of SOP development

Action	**Designated person**	**Signature**	Date
Compile	H. Netsimbupfe		2015/09/03
Compile	C. Gouws		2016/06/30

Addendum A:

ADDENDUM A: "Chemical, pharmaceutical and medical waste procedure"

ORICOL ENVIRONMENTAL SERVICES

Chemical, pharmaceutical and medical waste procedure

Aim

To control and manage the booking, collecting, handling and disposal of all chemical, pharmaceutical and medical waste originating from clients. To prevent incompatible chemicals being stored and transported together.

Scope

This procedure applies to all Oricol and client personnel requesting the assistance of Oricol staff in the disposal of chemical, pharmaceutical and medical waste.

Responsibility

- Oricol contract manager and/or sales representative
- Oricol drivers and assistants
- Client
- Booking clerk
- Hazardous waste clerk
- Logistics management

Method

Client responsibility

Chemical waste

- When the need to dispose of chemical waste arises, a TRF (treatment request form) must be completed and sent to the Oricol contract manager or sales representative. An example of a TRF can be seen in Appendix A and should be in Word format where possible.
- Campus name, Building number, building name and room number and date must be completed in full in order to ensure that the waste is collected from the right site.
- Chemical names must be written out in full on the TRF and the storage containers and no abbreviations or chemical formulas may be used (i.e. "Ether" instead of "Et2O and "Dimethylphosphinoethane" instead of "DMPE").
- A separate TRF must be completed for the following chemicals and/or their compounds as the incinerator is not permitted to accept and incinerate them. These will be collected and disposed of separately at the hazardous waste landfill:

    - Arsenic (As)
    - Astatine (At)
    - Cadmium (Cd)
    - Chromium (Cr)
    - Cesium (Cs)
    - Cyanide(CN^-)
    - Francium (Fr)
    - Iodine (I)
    - Krypton (Kr)
    - Lead (Pb)
    - Mercury (Hg)
    - Nickel (Ni)
    - Phosphorous (P)
    - Rubidium (Rb)
    - Selenium (Se)

Chemical, pharmaceutical and medical waste procedure

- The UN number and class of the chemicals must also be completed. The chemicals must then be grouped together in the following classes. Should the container be a mixture of chemicals, the highest concentration constituent applies:

  - Class 3 (Flammable Liquids); 4.1 (Flammable Solids); 4.2 (Spontaneously Combustible material); 4.3 (Dangerous when Wet material); 8.2 (Alkalis) and 9. (Miscellaneous materials not classified). A GREEN sticker must be placed on the box or container.

    Examples: Acetone, Magnesium, white phosphorous, sodium hydroxide, calcium

  - Class 6.1 (Toxic Substances). A **RED** sticker must be placed on the box or container

    Examples: Potassium cyanide, mercuric chloride

  - Class 5.1 (Oxidizing Agents) and 8.1. (Acids). A ORANGE sticker must be placed on the box or container

    Examples: Calcium hypochlorite, ammonium nitrate, hydrogen peroxide

  - Class 5.2 (Organic Peroxides) must be grouped separately and a **BLUE** sticker must be placed on the box or container. These chemicals will be transported with Class 5.1(Oxidizing Agents) and 8.1. (Acids).

    Examples: benzonyl peroxides, cumene hydroperoxide, hydrochloric acid.

  (Note: no collection will take place if chemicals aren't identified by means of a colour coded sticker & class)

- Each grouped class will be collected on different days/loads to ensure legal compliance.
- All chemical containers must be properly sealed. The bottle or jar must have a cap that fits tightly. If the chemical is a liquid, there must be at least 3 cm of room at the top of the container. The outside of the container must be clean and dry.
  (Note: Chemicals shall not be removed if they are in leaking or otherwise inappropriate containers)
- The chemical, pharmaceutical and medical waste that needs to be disposed of, must be kept until your TRF or DRF has been returned by Oricol indicating service date and reference number.
- When the need to dispose of medical or pharmaceutical waste, a DRF (disposal request form) must be completed and sent to the Oricol contract manager or sales representative. An example of a DRF can be seen in Appendix B and should be in Word format where possible.
- On the day of collection, all chemical, pharmaceutical and medical waste needs to be at a centralised area at the collection point. A customer copy of the TRF/DRF must be placed with the waste. This TRF/DRF will have a unique number. A TRF/DRF that has not got a unique number on that matches Oricol's TRF/DRF will not be collected
- This is important as the collection staff will use this TRF/DRF to match up the TRF/DRF that was received to ensure the correct waste is removed.

Chemical, pharmaceutical and medical waste procedure

- Medical waste containers must be properly sealed. Bio-hazardous tape must be used in the case of medical waste boxes.
- The maximum weights for containers are shown below. Under no circumstances must the weight of the containers exceed the maximum weight.

Container	*Maximum allowed weight in kg*
5L Sharps and Lids (Needles Ect)	2.5
10L Sharps and Lids (Needles Ect)	3.5
20L Sharps and Lids (Needles Ect)	8
5L Anatomical Waste	2.5
10L Anatomical Waste	5
20L Anatomical Waste	12
20L Pharmaceutical waste	12
50L Box & Liner	9
142L Box & Liner	15

Bookings (For Oricol ES use only)

- The sales representative or Oricol contract manager will forward the TRF/DRF received from the client to the Hazardous Waste Clerk to generate a unique number.
- In the case of TRF's, a quote will be requested from the disposal site by the hazardous waste clerk.
- Once the disposal site sent the quote, the Hazardous waste clerk will reply with an acceptance form to the disposal site.
- The Hazardous waste clerk will make a booking with the Booking clerk. More than one booking shall be made if the TRF contains more than one group of classes. The Booking Clerk will complete the booking ref number and service date on the TRF/DRF and send it to the Hazardous waste Clerk.
- The Hazardous waste clerk will print out the TRF/DRF with the reference number and service date and prepare the stock.
- The Hazardous waste clerk will request delivery notes on daily basis from the logistics department which he will then use to match up with the TRF's/DRF's. Special care must be taken to ensure the waste type is correct and reflects the classes for collection.
- The Bookings waste clerk will then communicate the service date and reference number to the sales representative or Oricol contract manager which in turn needs to communicate this information with the client.

