Characterisation of Pheroid® formulations with specific reference to azoxystrobin

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B.Pharm

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“God is our refuge and strength, a very present help in trouble.

Therefore will not we fear, though the earth be removed, and though the mountains be carried into the midst of the sea;

Though the waters thereof roar and be troubled, though the mountains shake with the swelling thereof.”

– Psalm 46: 1-3, KJV –

Dedicated to my loving parents,
Alex and Anje Peters

Declaration

I, Andri Peters, hereby declare that this dissertation is a record of my own work (except where citations or acknowledgements indicate otherwise) and that the study in part or as a whole has not been submitted to any other university.

I would like to acknowledge the following individuals for their contributions to my study:

- The confocal analyses for the azoxystrobin and chlorpyrifos formulations were carried out by Dr. Matthew Glynn. While the confocal analyses for the reference and anti-infective Pheroid® formulations (from 2008 to 2015) were performed by both Ms. Liezl-Marie Scholtz and Dr. Matthew Glynn.
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- The field trials for the azoxystrobin and chlorpyrifos formulations were contracted to Agricultural Science Consultants™ Research which included the statistical analysis of the results that were obtained.

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Abstract

Title

Characterisation of Pheroid® formulations with specific reference to azoxystrobin

Aim

The aim of this project was to set standardized specifications for anti-infective Pheroid® products that would ultimately promote quality control and the reproducibility of formulations.

Background and rationale

Standard specifications are a set of evaluations, references to analytical processes and acceptance criteria that are converted into the numerical ranges that provide the outline of a product. An acceptable product abides by these ranges as it serves as the most important regulatory measures.

The Pheroid® delivery system is a colloidal delivery system that comprises of a unique submicron type of formulation that is environmentally safe and contains non-toxic ingredients. The system has successfully been applied in several applications and has been shown to provide promising results – especially in anti-infective formulations, some of the most commonly used agents in the world. During this study, it was sought to describe and formalize existing ranges for anti-infective Pheroid® products that would ultimately promote quality control and the reproducibility of formulations. The results would then be verified through the formulation of the fungicide, azoxystrobin, in the Pheroid® delivery system according to the determined ranges. The use of a plant model to verify the formalized ranges is effective and much less time consuming than an animal or human model.

To achieve the stated aim, data was collected from previously manufactured anti-infective Pheroid® formulations, grouped according to the type of Pheroid® used and subdivided according to the variables in the formulations (e.g. active ingredient or additives). The data was subsequently statistically analysed with regards to the characterization results that were obtained for the various formulations (i.e. confocal laser scanning microscopy, Mastersize analysis, and zeta potential measurements). Unfortunately, due to insufficient data and the unpredictable behaviour observed with several formulations, the establishment of specification ranges was not feasible and the aim could not be met. The azoxystrobin/Pheroid® formulation could not be compiled with the use of the formalized ranges as intended but was manufactured nonetheless through solubility testing. In addition, an insecticide, chlorpyrifos, was also manufactured using Pheroid® technology.

Results

Two azoxystrobin/pro-Pheroid® formulations (6.25% and 12.5% in turn) were manufactured and tested for their efficacy and possible phytotoxicity against late blight (*Phytophthora infestans*) on tomatoes and white blister (*Albugo candida*) on cabbage. Relative to the control, the comparator (0.15 µm /mL) was slightly more effective than the same-strength azoxystrobin/pro-Pheroid® formulations (71.64% compared to equal effectiveness of 70.15%) against late blight on tomatoes. In the control of white blister on cabbage, the comparator (0.94 µm /mL) was more effective than the double strength azoxystrobin/pro-Pheroid® formulations and significantly more effective than the same-strength azoxystrobin/pro-Pheroid® formulations relative to the control.

The efficacy and possible phytotoxicity of the chlorpyrifos/pro-Pheroid® formulation were tested against African Bollworm (*Helicoverpa armigera)* on tomatoes and cabbage aphids (*Brevicoryne brassicae*). In the control of African bollworm in tomatoes, half the standard strength chlorpyrifos/pro-Pheroid® (0.36 µg /mL) was 15.3% more effective than the comparator (0.72 µg /mL). At the same strength than the comparator (0.24 µg /mL), it was 14.9% more effective in controlling cabbage aphids than the comparator.

Conclusion

The incorporation of the two anti-infective compounds, azoxystrobin (fungicide) and chlorpyrifos (insecticide) in Pheroid® technology was not equally successful. The azoxystrobin/pro-Pheroid® formulations did not provide increased efficacy compared to the comparator whereas the chlorpyrifos/pro-Pheroid® formulation delivered results that were superior to the comparators in the control of late blight on tomatoes (at half the concentration) as well as white blister on cabbage (at the same concentration). Pheroid® technology's potential in the agricultural industry (with specific reference to anti-infective application) was observed in the chlorpyrifos/pro-Pheroid® formulation; therefore, it was concluded that the solubility studies for the azoxystrobin/pro-Pheroid® formulations should be revisited in the near future.

Keywords

Pheroid®, agriculture, specifications, anti-infective, delivery system, azoxystrobin, chlorpyrifos

Opsomming

Titel:

Karakterisering van Pheroid® formulerings met spesifieke verwysing na azoksistrobien

Doel:

Die doel van hierdie projek was om gestandaardiseerde spesifikasies vir anti-infektiewe Pheroid®-produkte, wat gehaltebeheer en die herhaalbaarheid van die formulerings sal bevorder, daar te stel.

Agtergrond en rasionaal:

Gestandaardiseerde spesifikasies is ’n stel evaluasies, verwysings na analitiese prosesse en aanvaardings kriteriums wat na die numeriese reekse, wat die raamwerk van ’n produk verskaf, omgeskakel is. ’n Aanvaarbare produk voldoen aan hierdie reekse aangesien dit as die belangrikste regulerende maatreël beskou word.

Die Pheroid® afleweringsisteem is ’n kolloïdale afleweringsisteem wat uit ’n unieke submikron-tipe formulering bestaan. Die omgewingsvriendelike sisteem, wat geen skadelike bestanddele bevat nie, is al in verskeie belowende formulerings suksesvol toegepas – insluitend anti-infektiewe middels wat van die mees algemeen gebruikte middels ter wêreld is.

Tydens hierdie studie was daar gepoog om die bestaande reekse vir anti-infektiewe Pheroid® formulerings te beskryf en te formaliseer om sodoende gehaltebeheer en die herhaalbaarheid van formulerings te bevorder. Die resultate sou dan gekontroleer word deur die swamdoder, azoksistrobien, in die Pheroid®-afleweringsisteem, volgens die vasgestelde reeks te formuleer. Die gebruik van ‘n plantmodel om die geformaliseerde reekse te verifieër is effektief en neem minder tyd in beslag in vergelyking met ‘n dier- of mensmodel.

Om die aangeduide doelwit te bereik, was data van voorheen vervaardigde anti-infektiewe Pheroid®-formulerings versamel, groepeer volgens die tipe Pheroid® wat gebruik is en onderverdeel na gelang van die veranderlikes in die formulerings (bv. aktiewe bestanddeel of bymiddels). Daarna was die data statisties geanaliseer ten opsigte van die resultate wat verkry is deur die verskeie formulerings te karakteriseer (d.m.v. deeltjiegrootte en deeltjiegrootte verspreiding, morfologiese eienskappe en stabiliteit). Weens ’n tekort aan beskikbare data en die onvoorspelbare gedrag van verskeie formulerings was die doelwit nie uitvoerbaar nie en die spesifikasiereeks kon nie saamgestel word nie. Alhoewel die azoksistrobien/Pheroid®-formulering nie op grond van die geformaliseerde reeks vervaardig kon word nie, is dit steeds

vervaardig deur die gebruik van oplosbaarheidstoetse. Daarbenewens is die insektisied, chloorpirifos, ook met die gebruik van Pheroid®-tegnologie vervaardig.

Resultate:

Twee azoksistrobien/pro-Pheroid®-formulerings (6.25% en 12.5% onderskeidelik) was vervaardig en getoets vir hul effektiwiteit en moontlike fitotoksisiteit teen laatroes (*Phytophthora infestans*) op tamaties en witroes (*Albugo candida*) op kool. Die markproduk (0.15 µm /mL) was effens meer effektief as beide azoksistrobien/pro-Pheroid®-formulerings teen dieselfde sterkte (71.64% teenoor die 70.15% ondervind met albei azoksistrobien/pro-Pheroid®-formulerings) in die beheer van laatroes. In vergelyking met die kontrole was die markproduk (0.94 µm /mL) meer effektief as die dubbel-sterkte formulerings en aansienlik meer effektief as die dieselfde sterkte azoksistrobien/pro-Pheroid®-formulerings in die beheer van witroes.

Die effektiwiteit en moontlike fitotoksisiteit van die chloorpirifos/pro-Pheroid®-formulering was teen die Afrika-bolwurm (*Helicoverpa armigera)* op tamaties en die koolplantluis *(Brevicoryne brassicae)* getoets. In die beheer van die Afrika bolwurm was die chloorpirifos/pro-Pheroid®-formulering teen die helfte van die konsentrasie (0.36 µg /mL) 15.3% meer effektief as die markproduk (0.72 µg /mL). Teen dieselfde sterkte as die markproduk (0.24 µg /mL) was dit 14.9% meer effektief in die beheer van die koolplantluis.

Gevolgtrekking:

Die integrasie van azoksistrobien en chloorpirifos in Pheroid®-tegnologie was nie ewe suksesvol nie. Die markproduk was meer effektief as albei azoksistrobien/pro-Pheroid®-formulerings terwyl die chloorpirifos/pro-Pheroid®-formulering resultate gelewer het wat die vergelykbare produkt op die mark oortref het in die beheer van die Afrika-bolwurm op tamaties sowel as plantluise op kool. Pheroid®-tegnologie se potensiaal in die landboubedryf (spesifiek op die anti-infektiewe toepassing daarvan gefokus) is met die chloorpirifos/pro-Pheroid®-formulering opgemerk. Daarvolgens is besluit dat die oplosbaarheidstoetse vir die azoksistrobien/pro-Pheroid®-formulerings binnekort opnuut aangepak moet word.

Sleutelterme:

Pheroid®, landbou, spesifikasies, anti-infektief, afleweringsisteem, azoksistrobien, chloorpirifos

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ABBREVIATIONS

($^{w}/_{w}$)	-	Weight per weight
°C	-	Degrees Celsius
µm	-	micrometre
5-FU	-	5-fluorouracil
ACN	-	acetonitrile
AChE	-	Acetyl cholinesterase
ASC	-	Agricultural Science Consultants
AUC	-	Area under the curve
AZ	-	Azoxystrobin
AZ/PPA	-	12.5% azoxystrobin/pro-Pheroid® formulation
AZ/PPB	-	6.25% azoxystrobin/pro-Pheroid® formulation
BBCH	-	Biologische Bundesanstalt, Bundessortenamt and Chemical Industry (German scale used to identify the phenological development stages of a plant)
BHA	-	Butylated Hydroxyanisole
BHT	-	Butylated Hydroxytoluene
BnOH	-	Benzyl alcohol
CLSM	-	Confocal laser scanning microscopy
C_{max}	-	Maximum concentration of active achieved
CPF	-	Chlorpyrifos 480 EC formulation
CPF/PP	-	Chlorpyrifos/pro-Pheroid® 480 EC formulation
d0.1	-	10% of particles in the formulation are smaller than the stated amount
d0.5	-	50% of particles in the formulation are smaller than the stated amount
$d_{0.9}$	-	90% of particles in the formulation are smaller than the stated amount
DCM	-	dichloromethane
ddH_2O	-	Deionized water
DMSO	-	Dimethylsulfoxide
DNA	-	Deoxyribonucleic acid

EC	-	Emulsifiable concentrate
EE	-	Entrapment efficacy
EFA	-	Essential fatty acid
EMA	-	European Medicines Agency
FAO	-	Food and Agriculture Organization of the United Nations
FDA	-	Food and Drug Administration
Freq.	-	Frequency
GLP	-	Good Laboratory Practise
GMP	-	Good Manufacture Practice
h	-	Hours
H_2O	-	Water
ha	-	Hectare
HCl	-	Hydrochloride
HIV/AIDS	-	Human immunodeficiency virus infection and acquired immune deficiency syndrome
HPMC	-	Hydroxypropylmethylcellulose
IC_{50}	-	Half maximal inhibitory concentration
ICH	-	The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IP	-	Intellectual Property
ISO	-	International Organization for Specifications
Log K_{ow}	-	Octanol-water partition coefficient
L	-	Litre
L/ha	-	Litre per hectare
LD_{50}	-	Median lethal dose
MCC	-	Medicines Control Council
mg	-	Milligram
MIC	-	Minimum inhibitory concentration
mL	-	Millilitre

mL/ha	-	Millilitre per hectare
mL/L	-	Millilitre per litre
mV	-	Millivolts
N_2O	-	Nitrous oxide
$N_2O \cdot H_2O$	-	Nitrous oxide gassed water
$N_2O \cdot PBS$	-	Nitrous oxide gassed phosphate buffered saline
NH_4OH	-	Ammonium Hydroxide
nm	-	nanometre
NP	-	nanoparticle
OECD	-	Organization for Economic Co-operation and Development
OP	-	Organophosphate
PBS	-	Phosphate buffered saline
PCDDP	-	Preclinical Drug Development Platform
PEG	-	Polyethylene Glycol
QA	-	Quality Assurance
Rpm	-	Revolutions per minute
SA	-	South Africa
SC	-	Suspension Concentrate
SOP	-	Standard Operating Procedure
$T_{1/2}$	-	Half-life
TB	-	Tuberculosis
TBHQ	-	Tetrahydroxy-1,4-benzoquinone
TEM	-	Transmission electron microscopy
THFA	-	Tetrahydrofurfuryl alcohol
T_{max}	-	Time to reach maximum plasma concentration
UK	-	United Kingdom
USA	-	United States of America
USP	-	United States Pharmacopoeia
WHO	-	World Health Organization

CHAPTER 1 STUDY RATIONALE, AIM AND OBJECTIVES

Pharmaceutical formulation is the process whereby active compounds are manipulated into preparations that are safe, effective and convenient to use (Fishburn *et al.*, 2013:1). An alternative approach to formulating is introduced with the use of colloidal delivery systems. The latter holds the potential to enhance solubility and dissolution as well as the ability to alter the pharmacokinetics and -dynamics of the formulation. (Misra *et al.*, 2016:3). In recent years, numerous studies have been focused on the investigation of colloidal particles as carrier systems for the targeted delivery of drugs in the body (Buszello & Muller, 2000:192). Liposomes, emulsions, micro-emulsions, microspheres, and micelles are commonly used colloidal systems (Sharma *et al.*, 2010).

Emulsions have received increasing attention as a carrier of lipophilic drugs due to their biocompatibility, long-term stability, and ease of manufacture. An emulsion-drug formulation is considered for use in drug delivery only when its benefits supersede those of conventional drug formulations. Promising results obtained from experimental animal and human studies with emulsion-based products could subsequently indicate a tendency towards the increased use of emulsions to formulate vital products such as anticancer therapy, antimicrobial therapy, and vaccines (Buszello & Muller, 2000:208).

Pheroid® technology comprises of a submicron emulsion type of formulation. The Pheroid® delivery system is composed through the distribution of stable and unique lipid-based vesicles in a dispersion medium and can be altered as required in terms of morphology, size, structure, and function (Grobler *et al.*, 2008; Grobler, 2009). The versatile carrier system consists mainly of modified essential fatty acids and has the ability to entrap, transport and deliver, amongst other, pharmacologically active compounds. Additionally, it is safe, stable, inexpensive and easy to manufacture. A few key advantages of the Pheroid® system include application in various routes of administration, compatibility with hydrophilic, lipophilic and amphiphilic drugs, penetration of most known barriers in the body and into cells, increased delivery, absorption and bioavailability of active compounds, enhancement of therapeutic efficacy, decreased side effects and reduction of drug resistance (Grobler, 2009).

Standard specifications serve as the framework of a product. Manufactured products should adhere to those standards to be regarded as satisfactory for its intended application and to ensure uniformity between batches produced at different time points (Gibson, 2016). Variables in the formulations such as the type of formulation, active ingredients, and administration routes are inter-dependable and therefore influence the outcome of the formulation as well as the concomitant specifications.

According to the online database OECD (Organization for Economic Co-operation and Development) health statistics, the mortality rate of South Africans in 2015 due to certain infectious and parasitic diseases were just short of a hundred thousand (21.8% of total deaths). Moreover, HIV/AIDS (Human Immunodeficiency Virus infection and Acquired Immune Deficiency Syndrome) and tuberculosis were responsible for 61.2% of those mortalities. Of the 41 countries listed, Germany had the second highest mortality rate due to infectious and parasitic diseases with almost eighteen thousand deaths – more than five times less than South Africa (OECD). Due to the gravity of the situation, and the fact that previous studies on anti-infective Pheroid formulations showed promising results, including escalated drug plasma levels at lower dosages, increased bioavailability, and reduced side effects, anti-infective Pheroid® formulations were selected for the purpose of this study.

It is necessary to refine Pheroid® specifications in such a manner that the impact of specific aspects (e.g. active ingredients, excipients, instruments etc.) is taken into account so that the results and products obtained with the inclusion of Pheroid® technology can be achieved repeatedly. These specifications will also enable better quality assurance and the prediction of the efficacy of formulations during the development phase. Moreover, the optimization of standardized product specifications will serve as a guideline to validate manufacturing methods, monitor the stability of formulations and for application in the compilation of dossiers if needed.

In developing countries such as South Africa, crop production reduced by more than 44% over the past two years (Crop Estimates Committee, 2015) while a 59% decrease is expected from 2016 to 2017 (Crop Estimates Committee, 2016) due to factors such as drought and pests. With an average annual growth of nearly 2% (Statistics South Africa, 2015), the population (~55 million in 2015) is expected to reach 82 million by 2035. By that time, it would be essential that the country's current food production is doubled to ensure adequate nourishment (WWF - SA, 2010).

In light of the abovementioned statistics, the study focussed on describing and formalizing existing ranges for different types of Pheroid® batches with anti-infective properties. The results would then be verified through the formulation of azoxystrobin (an anti-infective agent due to its fungicidal properties) in the Pheroid® delivery system according to the determined ranges. Chlorpyrifos, an insecticide and anti-infective agent from a different class as azoxystrobin, was also formulated with Pheroid® technology but was not meant to verify the specification ranges. The data obtained from the chlorpyrifos/pro-Pheroid® formulation is presented in article format for publication (chapter 5) but is not discussed in the other sections of the dissertation. The use of a plant model to verify the formalized ranges is effective and much less time consuming than an animal or human model.

The aim of this project was to set standardized specifications for anti-infective Pheroid® products that would ultimately promote quality control and the reproducibility of formulations.

To achieve this aim, the following objectives had to be met:

1) Collation, processing and statistical analysis of data and information obtained from previously manufactured anti-infective Pheroid® formulations to determine the ideal specification standards for each formulation - differentiation between the type of Pheroid® used, administration route, active ingredients, manufacturing methods and raw materials or equipment used.

2) Evaluation of the feasibility to use the formalized specifications to predict the outcome of future anti-infective formulations (or at least to formulate a well-informed hypothesis) by application of the above-mentioned objectives experimentally through the formulation of an azoxystrobin and Pheroid® formulation.

3) Evaluation of the efficacy of the azoxystrobin and Pheroid® formulation through trial field studies.

Chapter 2 is a literature review comprising of a basic overview of Pheroid® technology, the antifungal, azoxystrobin, and the application of standardized anti-infective Pheroid® specifications. Chapter 3 describes the research methodology behind the data possessing and statistical analysis, as well as the results obtained. Chapter 4 portrays the establishment, characterization and efficacy studies of the azoxystrobin/pro-Pheroid® formulation while Chapter 5 consists of an article on the efficacy of a Pheroid®-incorporated chlorpyrifos formulation. The conclusion and future prospects are presented in Chapter 6.