Collection and Handling (For Oricol ES use only)

- The collection staff will take the crates and a trolley with to ease loading and offloading of the waste
- When arriving at the site, the collection staff will confirm the TRF/DRF corresponds to the client's DRF/TRF. They will only check that the TRF/DRF is the same and not check each chemical item for item. It is still the client's responsibility to supply them with the correct waste.
- The collections staff will confirm containers are labelled sealed and have no residue.
- Oricol staff will not remove any waste that does not conform.

Chemical, pharmaceutical and medical waste procedure

- In the case of chemical waste, collection staff will collect chemicals as per the delivery note instruction of which colour to collect
- The client will then sign the delivery note to confirm collection. Should there be no one to sign for the collection; the waste will not be removed.
- The crate must then be sealed and the trolley used to transport the waste to the vehicle. When arriving at the vehicle, the boxes will be offloaded and placed inside the vehicle for chemical waste.
- In the case of medical waste, the trolley must be used to transport the containers or boxes to the vehicle.
- The waste will then be transported and disposed of at the appropriate disposal site.

ORICOL
ENVIRONMENTAL
SERVICES

Chemical, pharmaceutical and medical waste procedure

Treatment Request Form (TRF) for chemical waste

Generator Details				Waste Management Details
Company Name		Account Number		Oricol environmental Services
Contact Person		Department		JUAN-MARI DAVIES
Cell Number		Campus, Building Name, Room No:		083 305 4526
Tel Number				011 9225900
Fax Number				011 922 5901
Email Address				Juan-mari.davies@oricoles.co.za
*Booking can only be made once this form has been completed in full.				16 Vuurslag, Spartan ext7, Kempton park

Date booking made (Completed by Client):	Booking Reference number (Completed by booking clerk):	Service date: (Completed by booking clerk, Could be more than one):

Item No.	Chemical/Product/Shipping name	Container Capacity and Type	Number of Containers	PLEASE TICK ✓			Estimated Quantity and *Physical nature	UN NUMBER	CLASS
				EXCHANGE	REMOVE	PLACEMENT			

[2] Physical Nature to be specified according to symbol. **Solid = S, Sludge Solid = SS, Sludge Liquid = SL, Liquid = L, Powder = P, Gas Cylinder = G**

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Document: ……………………. Procedure

ORICOL ENVIRONMENTAL SERVICES

Chemical, pharmaceutical and medical waste procedure

Disposal Request Form (DRF) for medical and pharmaceutical waste

Generator Details				Waste Management Details
Company Name		Account Number		Oricol environmental Services
Contact Person		Department		JUAN-MARI DAVIES
Cell Number		Campus, Building Name, Room No:		083 305 4526
Tel Number				011 9225900
Fax Number				011 922 5901
Email Address				Juan-Marimari.davies@oricoles.co.za
Booking can only be made once this form has been completed in full.				16 Vuurslag, Spartan ext7, Kempton park

Date booking made (Completed by Client):	Booking Reference number (Completed by booking clerk):	Service date: (Completed by booking clerk):

Item No.	Product Name, Container Capacity and Type	Number of Containers	PLEASE TICK ✓			Estimated Quantity
			EXCHANGE	REMOVE	PLACEMENT	

Last printed	Date	Document Writer	Authorised By	Doc Rev No	Page No.
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ORICOL
ENVIRONMENTAL
SERVICES

Chemical, pharmaceutical and medical waste procedure

Revision Notes		
Date	**Rev: No**	**Notes**
26 January 2012	02	Added dangerous good load compatibility chart
24 September 2012	03	Added medical waste to procedure
9 October 2012	04	Added Account number and Site
31 January 2013	05	Update TRF document in landscape mode
23 May 2013	06	Update of collecting an packing of chemicals
22 October 2013	07	Update of classifying and packing
11 June 2014	08	Update checking of TRF/DRF

ADDENDUM B

ETHICS APPROVAL

Private Bag X1290, Potchefstroom
South Africa 2520

Tel: 086 016 9698
Web: http://www.nwu.ac.za/

North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC)

Tel: 018 299-1208
Email: Ethics-AnimCare@nwu.ac.za (for animal studies)

21 October 2019

ETHICS APPROVAL LETTER OF STUDY

Based on approval by the North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC) on 21/10/2019, the NWU-AnimCareREC hereby approves your study as indicated below. This implies that the NWU-AnimCareREC grants its permission that, provided the general conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: Extemporaneously prepared astaxanthin capsules for improved systemic delivery
Principal Investigator/Study Supervisor/Researcher: Prof JD Steyn
Student: V Lemmer - 24922846

Ethics number:

N	W	U	-	0	0	5	8	1	-	1	9	-	A	5

Institution — Study Number — Year — Status

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Application Type: Single study
Commencement date: 21/10/2019
Expiry date: 30/10/2020

Risk: **Category 0**

Approval of the study is provided for a year, after which continuation of the study is dependent on receipt and review of an annual monitoring report and the concomitant issuing of a letter of continuation. A monitoring report is due at the end of October annually until completion.

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, the following general terms and conditions will apply:

- *The principal investigator/study supervisor/researcher must report in the prescribed format to the NWU-AnimCareREC:*
  - *Annually on the monitoring of the study, whereby a letter of continuation will be provided annually, and upon completion of the study; and*
  - *without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.*
- *The approval applies strictly to the proposal as stipulated in the application form. Should any amendments to the proposal be deemed necessary during the course of the study, the principal investigator/study supervisor/researcher must apply for approval of these amendments at the NWU-AnimCareREC, prior to implementation. Should there be any deviations from the study proposal*

without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.

- *Annually a number of studies may be randomly selected for active monitoring.*
- *The date of approval indicates the first date that the study may be started.*
- *In the interest of ethical responsibility, the NWU-AnimCareREC reserves the right to:*
  - *request access to any information or data at any time during the course or after completion of the study;*
  - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process;*
  - *withdraw or postpone approval if:*
    - *any unethical principles or practices of the study are revealed or suspected;*
    - *it becomes apparent that any relevant information was withheld from the NWU-AnimCareREC or that information has been false or misrepresented;*
    - *submission of the annual monitoring report, the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and/or*
    - *new institutional rules, national legislation or international conventions deem it necessary.*
- *NWU-AnimCareREC can be contacted for further information via Ethics-AnimCare@nwu.ac.za or 018 299 1208*

NWU-AnimCareREC would like to remain at your service and wishes you well with your study. Please do not hesitate to contact the NWU-AnimCareREC for any further enquiries or requests for assistance.

Yours sincerely,

Digitally signed by Christiaan B Brink
Date: 2019.10.24 16:30:07 +02'00'

Chairperson: NWU-AnimCareREC

Current details:(23239522) G:\My Drive\9. Research and Postgraduate Education\9.1.5.4 Templates\9.1.5.4.2_NWU-AC_EAL.docm
20 August 2019

File Reference: 9.1.5.4.2

ADDENDUM C

EXPERIMENTAL DATA

Ex vivo transport data

Table C-1: Apical to basolateral transport of the control group

Control	% Transport				
Time	**1**	**2**	**3**	**Average**	**STDEV**
0	0	0	0	0	0
20	0	0	7.255389718	2.418463239	3.420223513
40	2.122369777	3.773178094	34.59453938	2.947773936	0.825404158
60	1.303741435	2.514670866	9.745956209	4.52145617	3.727209682
80	2.069815859	4.956690478	11.69875261	6.241752983	4.03465364
100	2.222424353	3.515383317	20.56444829	8.767418653	8.358443512
120	1.446243405	4.314547125	24.68635085	10.14904713	10.34590712

Table C-2: Basolateral to apical transport of the control group

Control	% Transport				
Time	**1**	**2**	**3**	**Average**	**STDEV**
0	0	0	0	0	0
20	3.996447602	1.792917974	1.923899102	2.571088226	1.009298775
40	7.716168559	2.640656364	3.063987459	4.473604127	2.299343439
60	5.815549892	6.062466302	4.597530222	5.491848805	0.640362514
80	6.688633185	6.565912472	3.101164253	5.451903303	1.662978383
100	8.653606885	6.117682978	3.513207056	6.094832307	2.098621645
120	10.48343501	6.452231079	4.820590987	7.252085693	2.380024674

Table C-3: Apical-to-basolateral transport of the formulation containing the low concentration *Aloe vera* gel

AVG	% Transport				
Time	**1**	**2**	**3**	**Average**	**STDEV**
0	0	0	0	0	0
20	0	0	6.941649899	2.3138833	3.272325144
40	3.711558855	1.822861893	27.55518619	11.02986898	11.71057573
60	9.299361872	3.987259998	37.64470883	16.97711023	14.7742305
80	15.43413146	5.735504742	43.50013122	21.55658914	16.0136425
100	9.074249781	4.778502249	49.60341761	21.15205655	20.19444348
120	15.10297483	6.727562273	40.29976963	20.71010224	14.26775351

Table C-4: Basolateral-to-apical transport of the formulation containing the low concentration Aloe vera gel

AVG	% Transport				
Time	**1**	**2**	**3**	**Average**	**STDEV**
0	0	0	0	0	0
20	8.709245199	4.366123093	5.397365066	6.157577786	1.852767289
40	11.86153239	9.509106572	11.46054682	10.94372859	1.027554577
60	16.2527267	15.8246232	14.69231385	15.58988791	0.658304666
80	24.19882064	30.90974239	14.52170217	23.2100884	6.726819909
100	26.12385187	21.14447773	18.70076436	21.98969799	3.088835272
120	23.29520814	23.29666308	12.76754313	19.78647145	4.963131847

Table C-5: Apical-to-basolateral transport of the formulation containing the medium concentration *Aloe vera* gel

AVG	% Transport				
Time	**1**	**2**	**3**	**Average**	**STDEV**
0	0	0	0	0	0
20	2.901998097	4.853041695	12.33283804	6.695959277	4.064680751
40	4.240957542	5.964516033	15.25292332	8.486132297	4.836304304
60	5.513727437	8.336057535	23.50496371	12.4515829	7.900392709
80	6.496918049	9.687261632	26.7879062	14.32402863	8.909012519
100	7.234655745	7.556440508	21.25567974	12.015592	6.53504922
120	11.44389746	15.98561621	38.81280014	22.08077127	11.97573644

Table C-6: Basolateral-to-apical transport of the formulation containing the medium concentration *Aloe vera* gel

AVG	% Transport				
Time	**1**	**2**	**3**	**Average**	**STDEV**
0	0	0	0	0	0
20	1.113262343	6.202531646	4.679376083	3.998390024	2.132755967
40	13.1760596	9.65694368	7.745376301	10.19279319	2.249211906
60	16.93253083	15.22564667	8.3733119	13.51049647	3.698771178
80	17.81851088	24.9357916	9.671045471	17.47511598	6.236535289
100	17.87954038	36.63089341	14.02640679	22.84561353	9.873773875
120	25.29497594	26.77949	9.282562647	20.45234286	7.921445072

Table C-7: Apical-to-basolateral transport of the formulation containing the high concentration *Aloe vera* gel

AVG	% Transport				
Time	**1**	**2**	**3**	**Average**	**STDEV**
0	0	0	0	0	0
20	0	0	0	0	0
40	0.945599105	1.015026978	1.22007547	1.060233851	0.116524861
60	2.647677493	1.296639726	0.993490026	1.645935748	0.719068762
80	5.981396785	1.941525288	0.01394372	2.645621931	2.486555594
100	5.285706015	2.324640734	1.403086791	3.004477847	1.656364872
120	7.706632702	2.864207707	1.97216485	4.181001753	2.519456331

Table C-8: Basolateral-to-apical transport of the formulation containing the high concentration *Aloe vera* gel

AVG	% Transport				
Time	**1**	**2**	**3**	**Average**	**STDEV**
0	0	0	0	0	0
20	5.693069307	2.981148619	2.160493827	3.611570584	1.509491365
40	5.983641842	4.001550299	3.726069064	4.570420402	1.005607149
60	9.563782465	4.859933032	4.561638934	6.328451477	2.290963377
80	10.80768403	6.917891339	0.064412238	5.929995869	4.441202937
100	11.89195006	6.546836183	6.387546967	8.275444405	2.558082375
120	12.72331037	6.452166289	4.537484344	7.904320336	3.496045431

Table C-9: Apical-to-basolateral transport of the formulation containing the low concentration *Aloe vera* whole leaf