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CHAPTER 2 LITERATURE REVIEW

2.1 Pheroid® specifications with specific reference to anti-infectives

2.1.1 Introduction

Pharmaceutical development is a descriptive term for the manufacture of a quality product that continuously delivers the desired outcome and is obtained through developmental studies. These studies enable the researcher to better understand and determine the study design, controls and specifications (ICH, 2009). The latter defines a product (Bharadwaj, 1998) and serves as a primary means of control (ICH, 2009:18).

Specifications comprise of a list of assessments, references to analytical procedures and acceptance criteria embodied by numerical limits or ranges to describe the size, shape, form and any other important information about the product. According to the Quality Assurance (QA) element of Good Manufacture Practice (GMP), a product or drug should adhere to these standards to be regarded as satisfactory for its intended application and ensure uniformity between batches produced at different time points (ICH, 1999:1; MCC, 2010:14; Gibson, 2016:3).

The first attempt to establish definite drug specifications was undertaken by the Florentine guild of physicians and pharmacists in 1499. Since there was no literature equivalent to a pharmacopoeia in existence, pharmacists applied various methods of preparation, manufacturing, care and protection of drugs that lead to numerous errors (Kremers *et al.*, 1986:66; Sonnedecker, 1993). A pharmaceutical formulary titled '*Nuovo receptario'* (New compound dispensary) was assembled to unite and define proper procedures. This was later regarded as the first European 'pharmacopoeia' (Anderson, 2005:51). Nowadays, 49 pharmacopoeias are in existence (e.g. British Pharmacopoeia, The International Pharmacopoeia and United States Pharmacopeia – National Formulary) that communicate a comprehensive collection of authoritative official standards for existing pharmaceutical substances (WHO, 2013; WHO, 2015). These are excellent sources of reference in terms of drug monographs, analysis, and medicinal preparations. However, with regards to new drug substances and products, reputable authoritative organizations such as the European Medicines Agency (EMA), Food and Drug Administration (FDA) and World Health Organization (WHO) refer to quality guideline Q6A drafted by The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). This guideline includes a comprehensive description of specification test procedures and acceptance criteria, which includes amongst others, identification, assay, and impurities. The South African counterpart,

Complementary Medicines - Health Supplements Quality, Safety, and Efficacy, compiled by the Medicines Control Council (MCC), is less detailed than Q6A although the main ideas correlate.

Product approval by regulatory authorities is dependent on specifications since it represents quality standards upon product release as well as throughout shelf life. Therefore, the aforementioned guidelines are used to set acceptance criteria that ensure the quality and consistency of new drug substances or products to subsequently gain approval by the regulatory authority (ICH, 1999). Specifications set during developmental procedures may not be absolute and require refinement as more information is gained through development and manufacture (Mollah *et al.*, 2013).

Specifications have several applications in the formulation industry, with the key functions including (ICH, 1999; FDA, 2000; MCC, 2010):

- behavioural prediction of a compound in a formulation or a formulation as a whole based on its physical and chemical traits,
- utilization of specifications as a standard to confirm the stability of a formulation, serving as method validation and to ensure quality control and reproducibility,
- writing of a collection of documents that verifies that a product is safe, effective, quality controlled and suitable for its intended purpose in order to be registered or marketed in a country, known as a dossier, and
- analysis of manufactured preparations to avoid distribution of cross-contaminated products.

In order to formalize specifications, it is necessary to provide and validate a list of measurements, analytical examinations, and acceptable standards expressed by concrete values or ranges that are used to characterize the formulation. Important information of the active ingredient(s) and/or final product such as shape, colour, viscosity, and water solubility, to name but a few, should be stated. A formulation can, therefore, be deemed as satisfactory for its intended use if it adheres to these standards (Gibson, 2016). Since specifications are fundamental to a product, the study sought to describe and formalize existing ranges for different types of Pheroid® batches. Pheroid® is a colloidal delivery system that is unique to our laboratories, the DST/NWU Preclinical Drug Development Platform (PCDDP), and will be described in greater detail in a consecutive section of this chapter.

One of the challenges experienced with the Pheroid® delivery system is the occasional requirement for alternative or additional product specifications than those standardized for most formulations. Nonetheless, the establishment of the shape, size and form of the vesicles are crucial when evaluating all types of Pheroid® formulations.

The scope of this study did not include all aspects of specifications as laid out in the ICH and MCC-guidelines but was restricted to the standard characterization tests that every manufactured batch containing the Pheroid® delivery system undergoes, which include morphology, standard particle size and size distribution tests, as well as colloidal stability testing. These have always formed the basis of analysis for Pheroid® formulations and, since this study utilized former Pheroid® formulations (2008 to 2015), available data were mostly limited to results obtained from the aforementioned tests. It is consequently possible to provide standard parameters for at least these standard analyses. A more detailed description of the standard characterization tests will appear in the next sections.

2.1.2 Pheroid® characterization

Characterization is the process of executing analytical examinations and measurements. The results can be used as a guide to obtain the optimal formulation and provide a means to predict the performance and stability of the final product (Ahuja & Scypinski, 2001; Trivedi *et al.*, 2011). The characterization of a formulation provides the manufacturer with vital information that enables him/her to pinpoint the influences that variables have on process outcomes, recognize the weight of main factors, determine acceptance criteria for a specific formulation and thus set or confirm the specifications thereof (Junker, 2007:343). Although it involves additional time and expenses, failure to run these tests may exacerbate the manufacturers' costs even more in the event that the products do not comply with specifications in addition to the risks associated with utilization (e.g. consuming or applying) of subpar formulations (Parikh, 1997; FDA, 2000).

Results obtained from characterization tests should be compared with those obtained from similar, previously manufactured formulations. Characterization of different formulations and manufacturing methods is an opportunity to obtain additional information that might improve or result in the desired formulation (ICH, 2009:2). Furthermore, sources of variability, which might influence the product quality, can be identified and subsequent measures can be introduced to control it (ICH, 2009:13).

When running an analysis, is important to note that it is insufficient to simply consult the results obtained from characterization methods without following proper protocol. The following key measures are obligatory and should be abided by at all times (Apostol *et al.*, 2012:119):

- adherence to authorized, written standard operating procedures (SOP's),
- use of equipment that fulfils required guidelines, undergo routine maintenance checks and regular calibration,
- the analysis should be exclusively run by experienced, qualified personnel,

- the process should be controlled, meticulously documented and kept for repeatability or referencing purposes,
- proper reference standard should be available for all conducted operations for evaluation purposes.

All formulations manufactured at the DST/NWU PCDDP undergo standard characterization tests comprising of zeta potential measurement, particle size distribution analysis, and CLSM-imaging. All of the tests are carried out in accordance with the Standard Operating Procedures (SOP's) of the DST/NWU PCDDP.

2.1.2.1 Mastersize analysis (particle size and particle size distribution)

The mean particle size and particle size distribution is determined by means of laser diffraction, using a Malvern Mastersizer 2000 (Malvern Instruments Ltd, Malvern, Worcestershire, UK). This analytical instrument uses laser diffraction to measure the intensity of light scattered as a laser beam passes through a particulate sample. During the measurement, particles pass through a focused laser beam, resulting in light scattered at an angle that is inversely equivalent to their size i.e. smaller particles scatter the light at a greater angle. The angular intensity of the scattered light is measured by a series of photosensitive detectors (Sochan *et al.*, 2012). The data is then analysed with the use of the Mie theory of light scattering (applied in the analyses software) and expressed as a volume equivalent sphere diameter. The technique, covered by the International Organization for Specifications (ISO13320), eliminates user variability through software-driven SOP's (Standard Operating Procedures) (ISO, 2009).

This technique, developed in the 1970's, remains popular due to its simplicity, rapid computerized analysis, versatility and ability to measure an extensive range of particle sizes (0.02µm to 2000µm (Sperazza *et al.*, 2004)) . Apart from its application as particle size and particle size distribution analyser, it can be used to detect variability between batches, set control limits and evaluate the influence of differences in manufacture on the particle sizes (Ali *et al.*, 2010; Huang *et al.*, 2010). There is theoretically no method of calibration since it is based on fundamental physical properties - instrumental performance can be verified with the use of standard referencing material (Syvitski, 2007:202; Rawle, 2009:199 - 200). The latter, described as well controlled, polydisperse, spherical particle sizing standards, are 10µm to 120µm sized glass beads and are used to certify the measurement of both wet and dry dispersion units (Campbell, 2003:52).

Since different particles sizing techniques produce varied results, it is imperative that a single technique is chosen to determine and evaluate specifications throughout (Xu & Di Guida, 2003). Internationally accepted standards (USP, 2006:2572) require that samples be measured in

triplicate (independently) and that reproducibility should meet the specified guidelines. The coefficient of variation parameters for $d_{0.1}$, $d_{0.5}$ and $d_{0.9}$ differ slightly between the ISO guidelines and the United States Pharmacopeia (USP), the latter of which allows more deviation.

The instrument is unable to differentiate between scattering produced by single particles and that of aggregated particles. It is therefore advised that the measurement is supplemented with a reliable microscopic analysis. Only particles within a specific size range are recognized by the instrument, therefore procedures should be in place to ensure that the particle sizes in the formulation adhere to that limit i.e. pre-screening of particles to remove unfit sized particles or use of more suitable lens (USP, 2006:163; Syvitski, 2007:202). The laser diffraction technique accepts that all particles are spherical (Allen, 2003:566; Syvitski, 2007:202). Analysis run by another laser diffraction instrument reported larger mean size and size distributions as the spherical shape of particles lessened (Xu & Di Guida, 2003; Blott & Pye, 2006). This finding might not be applicable to the Malvern Mastersizer 2000 since, although different laser diffraction instruments are alike in construction, their laser wavelengths differ and they operate under diverse techniques (Keck, 2006:52).

The particle sizes can have a significant effect on the physical- and chemical properties of certain formulations or compounds and might influence amongst other, the bioavailability, stability, dissolution rate, bulk flow, safety and mixing efficiency (Guo, 2008:257; Liltorp *et al.*, 2014:8-9,17; Niazi, 2016b:99-100; Niazi, 2016a:5, 32-33). It is a valuable indicator of quality and performance and is, therefore, important to decide upon, maintain, and control the desired size range for a specific Pheroid® application. The stability of suspensions and emulsions will typically increase with smaller droplet sizes and higher surface charges (zeta potential). The range typical for Pheroid® formulations is between 200 nm and 2 µm (Grobler, 2009).

2.1.2.2 CLSM (particle size and concentration)

The morphological characteristics are assessed by a non-destructive optical imaging technique known as Confocal Laser Scanning Microscopy (CLSM). High definition particles can be observed in optical segments without the need to physically section the sample or changing its characteristics in any way (Clarke & Eberhardt, 2002). A two-dimensional image is created by scanning the focal point of the laser across a sample while a three-dimensional image is compiled by taking a series of two-dimensional images at different focal depths. Point illumination by means of a spatial pinhole is used to disregard out-of-focus light rays from the image.

One of the key elements of this non-invasive technique is that, unlike other microscopy techniques such as Transmission Electron Microscopy (TEM), CLSM does not introduce artefacts to the sample (Shaw, 2005). Sample preparation is convenient in the sense that it is

not restricted to particular conditions such as low temperatures or pressure but carried out under environmental conditions (Morris, 1993:65).

CLSM has an additional application during the evaluation of formulations: to determine entrapment efficacy (EE) and to visually confirm the presence of an/the internal structure in the particles (refer to Figure 2-1 for an example). EE refers to the percentage of the initial amount of compound added to the formulation that is entrapped inside the vesicles (Maestrelli *et al.*, 2005) and is visualized by means of fluorescent labelling. Selected components fluoresce extemporaneously, but most are stained with a dye that enters an excited state during the laser interaction and emits light at a particular wavelength (Pawley, 2010:353).

Due to its lipophilic nature, Nile red (9-diethylamino-5H-benzo-α-phenoxazine-5-one, excitation/emission maxima ~552/636 nm) associates with the lipid molecules in Pheroid® formulations. The lipophilic Pheroid® bilayer, therefore, fluoresces with a prominent red colour while the non-fluorescent aqueous core emits no photons and is thus shows up dark. Free Nile red does not interfere with results as it only emits red fluorescence when bound (Greenspan *et al.*, 1985). The resultant fluorescence should also be easily distinguishable from the Nile red. For instance, to determine EE during his study, Oberholzer (2009) used fluorescein isothiocyanate labelled insulin (FITC-insulin). The fluorescent wavelength of the marker (488-494µm) was compatible with the detector and the EE could be visualized as yellow fluorescence. Fluorescence light emissions will be collected within three wavelength bands (above 650nm, 540 to 640nm, and 485 to 545nm) and merged to form an image.

2.1.2.3 Zeta potential (stability)

Zeta potential is used as an indicator of the stability of colloidal dispersions by measuring the degree of electrostatic repulsion between neighbouring, equally charged particles in a dispersion (Denton & Rostron, 2013:161; Hunter *et al.*, 2013:159-160). Zeta potential is measured with the Malvern Zetasizer Nano ZS (Malvern Instruments Ltd, Worcestershire, United Kingdom) with the application of the Laser Doppler Micro-electrophoresis principle. Stability is a key parameter in any formulation with the exact stability requirements dependent on the product or indication. Measurements of >±25mV are beneficial due to the fact that at this potential difference the repulsive forces exceed the attractive London forces, the system tends to be electrically stabilized and, therefore, particles are dispersed in the emulsion (Roland *et al.,* 2003). On the other hand, the attractive forces in colloids with a low zeta potential (0 to ±25mV) exceed the repulsive forces causing the particles to gather and flocculate (Denton & Rostron, 2013:161; Hunter *et al.*, 2013:160).

The zeta potential of Pheroid® formulations were previously measured with the Malvern Zetasizer 2000. The Malvern Zetasizer Nano ZS, however, has a much wider measurement

range (diameter of 0.3nm to 10 microns) and is more appropriate when Pheroid® technology is involved.

2.1.2.4 Conditions of the study

Available data and information obtained from former anti-infective Pheroid studies were used for the purpose of this study (PCDDP, 2008 - 2015).

Since the zeta potential instrument was not operational and available at all times, the analysis of several Pheroid formulations were not carried out. In addition, the completed zeta potential reports for some formulations could not be found since, in contradiction to the SOP, the reports were not filled.

The qualitative analysis attributes of the CLSM (i.e. evaluating the EE) was not relative to the anti-infective formulations and was therefore not included.

Furthermore, research carried out on behalf of a third party is often subject to Intellectual Property (IP) protection and would therefore not be available and/or accessible for the purpose of this study.

2.2 Pheroid® technology

2.2.1 Introduction

A delivery system is a device or a pharmaceutical formulation that facilitates the delivery of a therapeutically active ingredient to the target area in the body or organism and enhances its efficacy and safety (Vonarbourg *et al.*, 2006). This is done through the control of parameters like rate, time and place of release of the ingredient (Jain, 2008:1). These type of technologies are aimed at overcoming the limitations that conventional drug forms present, such as poor absorption profiles, noncompliance of patients (e.g. parenteral formulations) and inaccurate targeting of drugs and collateral exposure of other non-target organs (Gibson, 2016:6). An ideal carrier system should be stable, biodegradable, pharmaceutically acceptable and suited for targeted delivery. The characteristics of the active ingredient, therapeutic effect required and the route of administration determines which system are used for drug delivery (Buszello & Muller, 2000:194).

One of the most utilized drug carrier systems are colloids (Grobler, 2009). These delivery systems comprise of two phases, namely a dispersed phase and a continuous phase. Liposomes, emulsions, micro-emulsions, macromolecular microspheres and polymeric microspheres are commonly used colloidal systems (Sharma *et al.*, 2010). The following section, however, will focus on a colloidal system in use at the DST/NWU Preclinical Drug

Development Platform (PCDDP), known as Pheroid® technology. To simplify reading, the ®–symbol indicating a registered trademark for the Pheroid® will be omitted from this point forward.

2.2.2 Pheroid technology

The Pheroid delivery system is a delivery system that comprises of a unique submicron type of formulation. The dispersed vesicular structures found in the delivery system are termed Pheroids, and can be tailor made to fit the application that the formulation is developed for in terms of select aspects, i.e. morphology, size, structure and function (Grobler, 2009). The system, comprising mostly of modified essential fatty acids, is also unique due to its environmentally safe manufacturing method and non-toxic ingredients as well as being inexpensive and easy to manufacture. It has the ability to entrap, transfer and deliver compounds over most physiological barriers (including cellular membranes of plants or organisms) (Grobler *et al.*, 2008; Grobler, 2009; Grobler *et al.*, 2014b; Grobler & Zeevaart, 2015; Jacobs *et al.*, 2015). At room temperature, the Pheroid delivery system is capable of remaining stable (Slabbert *et al.*, 2011) and structurally sound for upwards of two years (Grobler, 2009; Steyn *et al.*, 2011). Several studies have shown the value of using Pheroid technology as a carrier system - especially when enhanced absorption of the active ingredient with a specific administration route or targeted delivery is required (Grobler, 2009; Steyn, 2009; Steyn *et al.*, 2010; Slabbert *et al.*, 2011).

2.2.2.1 Structural characteristics

During formulation, the choice of Pheroid utilized is based on factors such as required capacity to entrap the active ingredient, administration route, and rate of delivery. By altering the type, ratios and saturation state of the specific fatty acids used in the formulation, Pheroid of different sizes and with or without membrane pores can be obtained. The method of manufacturing, on the other hand, has a less prominent influence (Grobler, 2009).

Pheroid is mainly formulated in three different types of structures, namely (Grobler *et al.*, 2008):

- Lipid-bilayer Pheroid vesicles (Figure 2-1 A)
- Pheroid micro-sponges (Figure 2-1 C)
- Depots/reservoirs

An additional application of Pheroid technology is the packaging of the active ingredient (especially water liable molecules) in the precursor formulation, called pro-Pheroid (the formation of pro-Pheroid is displayed in Figure 2-1 B).

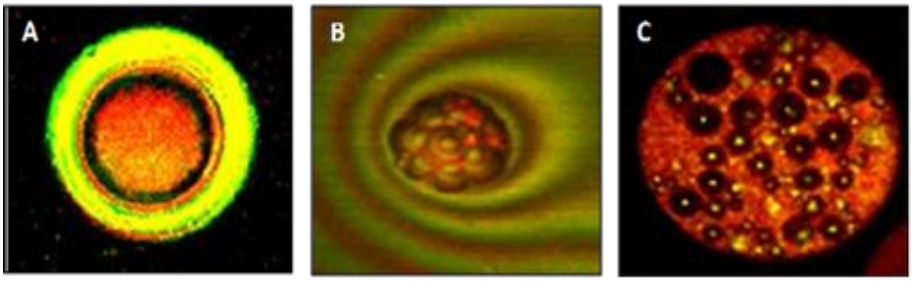

Figure 2-1: Different types of Pheroid formulations, with A) vesicle, B) pro-Pheroid C) micro-sponge (reprinted with permission from the author, Grobler (2009))

Pheroid vesicles are small structures (80-300 nm in size) that are uniform in both size and shape. They are structurally similar to liposomes but, contrary to the latter, do not contain cholesterol or phospholipids and also have a gas phase unique to the system (Grobler, 2009). The bilayer membrane of the vesicles, comprising of amongst other, modified essential fatty acids (ethyl esters), is easily crossed by water-soluble molecules. These Pheroid vesicles have therefore been frequently used to successfully entrap and deliver hydrophilic entities.

The larger Pheroid micro-sponges (0.5-5.0 nm in size) has a porous, sponge-like structure and is more suitable for the entrapment of lipid-soluble compounds (Grobler, 2009). Micro-sponges are used for the entrapment of insoluble compounds (e.g. nevirapine, a water-insoluble compound) and the size of these carriers is determined by the amount of compound contained within them. The micro-sponges can support sustained release according to the concentration gradient (Steyn, 2006; Uys, 2006).

The Pheroid depots or reservoirs contain pro-Pheroid and their sizes depend on the amount of the latter contained therein and provides extended release according to a concentration gradient (Grobler, 2009).

The formulation of pro-Pheroid, in contrast, excludes the aqueous phase (sterile or buffered water) in the manufacturing process to obtain only an oil-based system (see Figure 2-2 for a comparison). Upon contact with an aqueous media Pheroid vesicles forms instantaneously and active ingredient(s) present in the formulation are entrapped in the carrier system during this process (Grobler *et al.*, 2014a). This application has demonstrated improved stability, solubility, and bioavailability of some compounds (Grobler, 2009).

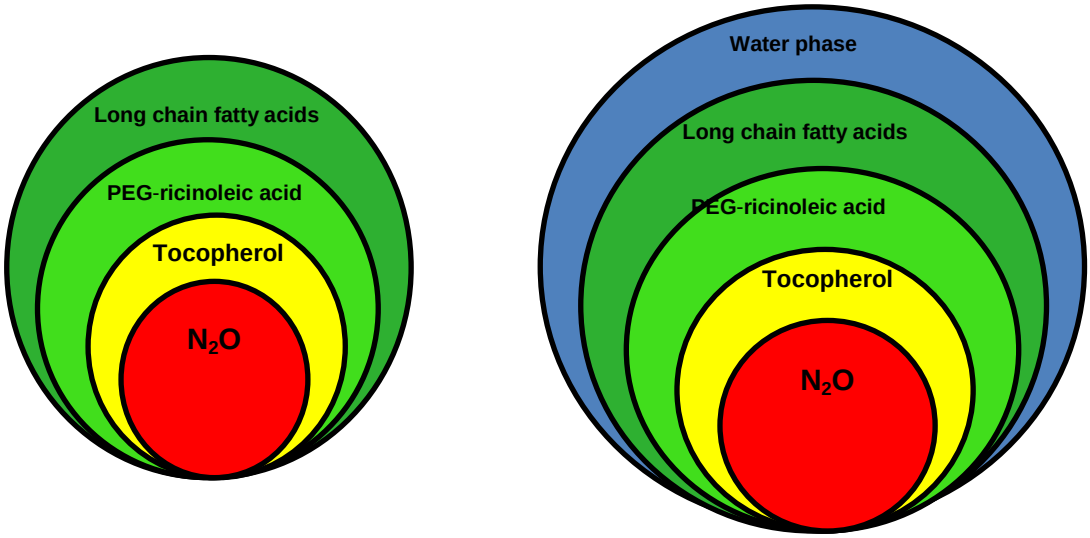

Figure 2-2: Illustration of the pro-Pheroid (left) and Pheroid (right) (adapted from Grobler (2009) with the permission of the author)

2.2.2.2 Pheroid ingredients

2.2.2.2.1 Essential fatty acid component

Essential fatty acids (EFAs) are critical for the proper functioning of mammalian cells but cannot be produced by the human body itself and have to be ingested from dietary sources (Dobryniewski *et al.*, 2006; Hibbeln, 2009; Forbes & Parsons, 2012). The shortage of these fatty acids in the average modern diet can be alleviated by supplementation with the Pheroid system as these EFAs are incorporated in its composition. The fatty acid mixture used in the manufacturing of Pheroid is obtained as vitamin F ethyl ester. The latter comprises mainly of ethylated polyunsaturated fatty acids, including linoleic acid (omega 6) and α-linolenic acid (omega 3) with the absence of arachidonic acid (Grobler *et al.*, 2008). The oleic acid incorporated in vitamin F ethyl ester disrupts the structure of membrane lipids temporarily (Ibarguren *et al.*, 2014), creating an enhanced absorption of Pheroid vesicles across biological membranes without causing permanent damage. The fatty acids present in Pheroids are formulated in a *cis*-formation to achieve compatibility with the fatty acids found in the human body (Grobler *et al.*, 2008).

Functions carried out by the fatty acid component of the Pheroid system, includes:

- Preservation of the membrane integrity of cells
- Energy homeostasis

- Adjustment of the immune system through leukotriene's and prostaglandins
- Regulatory aspects of apoptosis (Grobler, 2009)

2.2.2.2.2 DL-α-tocopherol

DL-α-tocopherol is the most biologically active form of vitamin E. It is used as an antioxidant in the Pheroid system for protection against atmospheric oxidation (Le & California, 2006). Vitamin E also acts as a membrane stabilizer through the formation of a complex with the products of membrane lipid hydrolysis to counteract their disruptive effects (Grobler *et al.*, 2008).

2.2.2.2.3 Kolliphor

Kolliphor EL® is an amphiphilic polyethoxylated castor oil derivative obtained by reacting castor oil with ethylene oxide and was previously known as Cremophor EL® (Lai *et al.*, 2013; Lamond *et al.*, 2013). Kolliphor RH-40® (previously Cremophor RH-40®), also used in some Pheroid formulations, differs from Kolliphor EL® in that the castor oil used for the reaction is hydrogenated and the mole ratios of the castor oil and ethylene oxide varies (Zhang, 2009:542; Christiansen *et al.*, 2010). Although chemically similar, physically and behaviourally (i.e. side effects and absorption) these excipients can be rather diverse (Kiss *et al.*, 2013; Berthelsen *et al.*, 2015). Kolliphor RH-40® (oral LD_{50} in rats of >16g/kg) has a higher daily dose limit than EL® (oral LD_{50} in rats of >6,4g/kg) making it the safer alternative (Zhang, 2009:548). Nevertheless, these non-ionic surfactants used in the production of Pheroid mediate solubilisation of an array of hydrophobic compounds, thus enhancing their bioavailability (Gelderblom *et al.*, 2001; Grube & Langguth, 2007:90; Liu *et al.*, 2008:120).

2.2.2.2.4 Nitrous oxide (N_2O)

This unique addition to the delivery system distinguishes Pheroid from other lipid-based delivery systems. N_2O is dispersed into the aqueous- and lipid phases to provide at least three known functions (Grobler *et al.*, 2008; Grobler, 2009):

- Helps with the miscibility of the dispersed fatty acids
- Promotes the self-assembly process of Pheroid (Uys, 2006)
- Contributes to the stability of Pheroid (Uys, 2006; Grobler *et al.*, 2008)

This compound, best known for its anaesthetic applications, is both hydro- and lipophilic (Eger, 2005), thus enabling it to move freely through the epidermal- and dermal layers of the skin. Lipid-rich membranes are an ideal site for N_2O to accumulate leading to an increase in the fluidity of cell membranes (Johansson & Linder, 1980; Auer *et al.*, 2013:103).

Studies have shown that the interaction between N_2O and the fatty acids generates stable vesicular Pheroid structure (Grobler, 2009). The significance of the combination of the types and amounts of each ingredient was proven during a controlled study where the stability and efficacy of various formulations dramatically decreased if either the essential fatty acids or the nitrous oxide was excluded from the formulation (Grobler *et al.*, 2008).

2.2.2.3 Pharmaceutical applicability of the Pheroid delivery system

Diligent research has been carried out on the Pheroid delivery system, which revealed several characteristics that contribute to its therapeutic applications. Some of the key characteristics are briefly discussed in the following section.

2.2.2.3.1 Decreased time to onset of action

Research proved that the Pheroid delivery system is able to deliver an active ingredient considerably quicker across biological barriers as opposed to conventional methods. Subsequently, the entrapped compound is delivered swiftly and effectively. This suggests that a patient could experience faster relief of symptoms with the application of Pheroid technology to the pharmaceutical product (Grobler, 2009).

2.2.2.3.2 Increased bioavailability

Bioavailability is the extent and rate at which an absorbed drug becomes available in the systemic circulation (Marshall *et al.*, 2014:768). During a previous *in vivo* murine study of tuberculosis (TB) drugs, the bioavailability of Pheroid-entrapped drugs was compared with that of the commercial product. Even though Matthee (2007) entrapped only 60.00% of the prescribed dosage in pro-Pheroid, a 205.00% increase in the bioavailability of rifampicin, 20.00% in isoniazid and 19.00% in pyrazinamide was obtained in comparison to the complete prescribed dosage (100.00%) of the commercial product.

2.2.2.3.3 Increased therapeutic effect

Entrapment of the active ingredient in Pheroid has shown increased therapeutic efficacy in most tested compounds. The *in vivo* study discussed in the previous paragraph (increased bioavailability) was reproduced in humans and also resulted in a higher therapeutic efficacy at 60.00% of the comparator's strength after Pheroid technology was applied (Grobler, 2009). This effect makes it possible to have a decreased frequency of dosing with improved patient compliance, an increase in dose to enhance the therapeutic effects, or a reduction in dose to reduce side effects without weakening the potency (Grobler, 2009).

2.2.2.3.4 Increased delivery of active ingredient(s)

In addition to fast delivery of active ingredients across biological barriers, *in vitro* and *in vivo* studies have shown that the percentage of active ingredient delivered to the desired site of action greatly increased when entrapped in Pheroid. The results obtained from an *in vitro* efficacy study indicated that Pheroid enhanced the flux and delivery of 5-fluorouracil into the skin. It was concluded that a lower concentration of 5-fluorouracil could be used to achieve a therapeutic effect due to the Pheroid delivery system (Chinembiri *et al.*, 2015).

2.2.2.3.5 Reduction in minimum inhibitory concentration (MIC)

MIC is the lowest amount of anti-microbial that prevents the visible growth of bacterium after overnight incubation (Andrews, 2001; Tripathi, 2013:696). A reduction in MIC would, therefore, enable the use of less active ingredient to achieve therapeutic results. Lesser amounts of active ingredients would, in turn, reduce adverse effects, manufacturing costs and treatment expenses. Previous *in vitro* studies revealed that an effective formulation could be manufactured with the use of as little as $1/40^{th}$ of the indicated amount of active compound when entrapped in Pheroid (Langley, 2007; Grobler, 2009).

2.2.2.3.6 Adaptability and flexibility

Pheroids have a polyphilic nature, meaning that hydrophilic, lipophilic and even insoluble drugs can be entrapped (Grobler, 2009). Most other delivery systems, on the other hand, are selective carriers of either hydrophilic or lipophilic drugs and are usually unable to carry insoluble drugs. Moreover, the size, morphology, and capacity of Pheroids can be adapted according to the indication or the active ingredient(s) via the modification of the ingredients or the method of preparation of the system (Grobler *et al.*, 2008).

2.2.2.3.7 Decline in cytotoxicity

Since Pheroid is capable of enhancing a cell's integrity, it could potentially reduce the cellular damage that commonly occurs due to exposure to the effects of active ingredients. The system comprises of ingredients that are natural in the body and no indication of cytotoxicity has been found. On the contrary, Du Plessis *et al.* (2014) found that the cytotoxicity against human neuroblastoma cells (SH-SY5Y) of the free drug (mefloquine) was reduced by 64.00% after entrapment in Pheroid. This finding suggests that the use of Pheroid technology will increase the tolerance and therapeutic index of mefloquine.

2.2.2.3.8 Reduction or invalidation of drug resistance

In previous studies, Pheroid has demonstrated a beneficial reduction or in some cases even the elimination of existing drug resistance. This can be due to the fact that Pheroid carries the drug over the membrane and releases the drug intracellularly. Thus, the effect of the drug efflux pumps present in some drug resistant organisms are negated (Grobler, 2009).

A Pheroid-entrapped rifampicin formulation exhibited a significant increase in effectiveness against the bacterial growth of multidrug resistant tuberculosis compared to standard rifampicin. A 1544.62% increase in efficacy of chloroquine on a chloroquine-resistant strain of malaria (RB-1) *in vivo* (mice) was demonstrated by chloroquine entrapped in Pheroid vesicles (Langley, 2007).

2.2.2.3.9 Absence of immunological responses

Since the fatty acid component of Pheroid is a natural product in the human body, it does not provoke an immune reaction. Additionally, it has the ability to mask the immunological effects caused by certain compounds like peptides or proteins. Entrapment of the latter in Pheroid would, therefore, reduce the immunological response that is provoked (Grobler, 2009).

2.2.2.3.10 Transdermal delivery

Pheroid-entrapped drugs have been formulated for administration through different routes. The topical route, however, holds a few advantages in comparison to other routes, for instance, localized delivery, bypassing of the first-pass effect of the liver that could metabolise drugs prematurely (Prausnitz & Langer, 2008), and negation of many of the unwanted side effects associated with other methods of administration (Grobler, 2009) .

Several studies proved that the incorporation of Pheroid is advantageous to transdermal formulations. Increases in delivery, permeation, and/or flux were found in amongst other, peptide drugs, anti-tubercular drugs, anti-fungal drugs and local anaesthetics (Botes, 2007; Coetzee, 2007; Campbell, 2010; Nell, 2012; Chinembiri *et al.*, 2015; Jacobs *et al.*, 2015).

2.2.2.3.11 Entrapment and transference of genes to nuclei and expression of proteins

Human and viral deoxyribonucleic acid (DNA) of various lengths has successfully been entrapped in Pheroid and these formulations demonstrated efficacy during *in vivo* studies. Furthermore, Pheroid has been proven to be compatible with both DNA vaccines and gene therapy. Reproducible expressions of suitable proteins were detected after transfection of cells via Pheroid-entrapped genes (Grobler, 2009).

2.2.2.3.12 Penetration of organisms and most barriers in the body and cells

The system's range is not limited to oral formulations. It has been proven to penetrate an array of barriers in the body, including skin, keratinized tissue, vascular walls, subcellular organelles and intestinal epithelium (Du Plessis *et al.*, 2010; Chinembiri *et al.*, 2015; Jacobs *et al.*, 2015). The Pheroid's diversity continues in the sense that it penetrates viruses, bacteria, fungi, and parasites in order to deliver the compounds and therefore terminate the organisms more effectively (Matthee, 2007; Du Plessis *et al.*, 2014; Grobler *et al.*, 2014b; Jacobs *et al.*, 2015).

2.2.2.4 Pheroid in the use of anti-infective formulations

Anti-infective agents is a general term used to describe a class of agents that includes antibacterials, antifungals, antivirals, antiprotozoals and antibiotics (McDonnell & Sheard, 2012:vii). It is estimated that ~17% of human deaths are due to infectious diseases (McDonnell & Sheard, 2012:1). The indiscriminate use of anti-infective agents are common practice and resistance to these drugs are therefore escalating (Roberts *et al.*, 2008; Leekha *et al.*, 2011). Mechanisms whereby resistance develop due to overexposure to anti-infective agents include diminished permeability of the organism, alteration of target proteins and inactivation of the drug (Hoffman, 2001).

Almost all microbes that infect humans have developed some degree of resistance to the anti-infective drugs which once was effective to combat these diseases (Heymann, 2006). Infections caused by drug-resistant pathogens lead to extended hospital stays, enhanced opportunity for the infection to spread, increased expenses and higher morbidity and mortality rates. New resistance mechanisms develop constantly and spread at a rapid pace. Furthermore the rise in incidence of anti-infective drug resistance is accompanied by a distressing decline in the research and development for new drugs against those resistant pathogens (Norrby *et al.*, 2005).

Pheroid technology has demonstrated unique advantages in previous studies particularly in the application of anti-infectives. When anti-infectives are used indiscriminately, the infectious organisms are not entirely eliminated and survival traits are passed on to other organisms which result in stronger infections (Weber, 2010:90). By formulating anti-infectives in Pheroid increased drug plasma levels at lower dosages can be obtained (Matthee, 2007; Grobler, 2009). This translates to a shorter treatment time, fewer side effects, better patient compliance, and increased exposure of the organisms to the drugs. Pheroid can also increase the amount of drug that penetrates the bacterial cell. This could counteract the reduced levels of the drug in the intracellular target – one of the mechanisms of resistance (Tenover, 2006).

In the following section, a brief overview is provided regarding the results obtained in previous studies with the use of Pheroid technology for anti-infective drugs.

2.2.2.4.1 Anti-bacterials

The gold standard of TB treatment, Rifafour® e-275, is a combination of four first-line drugs used to treat tuberculosis, namely rifampicin, isoniazid, pyrazinamide and ethambutol (Boeree *et al.*, 2015). An *in vivo* efficacy study in humans (phase 1) evaluating the effect of entrapment of first-line TB drugs (60.00% dose) in pro-Pheroid (referred to as Pyriftol) compared to the control Rifafour® e-275 formulation (100.00% dose) demonstrated favourable pharmacokinetic optimization. Compared to the standard formulation, Pyriftol displayed a significantly higher C_{max} (maximum concentration of active achieved) and AUC (area under the curve) while rifampicin and isoniazid reached the maximum concentration more rapidly. Confocal Laser Scanning Microscopy (CLSM) revealed that the Pheroid enabled the delivery of a higher concentration of active ingredient at the required site – the macrophage.

Pheroid and non-Pheroid isoniazid and rifampicin-combined lotions and emulgels were formulated for the treatment of dermal tuberculosis. Stability testing indicated that the Pheroid formulations were more stable than their non-Pheroid counterparts. Enhanced drug release was detected from the Pheroid formulations during *in vitro* permeation studies using vertical Franz diffusion cells although no transdermal delivery could be established with tape stripping (after 12 hours) when the membranes were substituted with female abdominal skin (Benade, 2009).

In another *in vitro* study on isoniazid (5mg/mL) and rifampicin (10mg/mL), Botes (2007) compared the transdermal delivery of the drugs with Pheroid and PBS (control) respectively. Pheroid delivered markedly better results than the comparator in terms of average flux - especially for rifampicin. No flux was observed for rifampicin in PBS (phosphate buffered saline) but in Pheroid a maximum average flux of 1.870 ± 2.080 µg/ cm^2 (between 0 and 1.5 hours) was detected. The maximum average flux (between 0 and 2 hours) for isoniazid obtained with Pheroid was more than nine times that of the PBS control.

Azelaic acid is used in the treatment of mild to moderate inflamed acne vulgaris. It has anti-bacterial, anti-inflammatory, and anti-keratinizing properties (Sieber & Hegel, 2013). A 20.00% azelaic acid cream and a 15.00% azelaic gel were formulated each with and without Pheroid. After 12 hours, the average cumulative concentrations of each across full thickness abdominal skin were noted. While substantially more active diffused from the Pheroid cream (38.48 µg/ cm^2) than its non-Pheroid counterpart (3.23 µg/ cm^2), the opposite was found with the Pheroid gel (11.03 µg/ cm^2 against 24.51 µg/ cm^2 from the non-Pheroid gel) (Moolman, 2010).

2.2.2.4.2 Anti-fungals

The anticancer chemotherapy drug, 5-fluorouracil (which has anti-fungal properties) is used as a topical treatment for certain skin cancers (Saif *et al.*, 2009). Transdermal permeation studies were carried out by Van Dyk (2008) to determine the Pheroid's effect on the transdermal flux of 5-fluorouracil (5-FU). The drug was formulated in PBS, HPLC-grade water (both serving as controls), PBS-based Pheroid or water-based Pheroid solutions. The Pheroid formulations had a greater permeation than the control formulations with the water-based Pheroid formulation being the most successful. Of the two 5-FU formulation strengths that were manufactured (0.50% and 1.00%), only the 0.50% water-based Pheroid solution delivered statistically significant results (yields, fluxes, and cumulative concentrations were compared by means of ANNOVA analysis). It was deduced that the 0.50% formulation would be as effective as the 1.00% formulation. This will reduce manufacturing and consumer costs, cause fewer side effects and consequently improve patient compliance.

A succeeding study was launched to determine the transdermal penetration of 5-FU on account of the aforementioned results. A 0.50% 5-FU cream, lotion, and emulgel were manufactured – each with and without Pheroid. Additionally, a 5.00% water and 5.00% Pheroid solution were prepared to serve as a comparator to the commercial product. Whilst the Pheroid cream, lotion, and emulgel displayed a greater transdermal delivery than the commercial product, it mostly resulted in lower % diffused values and reduced epidermis and dermis concentrations. As a result, the researcher concluded that 5-FU should not be formulated with Pheroid technology (Vermaas, 2010).