AVWL	% Transport				
Time	**1**	**2**	**3**	**Average**	**STDEV**
0	0	0	0	0	0
20	0	0	0	0	0
40	0	0	20.76914299	6.923047663	9.790667897
60	10.78552219	7.464495177	24.0613945	14.10380395	7.170425938
80	14.69819214	14.35703553	31.49321009	20.18281259	7.998871408
100	15.0787203	15.30221511	32.39758074	20.92617205	8.112024014
120	19.82248244	17.51975183	39.99771768	25.77998398	10.09731336

Table C-10: Basolateral-to-apical transport of the formulation containing the low concentration *Aloe vera* whole leaf

AVWL	% Transport				
Time	**1**	**2**	**3**	**Average**	**STDEV**
0	0	0	0	0	0
20	17.20714758	10.34985423	6.413612565	11.32353813	4.459906187
40	22.74144199	14.49486627	6.997875408	14.74472789	6.429712035
60	31.54164149	15.88076224	10.45602853	19.29281075	8.939885336
80	28.81286028	19.74479233	11.03649746	19.86471669	7.257665152
100	36.99680603	17.877213	12.23916837	22.37106247	10.5950041
120	37.78570675	22.87784679	13.05296305	24.5721722	10.1679302

Table C-11: Apical-to-basolateral transport of the formulation containing the medium concentration *Aloe vera* whole leaf

AVWL	% Transport				
Time	**1**	**2**	**3**	**Average**	**STDEV**
0	0	0	0	0	0
20	0	0	5.107003891	1.70233463	2,407464722
40	7.550519518	3.072710534	11.54626967	7.38983324	3,461181509
60	18.30337881	8.846796314	15.21351435	14.12122983	3,937135359
80	19.32190323	9.426933554	9.960243614	12.9030268	4,544050019
100	28.24636974	14.99814926	21.60343709	21.61598536	5,408570642
120	29.36391736	20.14345643	23.74457227	24.41731535	3,794176447

Table C-12: Basolateral-to-apical transport of the formulation containing the medium concentration *Aloe vera* whole leaf

AVWL	% Transport				
Time	**1**	**2**	**3**	**Average**	**STDEV**
0	0	0	0	0	0
20	44.17604236	35.42274052	9.947643979	29.84880895	14.51889226
40	41.76378058	35.71006042	5.854010168	27.77595039	15.69693132
60	64.87449525	44.42472641	10.83731694	40.04551287	22.27685336
80	49.09168513	46.1761102	22.59465817	39.2874845	11.86347286
100	45.33661363	54.33303756	12.53699067	37.40221395	17.96187458
120	47.80402651	78.70241264	18.76470142	48.42371352	24.47339121

Table C-13: Apical-to-basolateral transport of the formulation containing the high concentration *Aloe vera* whole leaf

AVWL	% Transport				
Time	**1**	**2**	**3**	**Average**	**STDEV**
0	0	0	0	0	0
20	4.016709512	3.430787589	9.836065574	5.761187558	2.891285672
40	3.970138967	3.530230708	9.289617486	5.596662387	2.617481976
60	3.478819716	3.378904223	10.94084105	5.932854998	3.541415822
80	3.960824857	3.376742416	10.54882395	5.962130408	3.252035906
100	4.456801162	4.890007264	12.82965075	7.392153058	3.848956805
120	4.300789836	6.122237211	8.496673794	6.306566947	1.717914169

Table C-14: Basolateral-to-apical transport of the formulation containing the high concentration Aloe vera whole leaf

AVWL	% Transport				
Time	**1**	**2**	**3**	**Average**	**STDEV**
0	0	0	0	0	0
20	8.130918073	10.41666667	6.976744186	8.508109642	1.429445528
40	6.904791738	8406929348	8.527131783	7.946284289	0.738079584
60	9.703104397	12.33676781	19.83725745	13.95904322	4.293335739
80	10.57869583	13.71433424	7.86508739	10.71937249	2.390015986
100	11.63477545	14.2285628	11.39920073	12.42084633	1.281861407
120	10.18788671	16.71242829	10.44104194	12.44711898	3.017799368

Table C-15: Apical-to-basolateral transport of the formulation containing the low concentration piperine

Piperine	% Transport				
Time	**1**	**2**	**3**	**Average**	**STDEV**
0	0	0	0	0	0
20	0	9.623259623	40.74074074	16.78800012	17.38681721
40	12.52684324	19.24948664	66.34460548	32.70697845	23.94320986
60	8.348635274	24.99139456	83.2259796	38.85533648	32.10203356
80	10.64651998	30.05673658	60.54213634	33.7484643	20.53638678
100	12.49572064	28.05077805	74.11701557	38.22117142	26.16452653
120	11.79546233	25.11305772	91.22383253	42.71078419	34.73208393

Table C-16: Basolateral-to-apical transport of the formulation containing the low concentration piperine

Piperine	% Transport				
Time	**1**	**2**	**3**	**Average**	**STDEV**
0	0	0	0	0	0
20	20.30019685	12.84385113	14.19129082	15.77844627	3.244335543
40	22.0119394	14.34471882	15.58872585	17.31512803	3.359753583
60	26.64612576	17.54139112	19.34996732	21.1791614	3.935607657
80	29.70764864	17.58682754	18.61462348	21.96969989	5.487621177
100	24.75785975	16.56084377	19.78666577	20.36845643	3.371609708
120	24.43690802	13.87862905	18.22582099	18.84711936	4.33272989

Table C-17: Apical-to-basolateral transport of the formulation containing the medium concentration piperine

Piperine		% Transport			
Time	**1**	**2**	**3**	**Average**	**STDEV**
0	0	0	0	0	0
20	0	7.283464567	24.34738956	10.54361804	10.20360378
40	26.50744026	11.68834874	34.98413946	24.39330949	9.627240102
60	23.91728467	16.54113888	38.18171236	26.21337863	8.98267372
80	35.53814648	20.20426794	32.87788837	29.54010093	6.690170723
100	31.25151471	18.85769714	29.74579477	26.61833554	5.521921735
120	57.48327759	22.43238617	40.14245387	40.01937254	14.30973117

Table C-18: Basolateral-to-apical transport of the formulation containing the medium concentration piperine