The *in vitro* effect of the Pheroid system on the topical delivery of ketoconazole was researched using vertical Franz diffusion cells and tape stripping. The formulations were intended for the treatment of fungal cutaneous manifestations due to HIV/AIDS -infection. A ketoconazole cream and emulgel with and without (control) Pheroid were formulated. Ketoconazole has a low aqueous solubility and high molecular weight and is usually delivered poorly but Pheroid (especially the emulgel formulation) still proved to enhance the transdermal penetration as well as delivery of the drug to the dermal and epidermal skin layers in comparison to control formulations. After 12 hours, 447.55 µg/cm^2 ketoconazole had diffused through the skin with the use of the Pheroid emulgel in comparison to the 25.68 µg/cm^2 from the control emulgel (Jacobs *et al.*, 2015).

2.2.2.4.3 Anti-virals

During an *in vitro* study, Franz cell skin diffusion studies were carried out on the epidermal layer of human abdominal skin to determine what effect Pheroid would have on the transdermal

diffusion of acyclovir (5.00% w/v). In comparison to the PBS control, the Pheroid formulation resulted in an increased average flux of acyclovir (Van der Walt, 2007).

The effect of Pheroid on the transdermal penetration of zalcitabine, lamivudine and synthesised *N*-acyl lamivudine esters was determined during an *in vitro* study by means of selected physicochemical properties. Entrapment was confirmed by CLSM, but no practical advantage in terms of the transdermal application was observed (Gerber *et al.*, 2008).

A preservative efficacy and stability study on nevirapine in pro-Pheroid was conducted by Britz (2009). It was concluded that a nevirapine-entrapped pro-Pheroid formulation, stable in pH and particle size, could successfully be manufactured with, in this case, butylparaben as a preservative.

A study was launched to determine the efficacy of three antiretrovirals, namely stavudine, lamivudine and nevirapine after entrapment in Pheroid. Ironically, Pheroid instigated toxicity properties in stavudine whereas protective properties were observed with lamivudine. Cell death occurred due to the formation of thymine from stavudine. After exposure to high concentrations of the lamivudine formulations, the cells were still viable and therefore, unlike stavudine, lamivudine showed a lack of toxicity. Due to solubility issues, the research on nevirapine was discontinued. Cytotoxic levels were reached after entrapment of the antiretrovirals (1:1000 dilution) in Pheroid and cell death occurred. The diluted Pheroid alone triggered no cell death in viability tests which could only imply that, in accordance with its characteristics, Pheroid enhanced the uptake of the drug into cells and resulted in toxic levels (Botha, 2007).

In a succeeding study, an exploratory topical acyclovir cream and emulgel with and without Pheroid was manufactured for HIV/AIDS patients with subsequent skin manifestations to determine whether the system would enhance delivery. The stratum corneum epidermis and epidermis-dermis delivery were tested by means of vertical Franz diffusion cells and tape stripping. An increase in transdermal, stratum corneum epidermis and epidermis–dermis delivery of the Pheroid formulations was noted with the best results obtained from the Pheroid cream (permeation of 2970.03 µg/cm^2 after 12 hours versus 2172.2 µg/cm^2 from the control cream). Stability was measured over six months with results indicating that the Pheroid formulations were more unstable than their drug-only counterparts and did not meet the criteria for stability as described by the MCC (Medicines Control Council) and ICH (International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use) guidelines (Jacobs *et al.*, 2015).

2.2.2.4.4 Anti-protozoans

Langley (2007) conducted an *in vitro* study that utilised light microscopy to investigate the efficacy of some antimalarial drugs entrapped in Pheroid vesicles against an RB-1 chloroquine-resistant strain of *Plasmodium falciparum.* Entrapment in Pheroid vesicles was determined to be more advantageous than entrapment in Pheroid micro-sponges. The MIC decreased and enhanced therapeutic efficacies of 314.00%, 238.00%, and 254.00% were found in mefloquine, artesunate, and artemether respectively. Chloroquine in combination with Pheroid vesicles displayed a 15-fold enhancement in efficacy. It was found that Pheroid poses the potential for treating malaria with a well-known drug, but with increased efficacy and reduced cytotoxicity and side effects.

Gibhard *et al.* (2012) orally administered normal chloroquine and chloroquine entrapped in Pheroid vesicles (20mg/kg) to vervet monkeys and analysed the chloroquine concentration in whole blood and plasma with the use of an adapted LC-MS/MS method. The C_{max} and AUC of chloroquine in whole blood were markedly enhanced and it was concluded that Pheroid improved the absorption of the drug. Conflicting results were obtained in a similar study when the same researcher investigated the effect of Pheroid on the bioavailability of amodiaquine and *N*-desethylamodiaquine (similar in structure and activity to chloroquine). The results reflected the fact that Pheroid does not have the same effect on all antimalarials or, for that matter, on all actives. It was found that contrary to previous studies with Pheroid and chloroquine or artemisone (Langley, 2007; Slabbert, 2008; Steyn *et al.*, 2011); the delivery system did not enhance the bioavailability of amodiaquine and *N*-desethylamodiaquine.

The *in vitro* efficacy of chloroquine and mefloquine entrapped in different Pheroid micro-sponge formulations against an RSA11 RB-1 chloroquine resistant strain of *Plasmodium falciparum* was evaluated. The parasitemia levels were analysed with the use of flow cytometry and colorimetry and compared through microscopic evaluation. Overall, Pheroid formulations displayed lower parasitemia levels than the controls. The most promising result, a 46.00% reduction in parasite growth at the peak concentration, was obtained when mefloquine was combined with Pheroid. A significant difference was observed between the Pheroid formulation and the mefloquine control (half maximal inhibitory concentrations (IC_{50}) of 70.53 nM and 15.95 nM respectively) and it was deduced that Pheroid micro-sponges were able to enhance the antimalarial effect of chloroquine and mefloquine *in vitro* (Slabbert, 2008).

During a study on Pheroid entrapped mefloquine, Du Plessis *et al.* (2014) saw a 63.00% EE and a 75.00% reduction in *in vitro* haemolytic activity in comparison to mefloquine alone. After 48 hour incubation, the IC_{50} were reduced by 50.00% and 30.00% respectively in chloroquine-

resistant and chloroquine-sensitive strains. Additionally, after entrapment in Pheroid, a 64.00% decrease was seen in the free drug cytotoxicity levels against human neuroblastoma cells.

A comparison was made between the physical properties and stability of mefloquine (0.50% m/v) when entrapped in Pheroid vesicles against entrapment in liposomes. Results showed that mefloquine hindered the formation of the liposome lipid bilayer and aggregation occurred during the accelerated stability studies. While the size of the Pheroid vesicles remained stable, the liposomes were double the size of the initial Pheroid vesicles and enlarged over time. Additionally, no structural changes were experienced with the Pheroid. Both formulations experienced a substantial decrease in pH towards the third month. Due to leakage of the drug from the lipid bilayer, the initial EE of 62.93% in liposomes could not be sustained whereas the EE of the Pheroid formulation (58.70%) remained stable over the three-month stability testing. It was found that, compared to Pheroid, liposomes are unstable (Slabbert *et al.*, 2011).

An *in vivo* murine study was carried out to determine what the pharmacokinetic implication would be if artemisinin derivatives were formulated with Pheroid technology. Only a marginal improvement was found with artemiside whereas a striking enhancement was observed in the C_{max} and $T_{1/2}$ (half-life) of the drug and a time delay in T_{max} (time to reach maximum plasma concentration) of the Pheroid entrapped artemisone. Moreover, the latter displayed a 4.57-fold increase in the AUC compared to the drug alone formulation (Steyn *et al.*, 2011). Therefore, entrapment of certain drugs in Pheroid could provide therapeutic drug plasma concentrations with the use of substantially lower drug concentrations.

During an *in vitro* antimalarial study, Grobler *et al.* (2014a) found that entrapment in Pheroid did not increase the activity of artemisone against multidrug-resistant *Plasmodium falciparum* lines. Furthermore, Pheroid entrapped artemisone, just like the drug-alone formulations, did not prevent the induction of dormant ring-stage parasites.

In another study carried out by the same researcher, the effect of Pheroid technology on the metabolism and pharmacokinetics of artemisone was determined in primates (*in vivo)* and in human and monkey liver and intestinal microsomes (*in vitro*). The latter indicated that Pheroid inhibits the microsomal metabolism of artemisone whereas the former signified an improvement in the pharmacokinetic profile due to the Pheroid system (Grobler *et al.*, 2014b).

In another study, formulation of the antimalarial, lumefantrine, with pro-Pheroid, resulted in increased solubility, absorption, and bioavailability following oral administration in mice. The therapeutic blood level was maintained for a longer duration, which would limit the chances of developing resistant infections. Although lumefantrine is known for its variable bioavailability (Ezzet *et al.*, 2000), entrapment in Pheroid delivered similar bioavailabilities in both fed and fasted states and reduced variability between subjects, which facilitates more consistent dosing

regardless of administration with or without food. Lastly, the *in vitro* antimalarial efficacy was enhanced by 46.00% with the use of Pheroid technology but, unfortunately, did not translate into improved antimalarial activity *in vivo* (Du Plessis *et al.*, 2015).

2.2.2.4.5 Antibiotics

An *in vitro* study to determine the activity of Pheroid vesicles containing antibiotics against *Plasmodium falciparum*, presented a decrease in the IC_{50} of several antibiotics with varied significance. After entrapment in Pheroid, azithromycin and erythromycin had significantly lower IC_{50} values and thus improved activity against a chloroquine-resistant strain of *Plasmodium falciparum.* The decrease in IC_{50} found with tetracycline and doxycycline, on the other hand, were not noteworthy (Du Plessis *et al.,* 2012).

2.3 Azoxystrobin

2.3.1 Introduction

There is a growing need for the enhancement of agricultural productivity and establishment of long-term food security so that especially poor communities will be less obliged to purchase food at highly inflated prices (Baiphethi & Jacobs, 2009). Maize is one of South Africa's (SA's) main food sources and is utilized by nine out of ten households (Kruger *et al.*, 2008). Many smallholders in Africa depend on its production for nutrition, income or animal feed and have few alternatives (Romney *et al.*, 2003; Baiphethi & Jacobs, 2009; Thornton, 2010; Fischer & Hajdu, 2015). According to the Crop Estimates Committee (2016), the total amount of maize produced in SA reduced by more than 50.00% from 2014 to 2016. SA experiences an annual population growth of nearly 2.00% (Statistics South Africa, 2015). The population (~55 million in 2015) is expected to reach 82 million by 2035. By then, it would be crucial that our country's current food production is increased almost twofold to feed the multiplying population (WWF - SA, 2010).

Malnutrition is highly prevalent in SA – especially in rural areas (Kruger *et al.*, 2008) and has a significant influence on clinical outcomes (Jensen *et al.*, 2010). Prevention of disease with the correct nutrition can reduce the impact of disease on the economy and increase life expectancy and productivity in the population. With the nutritional crisis in Africa continuing to spiral out of control, it is important to find new innovative ways to increase crop production without harming the environment further.

In an attempt to overcome the abovementioned agricultural hurdles, new formulations of pesticides will be addressed. Pesticides, such as the antifungal, azoxystrobin, are toxic formulations that are widely used to control a variety of harmful organisms in agriculture

(Schilter & Huggett, 1998), increase crop yield and quality (Koletzko *et al.*, 1999), and can be used in the prevention of certain vector-borne diseases like malaria. The formulation of azoxystrobin in Pheroid® aims to increase efficacy, decrease dosage, and thus decrease impact on the environment.

2.3.2 Azoxystrobin applications

Azoxystrobin, the systemic fungicide with the broadest spectrum of all known antifungals (Meyers, 2016), is used for its preventative and curative properties in plants (Clough *et al.*, 1996; Meister, 1997). This compound, derived from naturally occurring strobilurins, is effective at relatively low dosages (Ma, 2006) with activity against all four major fungal pathogens in plants, namely Ascomycetes (e.g., powdery mildews), Oomycetes (e.g., downy mildews), Deuteromycetes (e.g., leaf spots) and Basidiomycetes (e.g., rusts) (Bartlett *et al.*, 2002; Acton, 2013:366). It is absorbed through the roots of plants and translocate to the stems and leaves. Spore germination, mycelial growth, and spore production are inhibited by azoxystrobin through the inhibition of mitochondrial respiration in the fungi (Matheron & Porchas, 2000; Paranjape *et al.*, 2014:38).

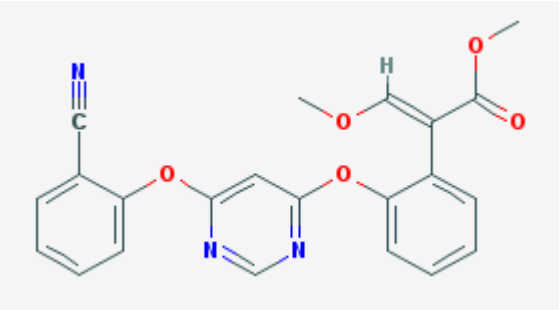

Figure 2-3: Chemical structure of azoxystrobin (obtained from the open chemistry database, Pubchem (2016)).

In addition to its use as a fungicide, it exhibits growth regulator, insecticidal and nematicidal properties (Anthony *et al.*, 1991). Azoxystrobin is used on vegetables, fruits, peanuts, rice and other crops to prevent or control diseases such as rice blast, apple scab and rusts (Anthony *et al.*, 1991; Meister, 1997). It is administered via foliar or stem application or seed treatment (Liu *et al.*, 2012) at a rate of 100 to 375g active ingredient per hectare (Hayes, 2007:740).

2.3.3 Characteristics

Azoxystrobin has been shown to have an oral median lethal dose (LD_{50}) of >5000 mg/kg, and a dermal LD_{50} of >2000 mg/kg, which signifies a low acute toxicity (FAO & WHO, 2009:56). It also proved to have a low chronic toxicity towards amongst other humans and mammals (Hollingworth, 2001:1200; US EPA, 2009). Contact with skin or eyes may cause irritation and inhalation could be toxic and should be avoided (FAO & WHO, 2009:56). Ingestion is concomitant with gastrointestinal disruption and burning of mucous membranes. It was

determined from studies carried out on small mammals that azoxystrobin would not have an embryotoxic, fetotoxic, teratogenic or carcinogenic effect in humans (FAO & WHO, 2009:57). After results from numerous trials were analysed, the FAO and WHO (2009:95) stated that the short and long term dietary intake of azoxystrobin residues would rarely cause a public health concern.

Requisite wear for handlers of this fungicide is strongly encouraged and consist of a coverall, long sleeved shirt and pants, shoes with socks, chemical resistant gloves and headwear (US EPA, 1997; Pohanish, 2014:52).

Azoxystrobin has a low solubility in water (6.7 mg/L at 20˚C). At the same temperature, however, the highest solubility is obtained with organic solvents like ethyl acetate and acetonitrile with the most effective solvent being dichloromethane (DCM) (400 mg/L) (MacBean, 2010; Paranjape *et al.*, 2014:38). Water solubility is indicated by an octanol-water partition coefficient (log K_{ow}) of <2 whereas fat solubility is indicated by a log K_{ow} of >4. Compounds that fall into the former classification have the potential to leach through soil into the groundwater and may pose an environmental risk (Carlile, 2006:192). The compound has a log K_{ow} of 2.5 which indicates an inclination to be water soluble (Pesticide Properties DataBase, 2016). Under field conditions, rapid degradation was noted and no residues of the compound or its metabolites were noticed at a depth of more than 10 cm into the soil (FAO & WHO, 2009:65).

In addition to the solubility requirements, an agricultural solvent has to be safe, environmentally suitable, and cost effective. Furthermore, the efficacy of the active ingredient should not be lowered and the final formulation has to be stable for the required amount of time.

2.3.4 Azoxystrobin and Pheroid®

A successful formulation of azoxystrobin in the Pheroid® system will provide a system that is an efficacious antifungal but that is also safe for the environment and cost effective. In agriculture, Pheroid® technology also has the potential to modify the toxicity of the active compounds, improve their target specificity on pests and reduce the development or exacerbation of resistance – a phenomenon with increasing incidence (Ma, 2006; Ma & Uddin, 2009; Thind, 2012:38-39; Ishii & Hollomon, 2015). This application of the delivery system is known as ‘enabling technology’, and is used to formulate non-toxic, user-friendly and effective products. Pheroid®, unlike many additives currently used in the industry, are completely harmless by itself and exhibit a peculiar ability to combine an array of functions of additives (Grobler, 2009).

A study was launched to formulate an azoxystrobin/Pheroid® formulation for use as a fungicide on crops. The standard specifications derived from the processing of data and information obtained from former studies was used to develop the formulation and predict its efficacy. The

results were then used to verify the specifications obtained from the data processing phase of the study and thus served to validate or discredit the results. Since azoxystrobin is a fungicide, it can be classified as an anti-infective - a general term that refers to antibacterials, antifungals, antivirals, antiprotozoans and antibiotics. Therefore, only data and information obtained from anti-infective Pheroid® formulations were used for the purpose of this study.

2.4 Conclusion

Standard specifications describe a product and are fundamental to the production process as it forms the basis of quality control. Consequently, it was decided to describe and formalize the existing ranges of the Pheroid delivery system. Due to the versatility of the system, and the favourable results that have been obtained with it, optimization of the results through formalizing specification ranges could only be beneficial to the system and possibly the pharmaceutical industry. The standardized characterization analyses that are applied to all Pheroid formulations were used as the focal point of the study. These analyses reflect the particle sizes and particle size distributions, morphology characteristics and the stability of the formulations. Data would therefore be grouped according to variables in the formulations (e.g. active ingredients or additives used) and then compared to the characterization reports obtained for the respective formulations. The results would then be used to reinforce the Pheroid specification ranges and subsequently to manufacture an azoxystrobin and Pheroid formulation in accord with the ranges. Azoxystrobin is not only ideal for this application because of its fungicidal (i.e. anti-infective) properties. The use of a plant model to verify the formalized ranges is effective and much less time consuming than an animal or human model.

In the subsequent chapter, the methodology used to statistically analyse the data and information, as well as the results that were obtained are discussed.

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CHAPTER 3 STATISTICAL DATA ANALYSIS

3.1 Introduction

This chapter includes an explanation of the experimental procedures that were followed to process and analyse the data and information obtained from previously manufactured reference and anti-infective Pheroid formulations as well as a detailed discussion of the results.

3.2 Statistical Data Analysis

3.2.1 Data processing: overview

Available data and information obtained from former anti-infective Pheroid studies were used for the purpose of this study (PCDDP, 2008 - 2015). Data and information were categorized in a logical manner to improve ease of use and ensure the accuracy of results. Broadly described, the data and information was separated dependant on:

- type of Pheroid used in the formulation (e.g. pro-Pheroid or micro-sponges),
- Active ingredient(s) in the formulation,
- raw materials,
- antioxidants used,
- preservatives used,
- other excipients,
- ingredient amounts,
- type of analysis,
- manufacturing methods,
- variation in the version/type of analytical instrument used,
- type of formulation (e.g. oral, topical, nasal),
- ratio of oil phase components, and
- results obtained.

After the statistical analysis was carried out, the need for a reference formulation was recognized. Several pro-Pheroid formulations with component ratios corresponding with that of the formulations used for the data processing were also processed (e.g. vitamin F ethyl ester to Kolliphor RH40 ratio of 2.8:1). These pro-Pheroid formulations comprised solely of pro-Pheroid ingredients, were manufactured with the same method, and contained no active ingredients, preservatives or any other excipients.

3.2.2 Data statistically processed for Pheroid characterization

Assistance with statistical data analysis and interpretation of processed results was obtained from the North-West University's Statistical Consultation Services (building G3, North-West University Campus).

For the purpose of this study, the following software and functions were used: IBM SPSS Statistics (version 23), STATISTICA (version 12), Descriptive Statistics, box plots, cross tabulation and frequencies.

- IBM SPSS statistics is software developed for managing data and calculating a wide variety of statistics.
- Descriptive Statistics was used to summarize and describe the basic features of the data in a meaningful and useful manner (De Muth, 2014:1). It does not infer the properties of the population from which the sample was drawn. Possible functions of descriptive statistics include mean, standard deviation, variation, minimum, maximum, median, range, and quartiles (De Muth, 2014:719).
- Box plots were used to graphically present the distribution of data in with regards to five factors: the minimum, first quartile, median, third quartile, and maximum. This facilitated comparison of data distributions between the different data sets as well as the identification of outliers.
- Cross tabulation was used to compare the relationship between the variables in the data.
- The frequencies were presented in a frequency distribution table. The latter displays the frequency of various outcomes within a sample.

3.3 Statistical analysis of data

The data used for the statistical analysis comprised of reference formulations, which consisted of Pheroid ingredients only, and complete anti-infective formulations (Pheroid, active ingredients, and other excipients). The reference and anti-infective formulations were further separated into the three types of Pheroid (vesicles, pro-Pheroid and micro-sponges) and discussed on the basis of the various characterization analyses results (Malvern Mastersizer, CLSM, and zeta potential). Before the characterization data of the anti-infective formulations are discussed, the reference formulations are statistically described in the form of box plots. This is included to provide an overview of the reference data and to supply important information such as minimum, maximum, and median values as well as outliers and extreme values. The reference data is also presented in the form of scatter plots amid the discussion of the anti-

infective formulations. These types of figures (also mainly used for the anti-infective data) enable a better understanding of data distribution through visual representation and facilitated the process of comparison. Unfortunately, the characterization analysis results (particle size distribution, CLSM, and zeta potential) for all the formulations were not obtainable. Insufficient data complicated the formation of trends in the data and subsequently resulted in several statistically insignificant observations.

3.3.1 Pheroid vesicles

38 Pheroid vesicle formulations were utilized for the reference data analysis. While the particle size distribution analysis for all the formulations was accessible, only 12 confocal reports and eight zeta potential measurements could be obtained. The anti-infective Pheroid vesicle data were compiled from 88 formulations. 67 had results from the Mastersize analysis; 51 had CLSM reports and ten included zeta potential measurements.

3.3.1.1 Particle size and particle size distribution

3.3.1.1.1 Statistical description of reference formulations

Most of the formulations had between 2.43 and 78.20% submicron sized particles although the interquartile range (25.00 to 75.00%) were more tightly spread and ranged between 19.74 and 44.47% submicron (see Figure 3-1). The minimum, average, and maximum values were 2.43, 36.68, and 100% respectively. Pheroid particles are preferred to be between 200 nm and 2 µm in size.

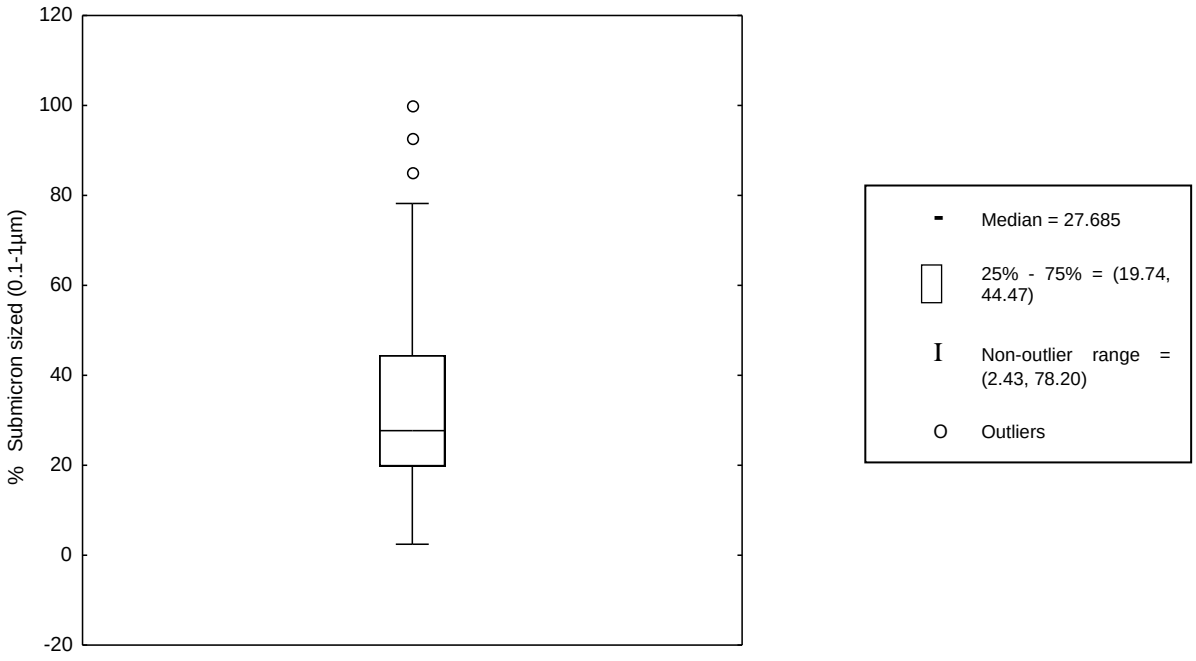

Figure 3-1: Percentage submicron sized particles (0.1 to 1 µm) in the Pheroid vesicle reference formulations (obtained from Mastersize analysis)

An average of 41.45% of the particles in the formulations was micron sized. Since some formulations were completely submicron sized, the minimum micro-sized value was 0.00%. The percentage micron sized particles in all of the formulations ranged from 0 to 75.28% (i.e. no outliers or extremes were present). The centric 50.00% contained 31.68 to 52.85% micron sized particles (see Figure 3-2).

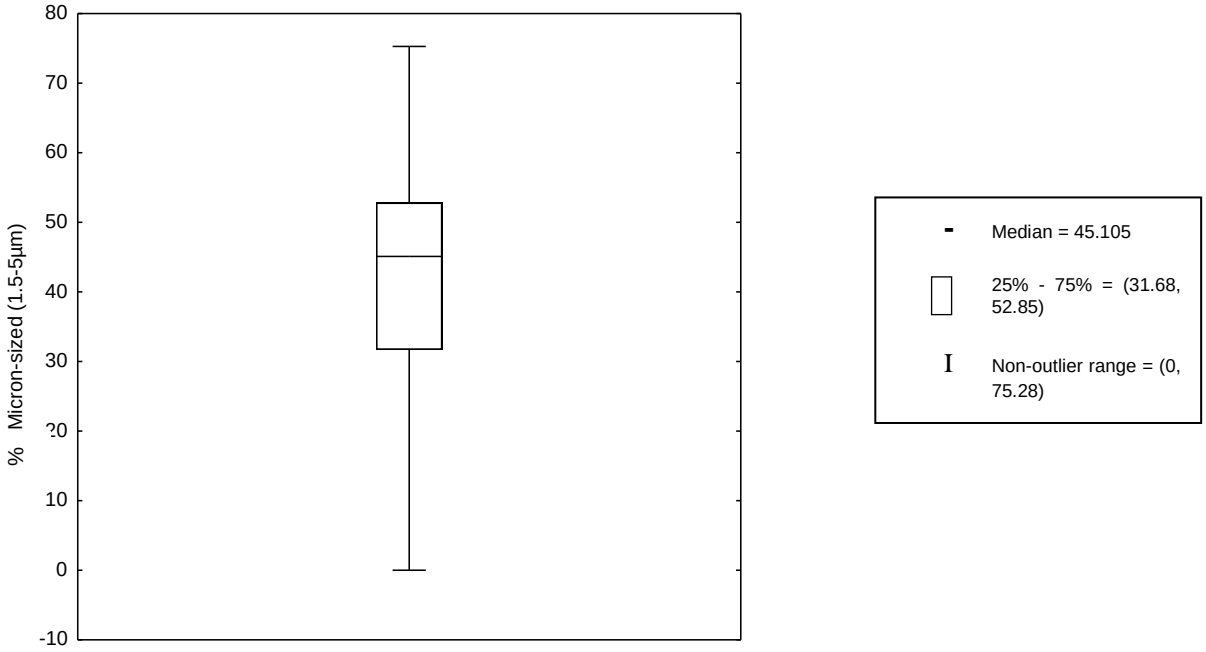

Figure 3-2: Percentage micron sized particles (1.5 to 5 µm) in the Pheroid vesicle reference formulations (obtained from Mastersize analysis)

3.3.1.1.2 Anti-infective formulations

Figures 3-3 and 3-4 are visual representations of the particle size distribution in the various vesicle reference and anti-infective formulations respectively. Submicron particles are between 0.1 and 1 µm in size whereas micron sized particles range from 1.5 to 5 µm. The figures only display the percentage particles in each formulation that falls within the stated size ranges. The rest of the percentage particles (which makes up 100.00%) are mainly larger than 5 µm. The latter is not presented on the figures as the desired particle sizes for Pheroid formulations (between 200 nm and 2 µm) do not exceed those ranges. No trend is visible for the percentage submicron and micron sized particles in both the reference and the anti-infective formulations except for the fact that most reference formulations manufactured during 2015 and 2016 contained a high fraction of submicron sized particles. During that timeframe, the manufacturers varied and some of them also manufactured several of the former formulations. The scattered distribution seen with the reference formulations (i.e. no actives or other ingredients) indicates that the method of preparation (e.g. mixing method) influenced the particle sizes. This undesirable phenomenon underlines the importance of having standardized manufacturing

guidelines in place and the concomitant adherence of the applicable personnel to those guidelines.

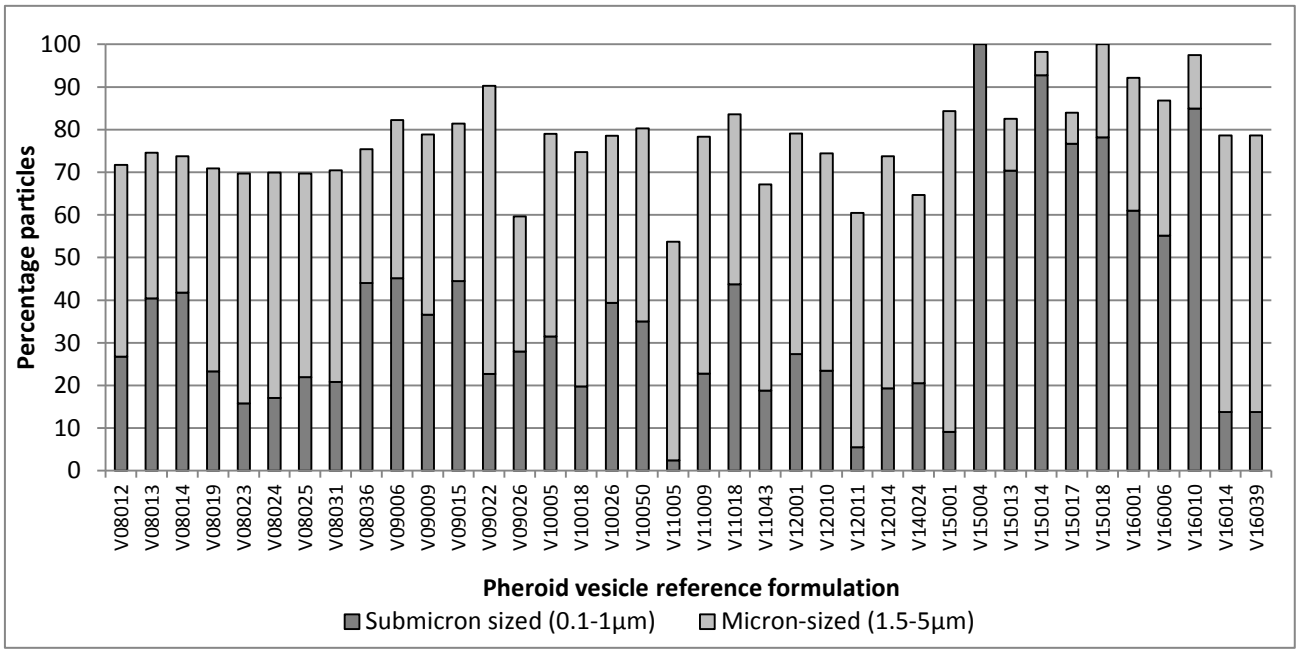

Figure 3-3: Percentage submicron and micron sized particles in the respective Pheroid vesicle reference formulations (obtained by Mastersize analysis)

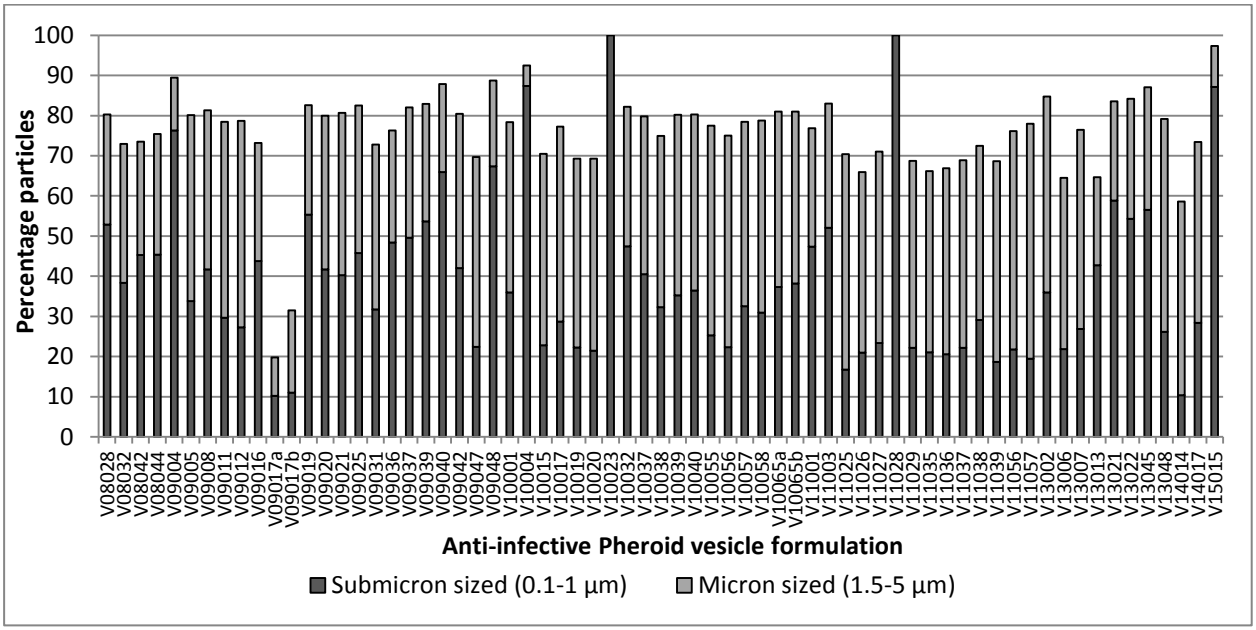

Figure 3-4 Percentage submicron and micron sized particles in the respective anti-infective Pheroid vesicle formulations (obtained by Mastersize analysis)

An outline of the ingredients used in each anti-infective formulation (identified by means of the batch number (e.g. V08028)) is presented in Table 3-1 (see Table 3-2 to 3-6 for a description of the values indicated for every ingredient). Since the reference formulations were identical and comprised exclusively of Pheroid ingredients at the standard ratios, an outline of the ingredients were not deemed necessary. The impact of the ingredients in the anti-infective formulation on the particles sizes is individually discussed.

Table 3-1: Ingredients present in the various anti-infective Pheroid vesicle formulations

BATCH NO.	OIL PHASE	WATER PHASE	ACTIVE INGREDIENT	PRESERVATIVE	ANTIOXIDANT	OTHER EXCIPIENTS
V08028	1	1	2	0	0	0
V08032	1	4	3	0	0	0
V08042	1	1	4	0	0	2
V08044	1	4	5	0	0	0
V09002	4	2	45	2	2	3
V09003	4	2	45	2	2	4
V09004	1	1	6	0	0	0
V09005	1	1	7	0	0	0
V09008	1	1	2	0	0	0
V09011	1	1	8	0	0	0
V09012	1	1	9	0	0	0
V09016	1	1	10	0	0	0
V09017a	1	1	11	0	0	5
V09017b	1	1	11	0	0	2
V09019	1	1	12	0	0	0
V09020	1	1	13	0	2	0
V09021	1	1	14	0	2	0
V09025	1	1	10	0	0	0
V09031	1	1	10	0	0	6
V09036	1	1	15	0	0	0
V09037	1	1	16	0	0	0
V09039	1	1	17	0	0	0
V09040	1	1	18	0	0	0
V09042	1	1	15	0	0	0
V09047	1	1	17	0	0	0
V09048	1	1	18	0	0	0
V10001	1	1	43	0	2	0
V10004	1	1	19	0	0	0
V10010	4	2	46	3	2	7
V10015	1	1	20	0	0	0
V10017	1	1	21	0	0	0
V10019	1	1	22	0	0	0
V10020	1	1	23	0	0	0
V10023	1	1	19	0	0	0
V10032	1	1	43	0	2	0
V10037	1	1	22	0	0	0
V10038	1	1	24	0	0	0
V10039	1	1	21	0	0	0
V10040	1	1	13	0	0	0
V10055	1	1	25	0	0	0
V10056	1	1	22	0	0	0
V10057	1	1	21	0	0	0
V10058	1	1	21	0	0	0

Table 3-1: Ingredients present in the various anti-infective Pheroid vesicle formulations (continued)

BATCH NO.	OIL PHASE	WATER PHASE	ACTIVE INGREDIENT	PRESERVATIVE	ANTIOXIDANT	OTHER EXCIPIENTS
V10065a	1	1	43	0	2	0
V10065b	1	1	43	0	2	0
V11001	1	1	26	0	2	0
V11003	1	1	27	0	0	0
V11025	1	1	28	0	0	0
V11026	1	1	29	0	0	0
V11027	1	1	30	0	0	8
V11028	1	1	31	0	0	9
V11029	1	1	1	0	0	0
V11035	1	1	28	0	0	0
V11036	1	1	32	0	0	0
V11037	1	1	7	0	0	0
V11038	1	1	15	0	3	0
V11039	1	1	15	0	3	0
V11056	1	1	32	0	0	0
V11057	1	1	7	0	0	0
V12006	4	3	46	4	4	10
V13002	2	1	33	0	3	5
V13006	1	1	34	0	0	0
V13007	1	1	35	0	0	0
V13013	1	4	36	3	0	11
V13021	1	1	38	0	0	0
V13022	1	1	39	0	0	0
V13030	1	1	35	0	0	0
V13031	1	1	44	0	0	0
V13045	1	4	37	3	0	11
V13048	1	1	41	0	0	0
V14014	3	3	42	2	3	13
V14017	1	1	40	0	0	0
V15015	1	5	37	3	0	12

3.3.1.1.2.1 Oil phase

Only two formulations (with Mastersize analysis results) did not include oil phase 1 in the formulation. Additionally, oil phase 1 is the standard oil phase (with specific ingredient ratios). In point of fact, it is the most likely used oil phase and only its effect on the formulation is crucial to be determined (it is also the only oil phase present in reference formulations). The wide spread of the percentage particles that made use of oil phase 1 (Figures 3-5 and 3-6) indicate that the oil phase did not influence the percentage submicron and micron sized particles in the formulations and therefore did not influence the particle sizes. The merging of oil phase 1 markers from Figure 3-5 and 3-6, would result in a graph that is very similar to that of the

reference formulation (see Figure 3-7). If a variable did have an influence on a formulation's parameters, a trend (e.g. clustering of data) would have been noticeable. No trends could be observed for oil phase 2 and 3 since Mastersize analysis data were only available for one formulation each.