Piperine	% Transport				
Time	1	2	3	Average	STDEV
0	0	0	0	0	0
20	12.37133808	20.18229167	13.12849162	15.22737379	3.517264917
40	16.54531998	24.59805254	18.60982916	19.91773389	3.415121911
60	16.80063571	20.42572464	26.33390009	21.18675348	3.928965607
80	19.11282087	21.29000604	35.22791677	25.21024789	7.139108306
100	17.03730478	21.30321558	33.93652336	24.09234791	7.175436872
120	28.95538573	24.0772192	38.16695004	30.39985166	5.842088107

Table C-19: Apical-to-basolateral transport of the formulation containing the high concentration piperine

Piperine	% Transport				
Time	1	2	3	Average	STDEV
0	0	0	0	0	0
20	23.04469274	30.46218487	3.869303525	19.12539371	11.20464229
40	16.27398591	32.76600495	12.48644809	20.50881299	8.803992537
60	17.93883086	33.49673203	12.82602465	21.42052918	8.790570829
80	26.11124605	35.28295376	13.91952347	25.10457443	8.750583952
100	20.20585378	31.04575163	15.4616372	22.23774754	6.522402415
120	19.76560602	30.27442861	13.95690805	21.33231423	6.753088197

Table C-20: Basolateral-to-apical transport of the formulation containing the high concentration piperine

Piperine	% Transport				
Time	1	2	3	Average	STDEV
0	0	0	0	0	0
20	12.43169399	5.42041248	4.963898917	7.605335129	3.417836162
40	27.62136691	15.36837345	22.80951883	21.9330864	5.040506755
60	18.1317811	19.12951509	14.9745374	17.41194453	1.770985305
80	24.92278451	19.31536876	22.18385392	22.14066906	2.289421551
100	2377445157	17.17134558	20.45728039	20.46769251	2.695716787
120	33.18088224	17.40893171	21.19412616	23.92798003	6.722800688

Dissolution studies

Table C-21: Dissolution data of astaxanthin only formulation

	% Dissolution				
Time	**1**	**2**	**3**	**Average**	**STDEV**
0	0	0	0	0	0
15	15.61884738	16.98772095	16.40823114	16.33826649	0.561025845
30	15.46334335	19.88480496	18.16422215	17.83745682	1.819782477
60	16.4587882	19.80924314	19.1983605	18.48879728	1.456936751
90	17.62233073	21.40425462	20.41976075	19.8154487	1.602005381
120	15.92876036	20.94814595	19.74152953	18.87281195	2.139246068
180	15.21585101	20.24290229	19.66231738	18.37369023	2.245474131
240	15.39544722	21.18742505	20.20329621	18.92872283	2.53050124

Table C-22: Dissolution data of formulation containing astaxanthin and low concentration piperine

	% Dissolution				
Time	**1**	**2**	**3**	**Average**	**STDEV**
0	0	0	0	0	0
15	34.77289198	35.88817374	39.38006782	36.68037785	1.962516719
30	40.79753783	40.8421491	36.6401923	39.42662641	1.970390625
60	37.13250953	34.47707677	39.7204943	37.11002687	2.140675274
90	35.62422372	35.69060954	31.43288866	34.24924064	1.991645993
120	31.3877463	35.63006568	38.99609224	35.33796807	3.112953876
180	35.40276063	32.43876659	45.64529587	37.82894103	5.657907
240	30.64422513	30.53216587	45.38081477	35.51906859	6.973457661

Table C-23: Dissolution data of formulation containing astaxanthin and medium concentration piperine

	% Dissolution				
Time	**1**	**2**	**3**	**Average**	**STDEV**
0	0	0	0	0	0
15	32.37743148	32.32382646	32.23001768	32.31042521	0.060922916
30	35.36108684	36.32383298	35.55620911	35.74704298	0.415558425
60	36.01828437	37.74543808	36.9510117	36.90491139	0.705860664
90	33.81726229	36.05580789	36.41388941	35.42898653	1.148998654
120	36.01989252	31.63071357	34.99657271	34.21572627	1.875013574
180	30.46962885	32.75641897	35.08823729	32.77142837	1.885568869
240	31.82529978	30.26217743	32.50769168	31.53172296	0.939937611

Table C-24: Dissolution data of formulation containing astaxanthin and high concentration piperine

	% Dissolution				
Time	**1**	**2**	**3**	**Average**	**STDEV**
0	0	0	0	0	0
15	23.33953631	21.24610461	22.95351344	22.51305145	0.909622315
30	26.17353802	27.73782144	27.2270835	27.04614765	0.651305865
60	27.34823529	29.18095308	29.6477438	28.72564406	0.992442972
90	26.60528974	29.03663991	33.34703061	29.66298676	2.787711159
120	25.8463094	29.14769572	33.40226158	29.46542223	3.092875244
180	25.20848085	30.44413867	32.56726442	29.40662798	3.092490391
240	24.23392159	28.72900938	31.65803211	28.20698769	3.053275206

ADDENDUM D

STATISTICAL ANALYSIS

Table D-1.1: Tukey HSD post hoc multiple comparison of apical-to-basolateral transport of astaxanthin in combination with different bioenhancers (significant differences markes as p < 0.05000)

Name	Tukey HSD test; Variable: Papp Marked differences are significant at p < .05000			
	1 M=.00001	2 M=0.0000	3 M=.00002	4 M=0.0000
Astaxanthin/FeSSIF 1			0.928929	
Astaxanthin/DMSO 2				
Astaxanthin/Aloe vera gel/FeSSIF-Low 3	0.928929			
Astaxanthin/Aloe vera gel/DMSO-Low 4				
Astaxanthin/Aloe vera gel/FeSSIF-Medium 5	0.996533		0.999948	
Astaxanthin/Aloe vera gel/DMSO-Medium 6				
Astaxanthin/Aloe vera gel/FeSSIF-High 7	1.000000		0.816488	
Astaxanthin/Aloe vera gel/DMSO-High 8				
Astaxanthin/Aloe vera whole leaf/FeSSIF-Low 9	0.755989		0.999992	
Astaxanthin/Aloe vera whole leaf/DMSO-Low 10				
Astaxanthin/Aloe vera whole leaf/FeSSIF-Medium 11	0.877323		1.000000	
Astaxanthin/Aloe vera whole leaf/DMSO-Medium 12				
Astaxanthin/Aloe vera whole leaf/FeSSIF-High 13	1.000000		0.837398	
Astaxanthin/Aloe vera whole leaf/DMSO-High 14				
Astaxanthin/Piperine/FeSSIF-Low 15	0.340398		0.976660	
Astaxanthin/Piperine/DMSO-Low 16				
Astaxanthin/Piperine/FeSSIF-Medium 17	0.486050		0.996428	
Astaxanthin/Piperine/DMSO-Medium 18				
Astaxanthin/Piperine/FeSSIF-High 19	0.999158		0.999592	
Astaxanthin/Piperine/DMSO-High 20				