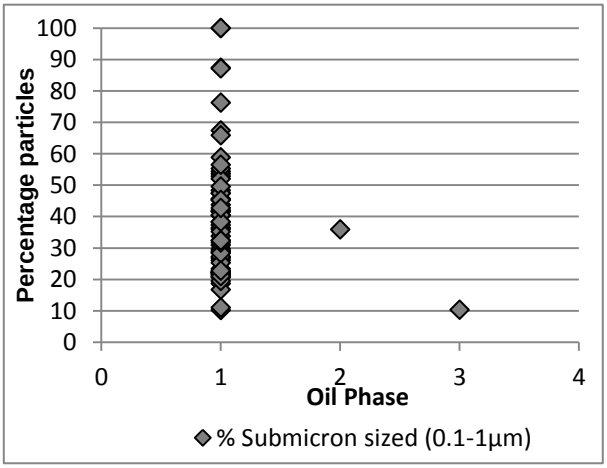

Figure 3-5: Percentage submicron sized particles against the oil phase used in the respective anti-infective Pheroid vesicle formulations (obtained by Mastersize analysis)

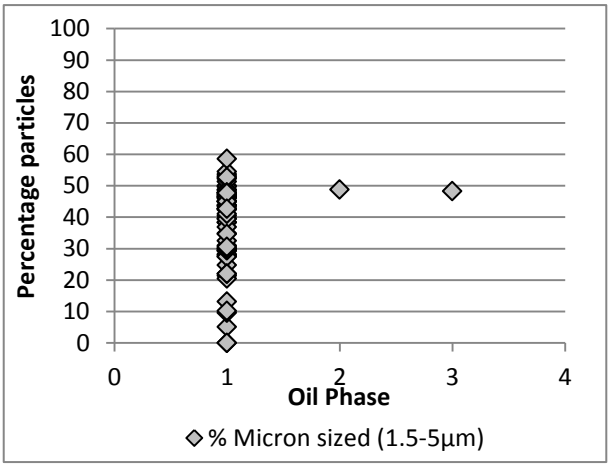

Figure 3-6: Percentage micron sized particles against the oil phase used in the respective anti-infective Pheroid vesicle formulations (obtained by Mastersize analysis)

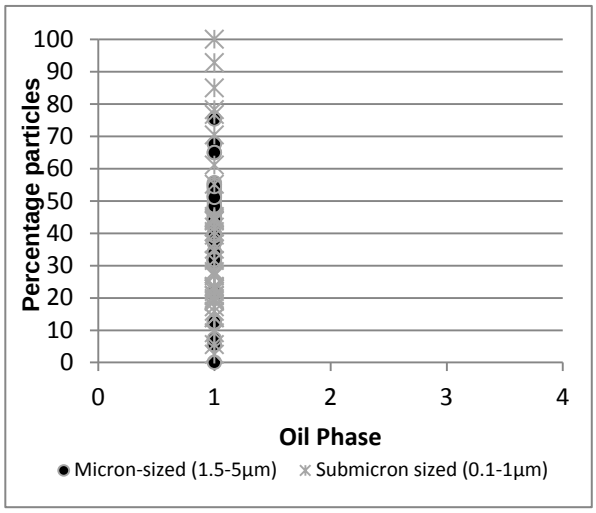

Figure 3-7: Percentage submicron and micron sized particles against the oil phase used in the Pheroid vesicle reference formulations (obtained by Mastersize analysis)

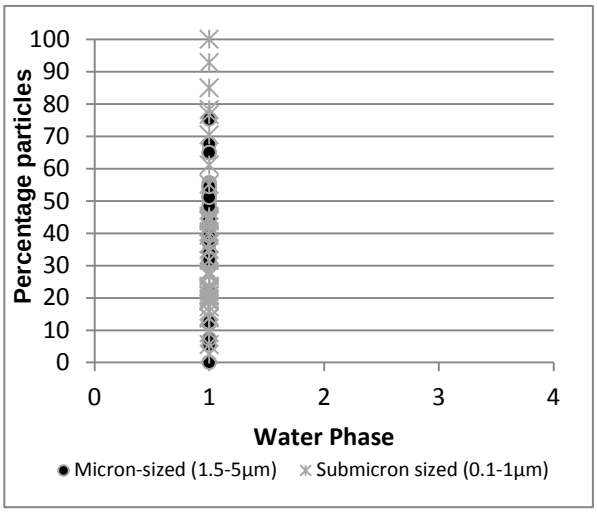

Figure 3-8: Percentage submicron and micron sized particles against the water phase used in the Pheroid vesicle reference formulations (obtained by Mastersize analysis)

3.3.1.1.2.2 Water phase

The water phases that made up the various anti-infective vesicle formulations were divided into five groups based on the volume and type of water used (deionized or phosphate-buffered saline (PBS)). A few groups were sufficient as the volumes of water in the phases were very

similar and could easily be grouped. All of the water phases were gassed with N_2O before and/or after the formulations was manufactured (see Table 3.2).

Table 3-2: Description of the various water phases used in the anti-infective Pheroid vesicle formulations

Water Phase	% of formulation	Description	Frequency
1	80-100	$N_2O{\cdot}H_2O$	69
2	60-79	$N_2O{\cdot}H_2O$	3
3	40-59	$N_2O{\cdot}\ H_2O$	3
4	80-100	$N_2O{\cdot}$PBS	12
5	40-59	$N_2O{\cdot}$PBS	1

Water phase 1 comprised of 80-100% $N_2O{\cdot}H_2O$ (nitrous oxide gassed water), and occurred in 69 formulations. Water phase 4 ($N_2O{\cdot}$PBS) was used in 12 formulations and made up 80-100% of every formulation. The other water phases were not used in a sufficient number of formulations to provide significant results and will subsequently not be discussed. From Figure 3-9 and 3-10, it is clear that water phase 1 did not influence the percentage submicron and micron sized particles in the formulations because, regardless of its presence in the formulation, a wide variety of particle sizes was obtained. Moreover, combining the graphs would result in a graph that resembles Figure 3-8 (the reference formulations). Figure 3-7 and 3-8 are identical since the same oil- and water phases were used in all the reference formulations.

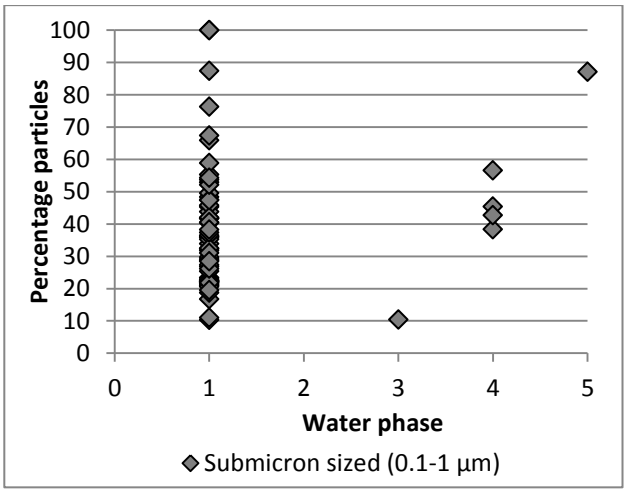

Figure 3-9: Percentage submicron sized particles against the water phase used in the respective anti-infective Pheroid vesicle formulations (obtained by Mastersize analysis)

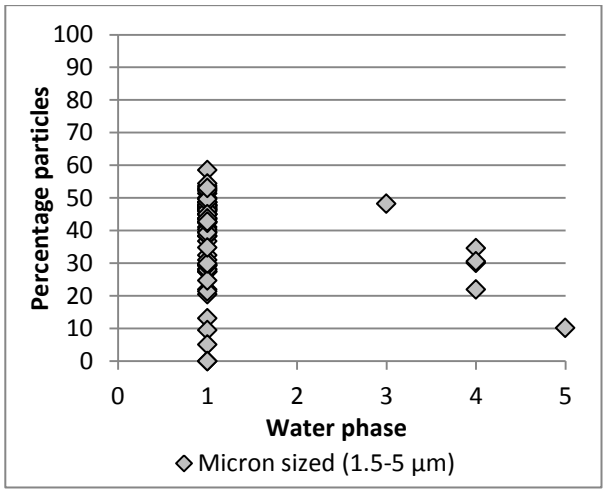

Figure 3-10: Percentage micron sized particles against the water phase used in the respective anti-infective Pheroid vesicle formulations (obtained by Mastersize analysis)

3.3.1.1.2.3 Preservatives

Table 3-3 summarizes the various preservative combinations found in the anti-infective Pheroid vesicle formulations. Of the 67 formulations that had Mastersize analysis results available, 63 did not contain any preservatives while preservative combination 2 and 3 was only present in

one and three formulations respectively (see Figure 3-11 and 3-12). It goes without saying that the omission of preservatives would not influence the particle size distribution in comparison with the reference (that did not contain any preservatives). Combination number 3 was unfortunately only present in a few formulations, but those formulations contained a range of particle sizes and did not likely influence the particle sizes of the formulation. Since there was not enough data available, this is only a vague prediction.

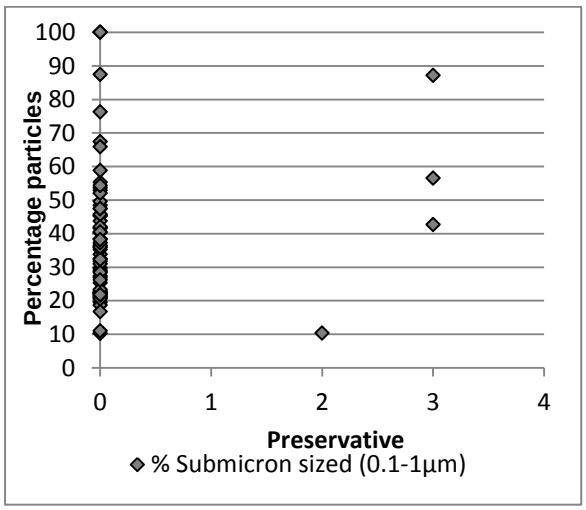

Figure 3-11: Percentage submicron sized particles against the preservative used in the respective anti-infective Pheroid vesicle formulations (obtained by Mastersize analysis)

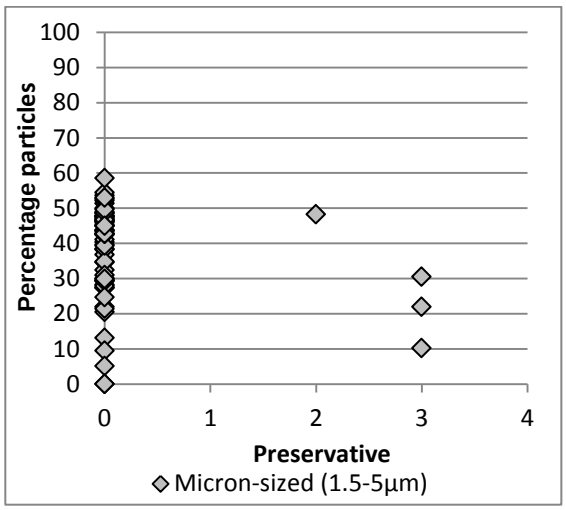

Figure 3-12: Percentage micron sized particles against the preservative used in the respective anti-infective Pheroid vesicle formulations (obtained by Mastersize analysis)

Table 3-3: Various preservative combinations used in the anti-infective Pheroid vesicle formulations

No.	Ingredient	%	Frequency
1	Methyl Paraben Propyl Paraben	0.50 0.02	1
2	Methyl Paraben Propyl Paraben	0.40 0.08	4
3	Methyl Paraben Propyl Paraben	0.20 0.04	5
4	Methyl Paraben Propyl Paraben	0.30 0.50	1

Table 3-4: Various antioxidant combinations used in the anti-infective Pheroid vesicle formulations

No.	Ingredient	%	Frequency
1	BHA BHT	0.02 0.10	1
2	BHA BHT	0.02 0.20	11
3	BHA BHT	0.01 0.01	4
4	BHT	0.01	1

3.3.1.1.2.4 Antioxidants

67 formulations had Mastersize analysis data available. Antioxidant combinations 2 and 3 were present in respectively seven and four formulations (see Table 3-4). No antioxidants were present in fifty-six formulations and subsequently had no influence on the particle sizes in these formulations. In contrast with the wide distribution of particle sizes seen with preservative

combination 3 in Figures 3-11 and 3-12, antioxidant combination 2 and 3 in Figures 3-13 and 3-14 were much more clustered between specific ranges. Formulations that included combination 2 had submicron and micron sized particles that comprised of 30.00 to 50.00% of every formulation. Formulations that contained antioxidant combination 3 had particles that were more distributed within the submicron particle range although the micron sized fraction was more clustered. However, the results obtained for combination 2 are statistically more significant than for combination 3 because more than twice the amount of data was available.

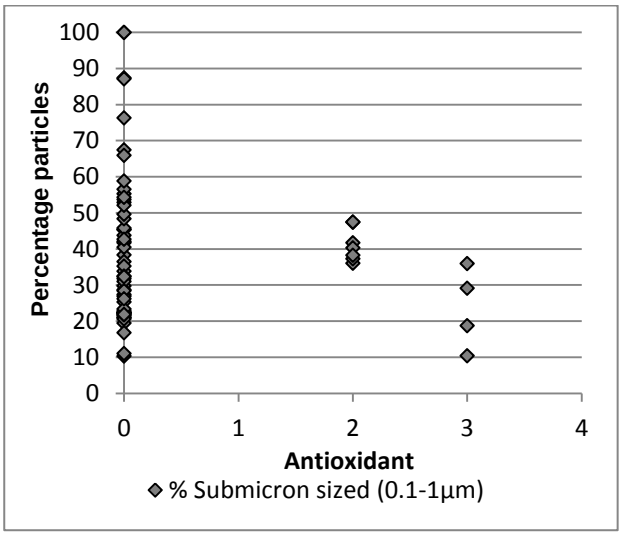

Figure 3-13: Percentage submicron sized particles against the antioxidant used in the respective anti-infective Pheroid vesicle formulations (obtained by Mastersize analysis)

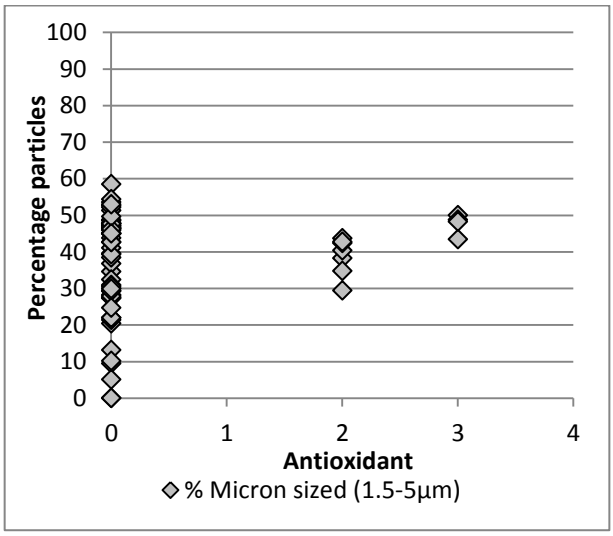

Figure 3-14: Percentage micron sized particles against the antioxidant used in the respective anti-infective Pheroid vesicle formulations (obtained by Mastersize analysis)

3.3.1.1.2.5 Other excipients

Fifty-six formulations that did not contain any other excipients had particle size distribution results available (see Figures 3-15 and 3-16). Any variations of the particle size distribution from the reference formulations could thus not be attributed to this factor. The various other excipient combinations that possessed Mastersize analysis data were present in two formulations at most. Combinations 2 and 5 were present in formulations that had a semi-wide distribution of particle sizes. Formulations that included combination 11, on the other hand, had values that were more closely distributed (see Table 3-5 for a discussion of excipient combinations). Significant conclusions could not be drawn from the limited data and are therefore not discussed further.

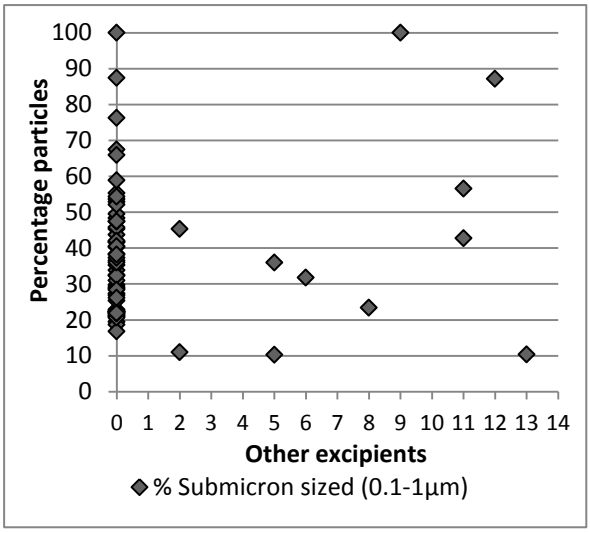

Figure 3-15: Percentage submicron sized particles against the other excipients used in the respective anti-infective Pheroid vesicle formulations (obtained by Mastersize analysis)

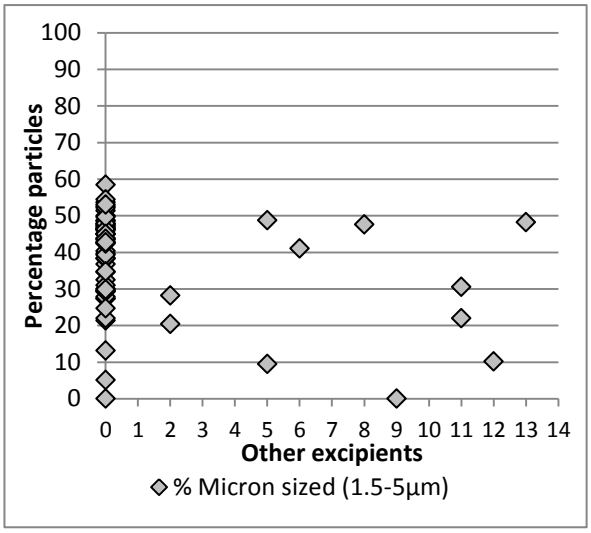

Figure 3-16: Percentage micron sized particles against the other excipients used in the respective anti-infective Pheroid vesicle formulations (obtained by Mastersize analysis)

Table 3-5: Various combinations of 'other excipients' used in the anti-infective Pheroid vesicle formulations

Nr.	Excipient	%	Frequency
1	Cetyl alcohol Liquid paraffin Span 60 Tween 80	10.00 20.00 0.50 4.50	1
2	PEG 400	0.40	2
3	Oleic acid Cetyl alcohol Tween 80 Span 60	14.00 2.00 5.00 1.50	1
4	Oleic acid HPMC Tween 80 Span 60 Glycerol	14.00 0.35 5.00 1.50 2.50	1
5	PEG 400	0.20	2
6	Glacial acetic acid	2.00	1
7	Liquid paraffin Cetyl alcohol Tween 80 Span 60 Propylene glycol	12.00 12.00 1.50 1.50 8.00	1
8	PEG 400	1.80	2
9	PEG 400	0.75	1
10	Liquid paraffin Cetyl alcohol Tween 80 Span 60 Propylene glycol	20.00 5.00 7.00 2.00 15.00	1
11	PEG 400 Xanthan Gum	1.25 1.00	3
12	PEG 400 Xanthan Gum	1.25 0.80	1
13	Cetearyl alcohol Oleic acid Isopropyl meristat Isostearyl isostearate Beeswax Liquid paraffin Glycerine Glyceryl stearate	3.00 9.00 8.00 4.00 6.00 2.00 1.00 2.00	1

3.3.1.1.2.6 Active ingredients

The percentage particles within the submicron and micron sized ranges obtained for the various anti-infective vesicle formulations are presented in Figure 3-17. The formulations are arranged by the different active ingredients contained in each Pheroid formulation (defined in Table 3-6). Only a few active ingredients were present in more than one formulation. For the most part, the formulations with analogous actives had similar particle size distribution in terms of percentage particles in the submicron and micron size ranges. It is important to note that, although the formulations had the same type and concentration of active ingredient, different additives may have been used. If that was the case, it may indicate that the active ingredients played a part in the particle sizes that were obtained. Nonetheless, most formulations had 70.00% or more particles in the submicron and micron size range combined. The formulation that contained active ingredient number 42 had a combined percentage just short of 60.00% while both formulations to which number 11 were added to had very low amounts of particles within the stated range (~ 20.00 to 30.00%).