Table D-1.2: Tukey HSD post hoc multiple comparison of apical-to-basolateral transport of astaxanthin in combination with different bioenhancers continues (significant differences markes as $p < 0.05000$)

Name	Tukey HSD test; Variable: Papp Marked differences are significant at p < .05000			
	5 M=.00001	6 M=0.0000	7 M=.00000	8 M=0.0000
Astaxanthin/FeSSIF 1	0.996533		1.000000	
Astaxanthin/DMSO 2				
Astaxanthin/Aloe vera gel/FeSSIF-Low 3	0.999948		0.816488	
Astaxanthin/Aloe vera gel/DMSO-Low 4				
Astaxanthin/Aloe vera gel/FeSSIF-Medium 5			0.974478	
Astaxanthin/Aloe vera gel/DMSO-Medium 6				
Astaxanthin/Aloe vera gel/FeSSIF-High 7	0.974478			
Astaxanthin/Aloe vera gel/DMSO-High 8				
Astaxanthin/Aloe vera whole leaf/FeSSIF-Low 9	0.994621		0.586275	
Astaxanthin/Aloe vera whole leaf/DMSO-Low 10				
Astaxanthin/Aloe vera whole leaf/FeSSIF-Medium 11	0.999558		0.736750	
Astaxanthin/Aloe vera whole leaf/DMSO-Medium 12				
Astaxanthin/Aloe vera whole leaf/FeSSIF-High 13	0.980157		1.000000	
Astaxanthin/Aloe vera whole leaf/DMSO-High 14				
Astaxanthin/Piperine/FeSSIF-Low 15	0.824221		0.217831	
Astaxanthin/Piperine/DMSO-Low 16				
Astaxanthin/Piperine/FeSSIF-Medium 17	0.927924		0.330918	
Astaxanthin/Piperine/DMSO-Medium 18				
Astaxanthin/Piperine/FeSSIF-High 19	1.000000		0.989511	
Astaxanthin/Piperine/DMSO-High 20				

Table D-1.3: Tukey HSD post hoc multiple comparison of apical-to-basolateral transport of astaxanthin in combination with different bioenhancers continues (significant differences markes as $p < 0.05000$)

Name	Tukey HSD test; Variable: Papp Marked differences are significant at p < .05000			
	9 M=.00002	10 M=0.0000	11 M=.00002	12 M=0.0000
Astaxanthin/FeSSIF 1	0.755989		0.877323	
Astaxanthin/DMSO 2				
Astaxanthin/Aloe vera gel/FeSSIF-Low 3	0.999992		1.000000	
Astaxanthin/Aloe vera gel/DMSO-Low 4				
Astaxanthin/Aloe vera gel/FeSSIF-Medium 5	0.994621		0.999558	
Astaxanthin/Aloe vera gel/DMSO-Medium 6				
Astaxanthin/Aloe vera gel/FeSSIF-High 7	0.586275		0.736750	
Astaxanthin/Aloe vera gel/DMSO-High 8				
Astaxanthin/Aloe vera whole leaf/FeSSIF-Low 9			1.000000	
Astaxanthin/Aloe vera whole leaf/DMSO-Low 10				
Astaxanthin/Aloe vera whole leaf/FeSSIF-Medium 11	1.000000			
Astaxanthin/Aloe vera whole leaf/DMSO-Medium 12				
Astaxanthin/Aloe vera whole leaf/FeSSIF-High 13	0.613049		0.761149	
Astaxanthin/Aloe vera whole leaf/DMSO-High 14				
Astaxanthin/Piperine/FeSSIF-Low 15	0.999041		0.991038	
Astaxanthin/Piperine/DMSO-Low 16				
Astaxanthin/Piperine/FeSSIF-Medium 17	0.999978		0.999186	
Astaxanthin/Piperine/DMSO-Medium 18				
Astaxanthin/Piperine/FeSSIF-High 19	0.985045		0.997910	
Astaxanthin/Piperine/DMSO-High 20				

Table D-1.4: Tukey HSD post hoc multiple comparison of apical-to-basolateral transport of astaxanthin in combination with different bioenhancers continues (significant differences markes as p < 0.05000)

Name	Tukey HSD test; Variable: Papp Marked differences are significant at p < .05000			
	13 M=.00000	14 M=0.0000	15 M=.00003	16 M=0.0000
Astaxanthin/FeSSIF 1	1.000000		0.340398	
Astaxanthin/DMSO 2				
Astaxanthin/Aloe vera gel/FeSSIF-Low 3	0.837398		0.976660	
Astaxanthin/Aloe vera gel/DMSO-Low 4				
Astaxanthin/Aloe vera gel/FeSSIF-Medium 5	0.980157		0.824221	
Astaxanthin/Aloe vera gel/DMSO-Medium 6				
Astaxanthin/Aloe vera gel/FeSSIF-High 7	1.000000		0.217831	
Astaxanthin/Aloe vera gel/DMSO-High 8				
Astaxanthin/Aloe vera whole leaf/FeSSIF-Low 9	0.613049		0.999041	
Astaxanthin/Aloe vera whole leaf/DMSO-Low 10				
Astaxanthin/Aloe vera whole leaf/FeSSIF-Medium 11	0.761149		0.991038	
Astaxanthin/Aloe vera whole leaf/DMSO-Medium 12				
Astaxanthin/Aloe vera whole leaf/FeSSIF-High 13			0.234097	
Astaxanthin/Aloe vera whole leaf/DMSO-High 14				
Astaxanthin/Piperine/FeSSIF-Low 15	0.234097			
Astaxanthin/Piperine/DMSO-Low 16				
Astaxanthin/Piperine/FeSSIF-Medium 17	0.352560		1.000000	
Astaxanthin/Piperine/DMSO-Medium 18				
Astaxanthin/Piperine/FeSSIF-High 19	0.992328		0.749326	
Astaxanthin/Piperine/DMSO-High 20				