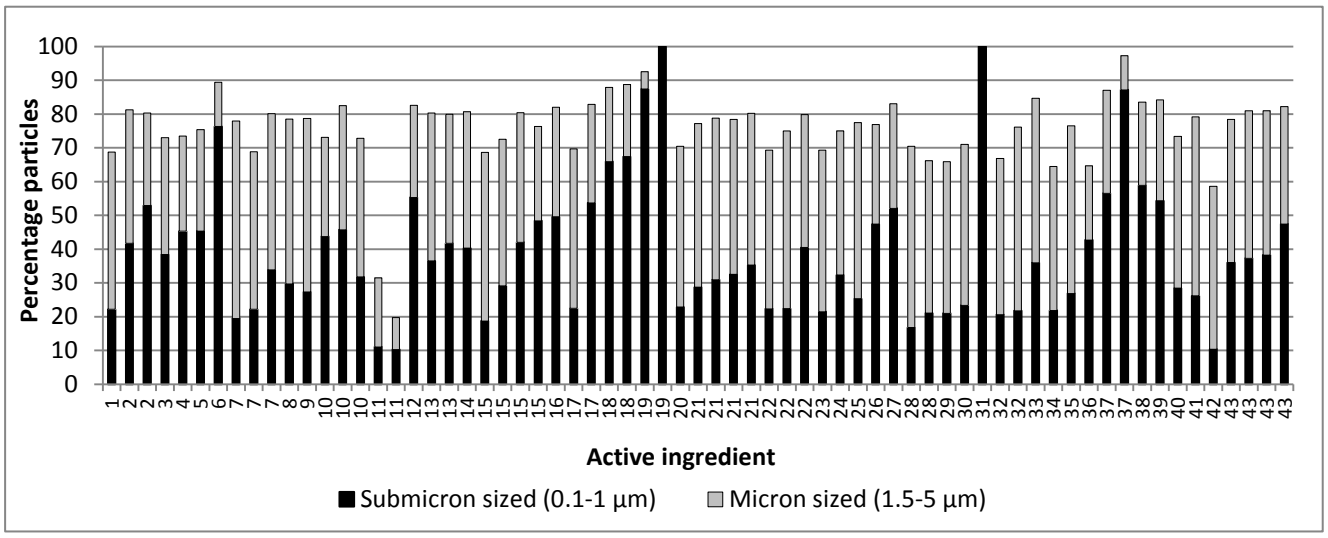

Figure 3-17: Percentage submicron and micron sized particles obtained with the various active ingredients included in the Pheroid vesicle formulations (obtained by Mastersize analysis)

Table 3-6: Active ingredients used in the anti-infective Pheroid vesicle formulations

Nr.	Active ingredient	%	Frequency
1	Chloroquine	1.50	1
2	Artesunate	0.08	2
3	Mefloquine	0.05	1
4	Artemiside	0.007	1
5	Artesunate	0.005	1
6	Mefloquine	0.20	1
7	Artemether	0.03	3
8	Chloroquine	0.07	1
9	Chloroquine	0.04	1
10	Quinine	1.25	3
11	Azithromycin	4.71	2
12	Triclosan	0.006	1
13	Chloroquine	0.25	2
14	Amodiaquine	0.25	1
15	Amodiaquine	0.05	4
16	Triclosan	0.40	1
17	Tetracycline	2.41	2
18	Doxycycline	2.57	2
19	Mefloquine	0.50	2
20	Chloroquine phosphate	5.56	1
21	Chloroquine phosphate	0.85	4
22	Amodiaquine	0.60	3
23	Amodiaquine	0.33	1
24	Amodiaquine	5.56	1
25	Amodiaquine	0.01	1
26	Amodiaquine	2.61	1
27	Streptomycin sulphate	1.00	1
28	Artemether	1.00	2
29	Amodiaquine	3.73	1
30	Artemisone	1.00	1
31	Artesunate	1.00	1
32	Artemether	0.30	2
33	Artemisone	0.40	1
34	Rifampicin	0.75	1
35	Isoniazid	0.625	2
36	Doxycycline	0.10	1
37	Doxycycline hyclate	0.10	2
38	Rifampicin NP	2.00	1
39	Isoniazid NP	1.725	1
40	Coumarin 6 NP	1.00	1
41	Isoniazid NP	2.50	1
42	Green tea active	0.25	1
43	Chloroquine	0.009	4
44	Rifampicin	1.50	1
45	Rifampicin Isoniazid	1.00 0.50	2
46	5-Fluorouracil	0.50	2

The particle size distribution of the anti-infective formulations that contained quinolones is presented in Figure 3-18. This serves to illustrate the particle size distributions that were obtained for formulations that contained active ingredients within the same drug class and to determine whether the recognition of a trend is simplified. The formulations in Figure 3-18 are positioned according to the active ingredient and the amount of drug in the formulation (in an ascending order).

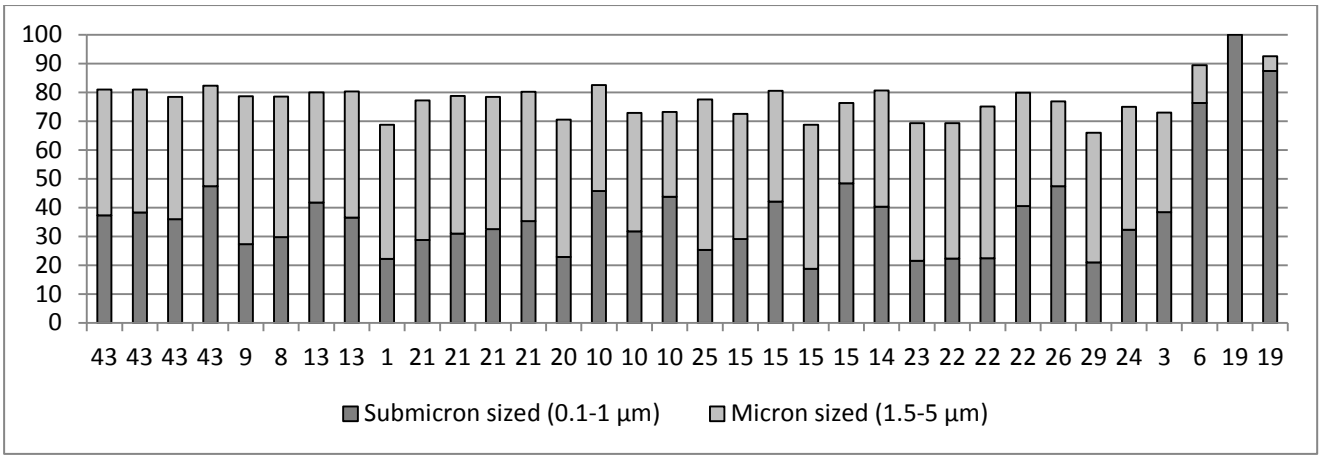

Figure 3-18: Percentage submicron and micron sized particles in the respective Pheroid vesicle formulations that contained quinolone anti-malarials (obtained by Mastersize analysis)

All but one formulation that contained chloroquine (present in active ingredient 43, 9, 8, 13 and 1) had a total of ~80.00% of particles in the submicron and micron size range. The formulation with the highest amount of chloroquine (active ingredient 1) had less than 70.00% of particles in those size ranges. Of the two formulations that contained active ingredient 13, one contained an antioxidant as well. The latter had slightly larger particles than the formulation that did not contain any additional excipients. The other chloroquine formulations that contained no additional excipients (active ingredient 9, 8 and 1) had larger particles than the other chloroquine formulations.

Formulations that contained chloroquine phosphate (active ingredient number 21 and 22) had particle size distributions that were similar to the chloroquine formulations but with smaller size variations between the formulations. No other excipients were present in any of the chloroquine phosphate formulations.

Quinine (active ingredient 10) was present in three formulations and only one formulation contained an extra excipient. The latter formulation had larger particles than the other quinine formulations.

The two formulations that contained amodiaquine (active ingredient 15) as well as an antioxidant had larger particles than the other two formulations that did not contain additional excipients. Active ingredient 14 and 26 were present in formulations that contained the same antioxidant and contained smaller particles than the surrounding formulations with similar amounts of amodiaquine and no additional excipients. Therefore, the smaller particle size distribution observed in the formulations that contained active ingredient 14 and 26 could be a result of antioxidant number 2 (see Table 3-4).

None of the formulations that contained mefloquine (active ingredient 3, 6 and 19) contained any additional excipients. The formulations that contained the highest concentration of the drug had the smallest particles. Active ingredient 3 signifies 0.05% mefloquine whereas active ingredient 6 and 19 indicates to 0.20% and 0.50% active respectively. The formulations that contained mefloquine contained the smallest particles whereas the other quinolones were present in formulations that had size distributions that were similar to each other (~80.00% of particles in the submicron and micron size range combined).

3.3.1.2 CLSM analysis: largest particle in each formulation

3.3.1.2.1 Statistical description of reference formulations

According to the confocal analysis, the size of the largest particle in each formulation ranged between 3 and 10 µm and averaged 6.12 µm (± 3.638). One outlier had a value of 18 µm (illustrated in Figure 3-19).

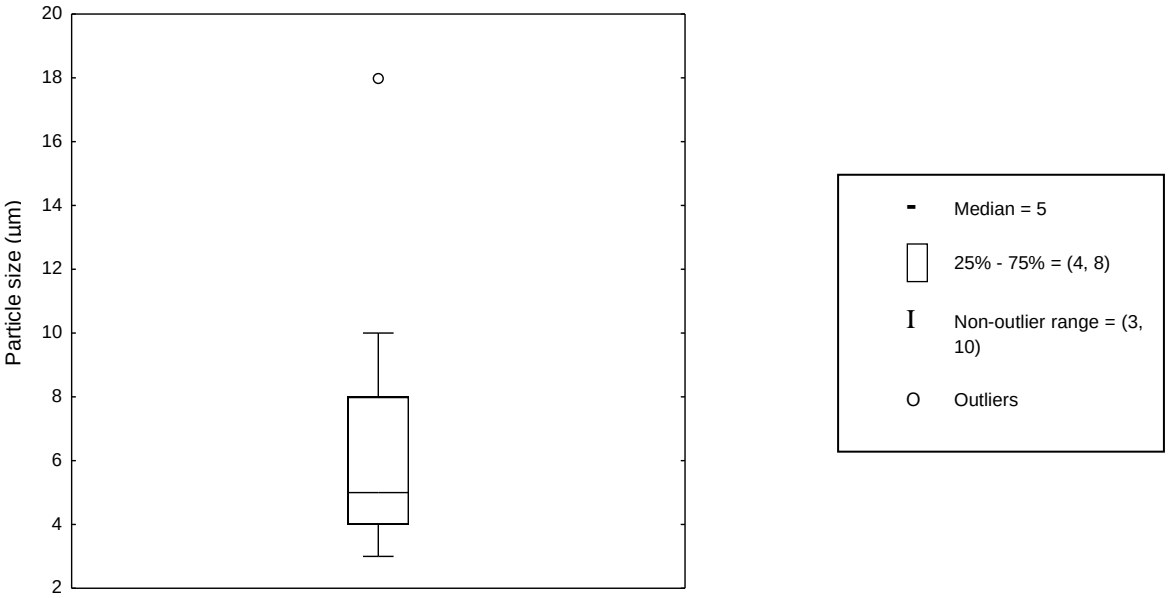

Figure 3-19: The largest particle in each Pheroid vesicle reference formulation (obtained from CLSM analysis)

The concentration of Pheroid within the 200 to 5000 nm diameter range for the various reference vesicle formulations are depicted in Figure 3-20 (displayed as concentration $x10^8$ per mL). The latter diameter range reflects the size span that is visible with the specific wavelengths that are used for the confocal microscopy analysis. The different formulations had Pheroid concentrations ranging from 122.72 $x10^8$ to 1614 $x10^8$ with an average mean concentration of 636.78 $x10^8$ per mL.

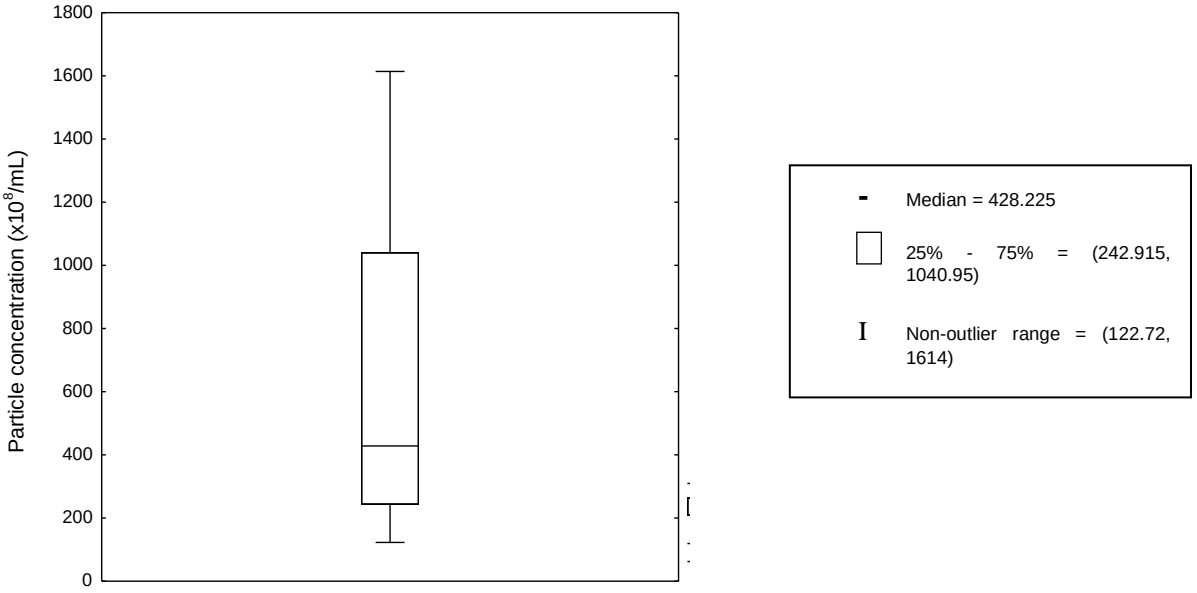

Figure 3-20: The Pheroid concentration (x10^8 per mL) in each Pheroid vesicle reference formulation (obtained from CLSM analysis)

A mean value of 1062.75 nm was obtained for the average Pheroid size (measured in nanometre) between the different Pheroid vesicle reference formulations. The interquartile range (25.00 to 75.00%) were 916 to 1147.5 nm in size although most of the formulations had an average Pheroid size ranging between 620 and 1175 nm (see Figure 3-21). One extreme value with an average size of 2048 nm was reported. None of the formulations contained any crystals.

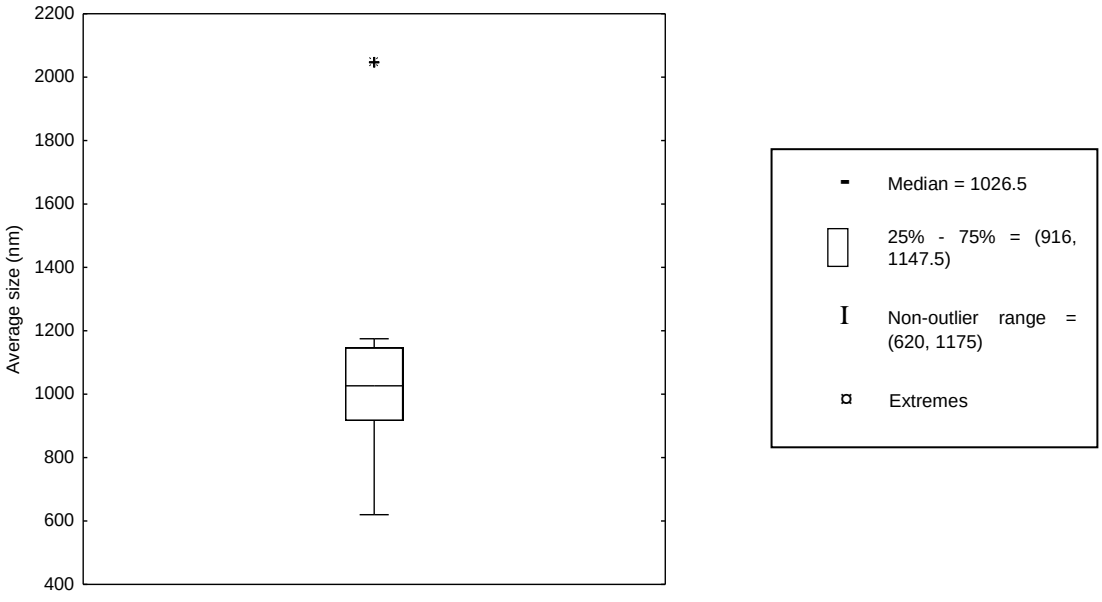

Figure 3-21: The average Pheroid size (nm) in each Pheroid vesicle reference formulation (obtained from CLSM analysis)

3.3.1.2.2 Anti-infective formulations

The largest particle in the various reference and anti-infective formulations are presented in a graph format for ease of comparison. Since submicron particles are always present in Pheroid

formulations (i.e. <1 µm), only the largest particle size in each formulation (measured in nanometre) is reflected in Figure 3-22 (for ease of visualisation) but represents the entire size span of each formulation. Therefore, the particles in the respective formulations are smaller than or equal to the stipulated value. The values between the anti-infective and the reference formulations were similar, although only twelve of the latter formulations were available for comparison. On the other hand, the anti-infective formulations did tend to have slightly larger particles. In an attempt to determine the causative factor, the data was compared to the various components in the anti-infective formulations.

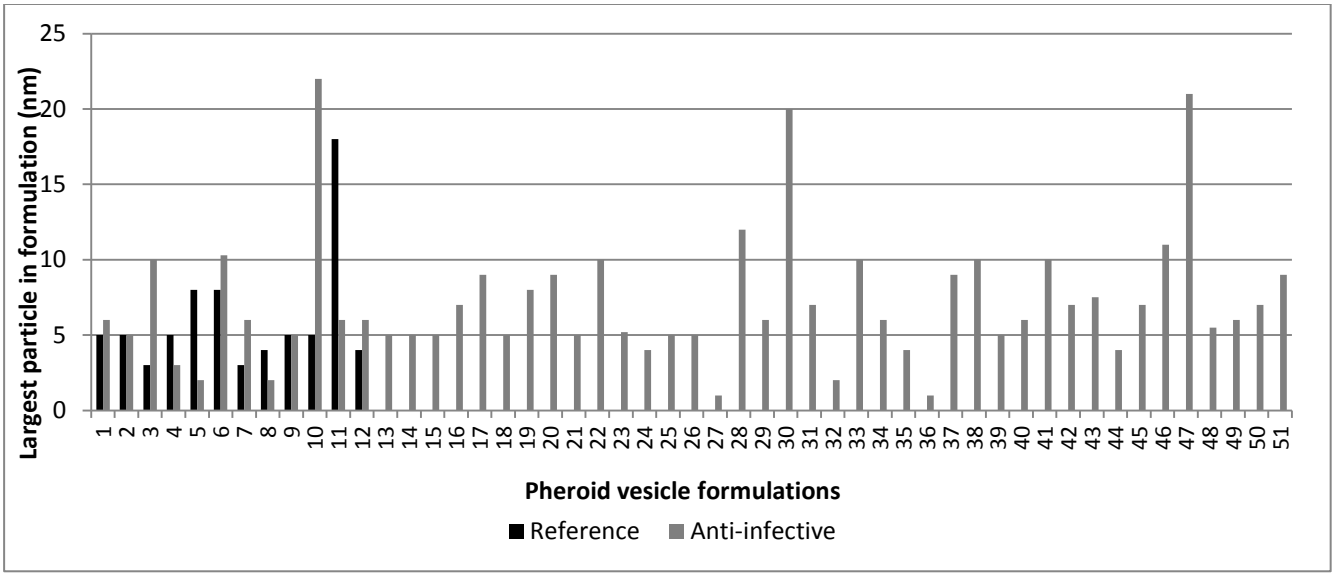

Figure 3-22: The largest particles in the reference and anti-infective vesicle formulations respectively (obtained by CLSM analysis)

3.3.1.2.2.1 Oil phase

A visual comparison of the average particle size in the Pheroid vesicle reference formulations against the anti-infective formulations is found in Figures 3-23 and 3-24. Oil phase 4 in the anti-infective formulations comprised of the exact same oil ratios as oil phase1 . The only difference between the two phases was the fact that no. 1 contained Kolliphor™ EL in contrast to the use of Kolliphor™ RH-40 in no. 4. Kolliphor™ EL is obtained through a reaction of castor oil with ethylene oxide at a molar ratio of 1:35. Instead of standard castor oil, hydrogenated castor oil is used in reaction with ethylene oxide (at a molar ratio of 1:40) to create Kolliphor™ RH40 (Berthelsen *et al.*, 2015). Limited data was available on the confocal analysis of the reference formulations. Particles were on average between 500 and 1250 nm in size although particles in some formulations averaged a size of ~2050 nm. The average particle sizes in the anti-infective formulations were mostly concentrated in the same range although some formulations had average particles sizes that were significantly larger. The scattered average size pattern seen in Figure 3-24 indicates that the oil phase did not influence the average particle size.