Table D-1.5: Tukey HSD post hoc multiple comparison of apical-to-basolateral transport of astaxanthin in combination with different bioenhancers continues (significant differences markes as $p < 0.05000$)

Name	Tukey HSD test; Variable: Papp Marked differences are significant at p < .05000			
	17 M=.00003	18 M=0.0000	19 M=.00001	20 M=0.0000
Astaxanthin/FeSSIF 1	0.486050		0.999158	
Astaxanthin/DMSO 2				
Astaxanthin/Aloe vera gel/FeSSIF-Low 3	0.996428		0.999592	
Astaxanthin/Aloe vera gel/DMSO-Low 4				
Astaxanthin/Aloe vera gel/FeSSIF-Medium 5	0.927924		1.000000	
Astaxanthin/Aloe vera gel/DMSO-Medium 6				
Astaxanthin/Aloe vera gel/FeSSIF-High 7	0.330918		0.989511	
Astaxanthin/Aloe vera gel/DMSO-High 8				
Astaxanthin/Aloe vera whole leaf/FeSSIF-Low 9	0.999978		0.985045	
Astaxanthin/Aloe vera whole leaf/DMSO-Low 10				
Astaxanthin/Aloe vera whole leaf/FeSSIF-Medium 11	0.999186		0.997910	
Astaxanthin/Aloe vera whole leaf/DMSO-Medium 12				
Astaxanthin/Aloe vera whole leaf/FeSSIF-High 13	0.352560		0.992328	
Astaxanthin/Aloe vera whole leaf/DMSO-High 14				
Astaxanthin/Piperine/FeSSIF-Low 15	1.000000		0.749326	
Astaxanthin/Piperine/DMSO-Low 16				
Astaxanthin/Piperine/FeSSIF-Medium 17			0.878521	
Astaxanthin/Piperine/DMSO-Medium 18				
Astaxanthin/Piperine/FeSSIF-High 19	0.878521			
Astaxanthin/Piperine/DMSO-High 20				

Table D-2.1: Tukey HSD post hoc multiple comparison of basolateral-to-apical transport of astaxanthin in combination with different bioenhancers (significant differences markes as p < 0.05000)

Name	Tukey HSD test; Variable: Papp Marked differences are significant at p < .05000			
	1 M=0.0000	2 M=.00000	3 M=0.0000	4 M=.00002
Astaxanthin/FeSSIF 1				
Astaxanthin/DMSO 2				0.652439
Astaxanthin/Aloe vera gel/FeSSIF-Low 3				
Astaxanthin/Aloe vera gel/DMSO-Low 4		0.652439		
Astaxanthin/Aloe vera gel/FeSSIF-Medium 5				
Astaxanthin/Aloe vera gel/DMSO-Medium 6		0.603718		1.000000
Astaxanthin/Aloe vera gel/FeSSIF-High 7				
Astaxanthin/Aloe vera gel/DMSO-High 8		1.000000		0.724258
Astaxanthin/Aloe vera whole leaf/FeSSIF-Low 9				
Astaxanthin/Aloe vera whole leaf/DMSO-Low 10		0.689573		1.000000
Astaxanthin/Aloe vera whole leaf/FeSSIF-Medium 11				
Astaxanthin/Aloe vera whole leaf/DMSO-Medium 12		0.034196		0.728629
Astaxanthin/Aloe vera whole leaf/FeSSIF-High 13				
Astaxanthin/Aloe vera whole leaf/DMSO-High 14		0.999966		0.895584
Astaxanthin/Piperine/FeSSIF-Low 15				
Astaxanthin/Piperine/DMSO-Low 16		0.983065		0.996084
Astaxanthin/Piperine/FeSSIF-Medium 17				
Astaxanthin/Piperine/DMSO-Medium 18		0.478674		1.000000
Astaxanthin/Piperine/FeSSIF-High 19				
Astaxanthin/Piperine/DMSO-High 20		0.738569		1.000000

Table D-2.2: Tukey HSD post hoc multiple comparison of basolateral-to-apical transport of astaxanthin in combination with different bioenhancers continues (significant differences markes as $p < 0.05000$)

Name	Tukey HSD test; Variable: Papp Marked differences are significant at p < .05000			
	5 M=0.0000	6 M=.00002	7 M=0.0000	8 M=.00001
Astaxanthin/FeSSIF 1				
Astaxanthin/DMSO 2		0.603718		1.000000
Astaxanthin/Aloe vera gel/FeSSIF-Low 3				
Astaxanthin/Aloe vera gel/DMSO-Low 4		1.000000		0.724258
Astaxanthin/Aloe vera gel/FeSSIF-Medium 5				
Astaxanthin/Aloe vera gel/DMSO-Medium 6				0.677317
Astaxanthin/Aloe vera gel/FeSSIF-High 7				
Astaxanthin/Aloe vera gel/DMSO-High 8		0.677317		
Astaxanthin/Aloe vera whole leaf/FeSSIF-Low 9				
Astaxanthin/Aloe vera whole leaf/DMSO-Low 10		1.000000		0.759124
Astaxanthin/Aloe vera whole leaf/FeSSIF-Medium 11				
Astaxanthin/Aloe vera whole leaf/DMSO-Medium 12		0.773058		0.043868
Astaxanthin/Aloe vera whole leaf/FeSSIF-High 13				
Astaxanthin/Aloe vera whole leaf/DMSO-High 14		0.864066		0.999997
Astaxanthin/Piperine/FeSSIF-Low 15				
Astaxanthin/Piperine/DMSO-Low 16		0.992588		0.992563
Astaxanthin/Piperine/FeSSIF-Medium 17				
Astaxanthin/Piperine/DMSO-Medium 18		1.000000		0.551234
Astaxanthin/Piperine/FeSSIF-High 19				
Astaxanthin/Piperine/DMSO-High 20		1.000000		0.803795

Table D-2.3: Tukey HSD post hoc multiple comparison of basolateral-to-apical transport of astaxanthin in combination with different bioenhancers continues (significant differences markes as $p < 0.05000$)

Name	Tukey HSD test; Variable: Papp Marked differences are significant at p < .05000			
	9 M=0.0000	10 M=.00002	11 M=0.0000	12 M=.00003
Astaxanthin/FeSSIF 1				
Astaxanthin/DMSO 2		0.689573		0.034196
Astaxanthin/Aloe vera gel/FeSSIF-Low 3				
Astaxanthin/Aloe vera gel/DMSO-Low 4		1.000000		0.728629
Astaxanthin/Aloe vera gel/FeSSIF-Medium 5				
Astaxanthin/Aloe vera gel/DMSO-Medium 6		1.000000		0.773058
Astaxanthin/Aloe vera gel/FeSSIF-High 7				
Astaxanthin/Aloe vera gel/DMSO-High 8		0.759124		0.043868
Astaxanthin/Aloe vera whole leaf/FeSSIF-Low 9				
Astaxanthin/Aloe vera whole leaf/DMSO-Low 10				0.692553
Astaxanthin/Aloe vera whole leaf/FeSSIF-Medium 11				
Astaxanthin/Aloe vera whole leaf/DMSO-Medium 12		0.692553		
Astaxanthin/Aloe vera whole leaf/FeSSIF-High 13				
Astaxanthin/Aloe vera whole leaf/DMSO-High 14		0.916728		0.088877
Astaxanthin/Piperine/FeSSIF-Low 15				
Astaxanthin/Piperine/DMSO-Low 16		0.997751		0.252913
Astaxanthin/Piperine/FeSSIF-Medium 17				
Astaxanthin/Piperine/DMSO-Medium 18		0.999998		0.872006
Astaxanthin/Piperine/FeSSIF-High 19				
Astaxanthin/Piperine/DMSO-High 20		1.000000		0.641843

Table D-2.4: Tukey HSD post hoc multiple comparison of basolateral-to-apical transport of astaxanthin in combination with different bioenhancers continues (significant differences markes as p < 0.05000)

Name	Tukey HSD test; Variable: Papp Marked differences are significant at p < .05000			
	13 M=0.0000	14 M=.00001	15 M=0.0000	16 M=.00001
Astaxanthin/FeSSIF 1				
Astaxanthin/DMSO 2		0.999966		0.983065
Astaxanthin/Aloe vera gel/FeSSIF-Low 3				
Astaxanthin/Aloe vera gel/DMSO-Low 4		0.895584		0.996084
Astaxanthin/Aloe vera gel/FeSSIF-Medium 5				
Astaxanthin/Aloe vera gel/DMSO-Medium 6		0.864066		0.992588
Astaxanthin/Aloe vera gel/FeSSIF-High 7				
Astaxanthin/Aloe vera gel/DMSO-High 8		0.999997		0.992563
Astaxanthin/Aloe vera whole leaf/FeSSIF-Low 9				
Astaxanthin/Aloe vera whole leaf/DMSO-Low 10		0.916728		0.997751
Astaxanthin/Aloe vera whole leaf/FeSSIF-Medium 11				
Astaxanthin/Aloe vera whole leaf/DMSO-Medium 12		0.088877		0.252913
Astaxanthin/Aloe vera whole leaf/FeSSIF-High 13				
Astaxanthin/Aloe vera whole leaf/DMSO-High 14				0.999803
Astaxanthin/Piperine/FeSSIF-Low 15				
Astaxanthin/Piperine/DMSO-Low 16		0.999803		
Astaxanthin/Piperine/FeSSIF-Medium 17				
Astaxanthin/Piperine/DMSO-Medium 18		0.762712		0.972229
Astaxanthin/Piperine/FeSSIF-High 19				
Astaxanthin/Piperine/DMSO-High 20		0.940924		0.999047

Table D-2.5: Tukey HSD post hoc multiple comparison of basolateral-to-apical transport of astaxanthin in combination with different bioenhancers continues (significant differences markes as $p < 0.05000$)

Name	Tukey HSD test; Variable: Papp Marked differences are significant at p < .05000			
	17 M=0.0000	18 M=.00002	19 M=0.0000	20 M=.00002
Astaxanthin/FeSSIF 1				
Astaxanthin/DMSO 2		0.478674		0.738569
Astaxanthin/Aloe vera gel/FeSSIF-Low 3				
Astaxanthin/Aloe vera gel/DMSO-Low 4		1.000000		1.000000
Astaxanthin/Aloe vera gel/FeSSIF-Medium 5				
Astaxanthin/Aloe vera gel/DMSO-Medium 6		1.000000		1.000000
Astaxanthin/Aloe vera gel/FeSSIF-High 7				
Astaxanthin/Aloe vera gel/DMSO-High 8		0.551234		0.803795
Astaxanthin/Aloe vera whole leaf/FeSSIF-Low 9				
Astaxanthin/Aloe vera whole leaf/DMSO-Low 10		0.999998		1.000000
Astaxanthin/Aloe vera whole leaf/FeSSIF-Medium 11				
Astaxanthin/Aloe vera whole leaf/DMSO-Medium 12		0.872006		0.641843
Astaxanthin/Aloe vera whole leaf/FeSSIF-High 13				
Astaxanthin/Aloe vera whole leaf/DMSO-High 14		0.762712		0.940924
Astaxanthin/Piperine/FeSSIF-Low 15				
Astaxanthin/Piperine/DMSO-Low 16		0.972229		0.999047
Astaxanthin/Piperine/FeSSIF-Medium 17				
Astaxanthin/Piperine/DMSO-Medium 18				0.999985
Astaxanthin/Piperine/FeSSIF-High 19				
Astaxanthin/Piperine/DMSO-High 20		0.999985		

Table D-3: Analysis of variance of apical-to-basolateral transport

Variable	Analysis of Variance Marked effects are significant at p < .05000							
	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p
Papp	0.000000	9	0.000000	0.000000	20	0.000000	1.962699	0.100354

Table D-4: Analysis of variance of basolateral-to-apical transport

Variable	Analysis of Variance Marked effects are significant at p < .05000							
	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p
Papp	0.000000	9	0.000000	0.000000	20	0.000000	2.558007	0.038496

Table D-5: Brown-Forsythe test of apical-to-basolateral transport

Variable	Brown-Forsythe Test of Homog. of Variances Marked effects are significant at p < .05000							
	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p
Papp	0.000000	9	0.000000	0.000000	20	0.000000	0.783076	0.634425

Table D-6: Brown-Forsythe test of basolateral-to-apical transport

Variable	Brown-Forsythe Test of Homog. of Variances Marked effects are significant at p < .05000							
	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p
Papp	0.000000	9	0.000000	0.000000	20	0.000000	1.167076	0.366097