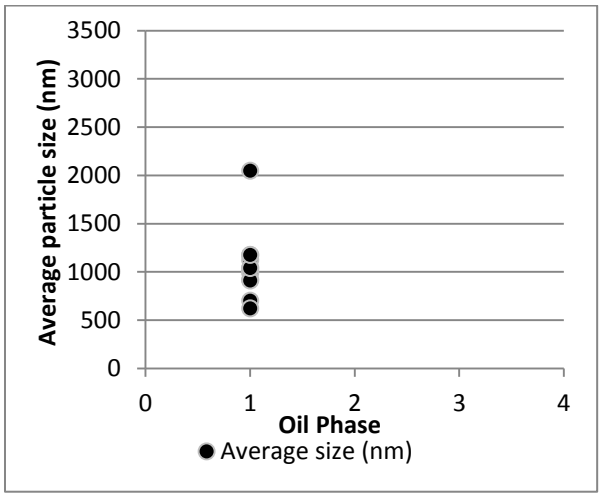

Figure 3-23: The average particle size obtained against the oil phase used in the Pheroid vesicle reference formulations (obtained by CLSM analysis)

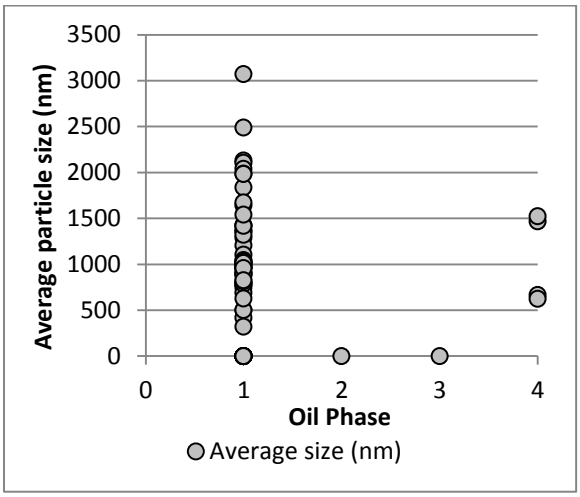

Figure 3-24: The average particle size obtained against the oil phase used in the respective anti-infective Pheroid vesicle formulations (obtained by CLSM analysis)

3.3.1.2.2.2 Water phase

Water phase 1 in the anti-infective formulations corresponds with the water phase in the reference formulations (displayed in Figures 3-25 and 3-26). Yet again, the reference data was limited but a trend (similar to the one found with the oil phases above) is seen between the average particle sizes in the reference and anti-infective formulations used in the anti-infective Pheroid vesicle formulations. See Table 3-2 for a description of the various water phases.

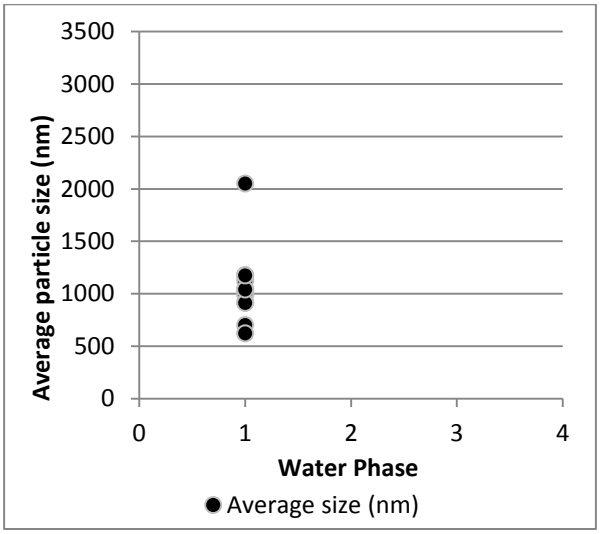

Figure 3-25: The average particle size obtained against the water phase used in the Pheroid vesicle reference formulations (obtained by CLSM analysis)

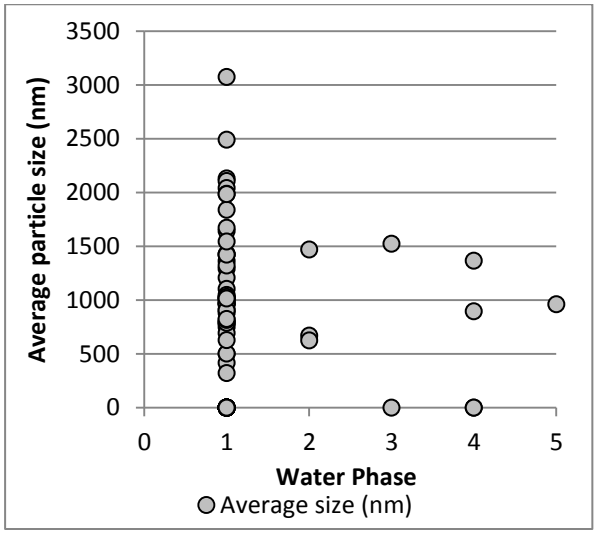

Figure 3-26: The average particle size obtained against the water phase used in the respective anti-infective Pheroid vesicle formulations (obtained by CLSM analysis)

3.3.1.2.2.3 Preservatives and antioxidants

The preservatives and antioxidants used in the anti-infective formulations are described in Tables 3-3 and 3-4. For the most part, no preservatives or antioxidants were added to the

formulations. Preservative combination 2 and 3 was only present in two formulations each while number 1 was found in only one formulation. Antioxidant combinations 2, 3 and 4 were utilized in four, two and one formulation(s) respectively. None of the formulations that included a preservative and/or antioxidant was over ~1500 nm in size (see Figures 3-27 and 3-28).

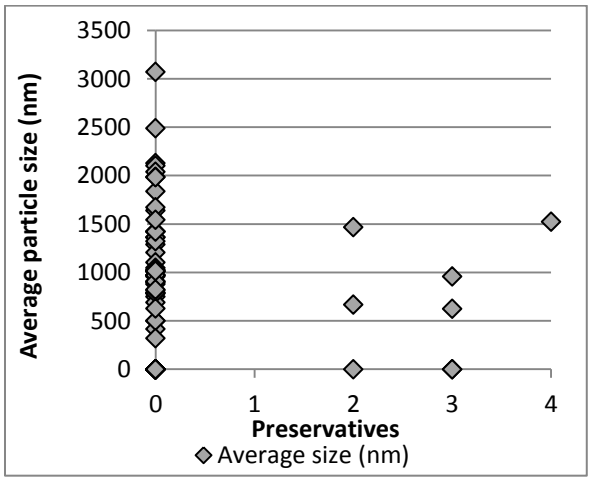

Figure 3-27: The average particle size obtained against the preservative used in the respective anti-infective Pheroid vesicle formulations (obtained by CLSM analysis)

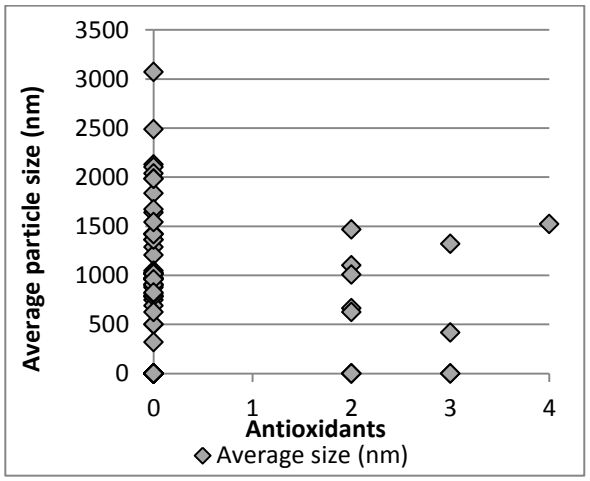

Figure 3-28: The average particle size obtained against the antioxidant used in the respective anti-infective Pheroid vesicle formulations (obtained by CLSM analysis)

3.3.1.2.2.4 Other excipients

Only one excipient combination that had the associated confocal reports available was present in more than two similar formulations. Combination 2 was contained in formulations that had an average particle size of between 700 and 1400 nm (refer to Figure 3-29). Since the other combinations were only present in one formulation each, no significant observations could be made. The formulations to which no excipients were added had no effect on the average particle size with regards to the reference formulations.

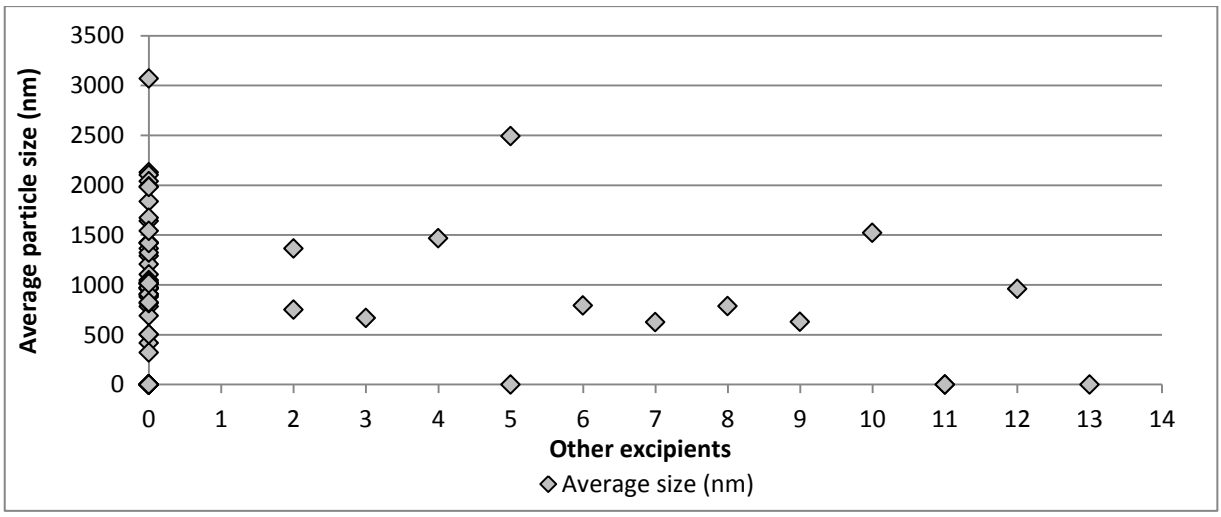

Figure 3-29: The average particle size obtained against the other excipients used in the respective anti-infective Pheroid vesicle formulations (obtained by CLSM analysis)

3.3.1.2.2.5 Active ingredients

The average particle sizes that were obtained with the inclusion of various active ingredients are displayed in Figure 3-30. Most of the active ingredients that were present in more than one formulation had varying average particle sizes (e.g. active ingredient number 11). This is contradictory to Figure 3-17 in which equivalent active ingredients had similar particle size distributions. The latter were Mastersize analysis data whereas the average particle sizes displayed in the graph below were determined via confocal microscopy. Active ingredient 20 was present in the formulation that contained the largest particles on average (3071 nm). The active ingredients are described in Table 3-6.

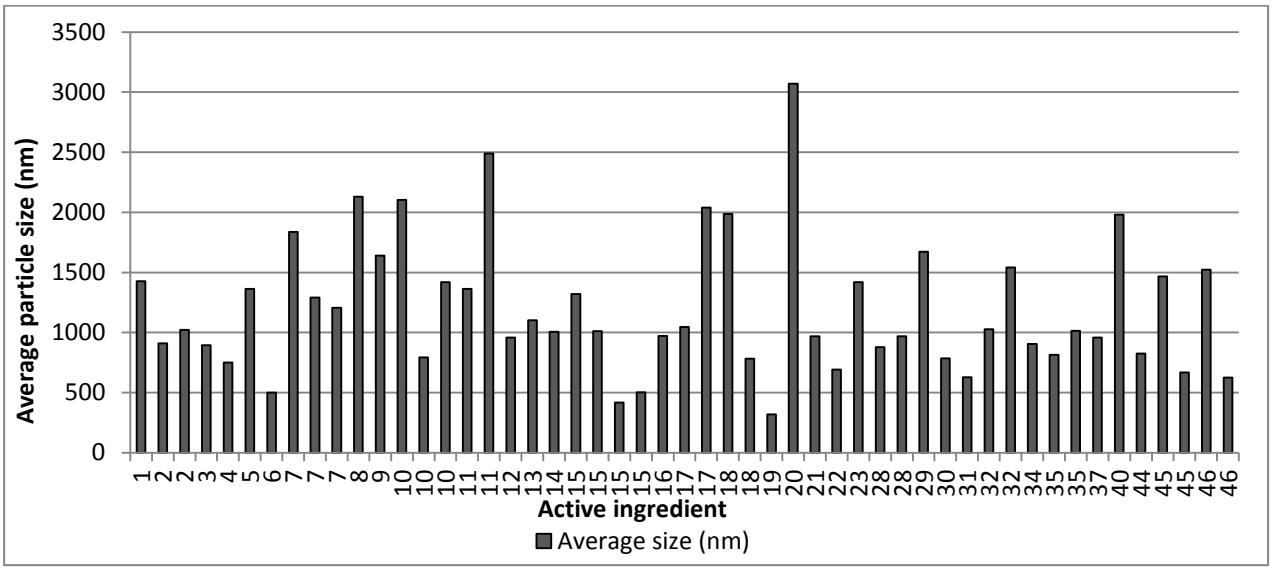

Figure 3-30: The average particle size obtained against the active ingredient used in the anti-infective Pheroid vesicle formulations (obtained by CLSM analysis)

3.3.1.3 CLSM analysis: particle concentration

3.3.1.3.1.1 Oil Phase

The formulations that had available confocal analysis reports generally contained oil phase 1. Four formulations contained oil phase 4, which comprises of the same ratios of ingredients but a different type of Kolliphor™ as oil phase 1. Both oil phases were present in formulations with a wide range of particle concentrations and followed the same trend as the reference formulations and were therefore not responsible for the concentration variations between formulations (see Figures 3-31 and 3-32). This statement is supported by the fact that the distributions that were obtained for the oil phases (and water phases) in the reference formulations for both Mastersize and CLSM analysis were nearly identical.

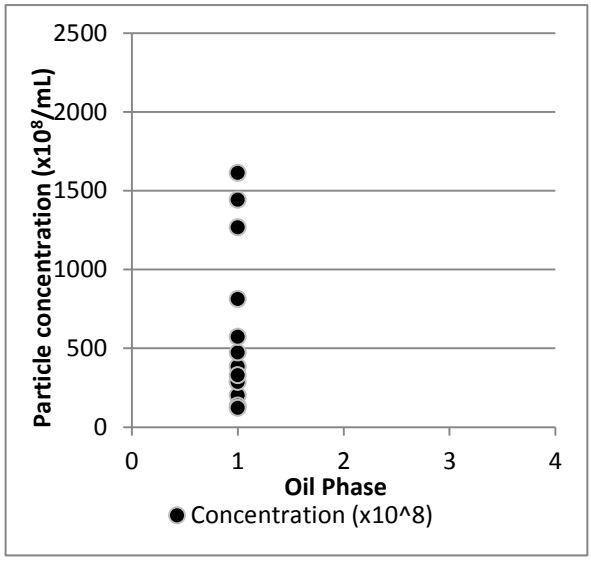

Figure 3-31: The particle concentration (per mL) against the oil phase used in the Pheroid vesicle reference formulations (obtained by CLSM analysis)

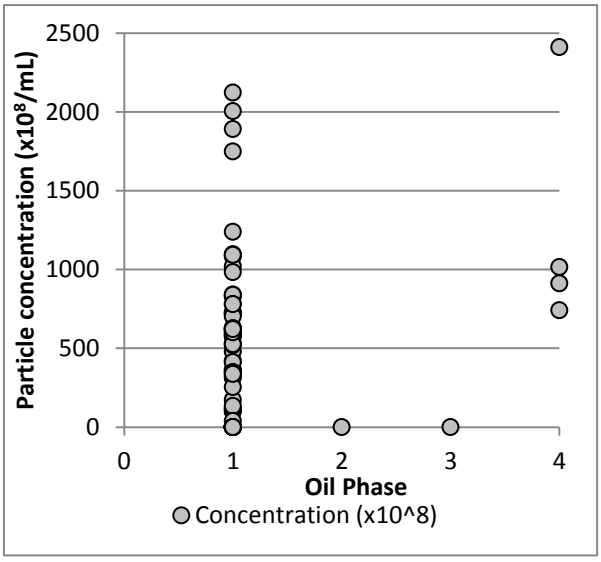

Figure 3-32: The particle concentration (per mL) against the oil phase used in the respective anti-infective Pheroid vesicle formulations (obtained by CLSM analysis)

3.3.1.3.1.2 Water phase

Water phase 1 is similar in the reference and anti-infective formulations. Formulations that included water phases 1 and 2 demonstrated a wide spread of particle concentrations and were therefore not responsible for the variations in particle concentrations between the formulations. On the other hand, the particle concentration of formulations that made use of water phases 3 and 4 were much smaller but the data was lacking and no significant deductions could be made (see Figures 3-33 and 3-34).

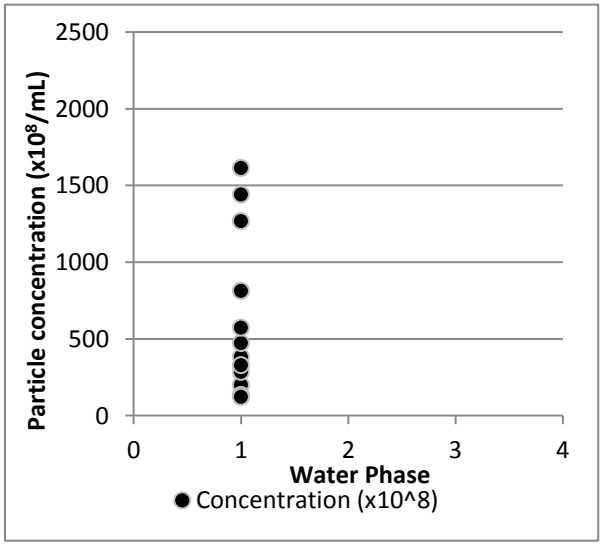

Figure 3-33: The particle concentration (per mL) against the water phase used in the Pheroid vesicle reference formulations (obtained by CLSM analysis)

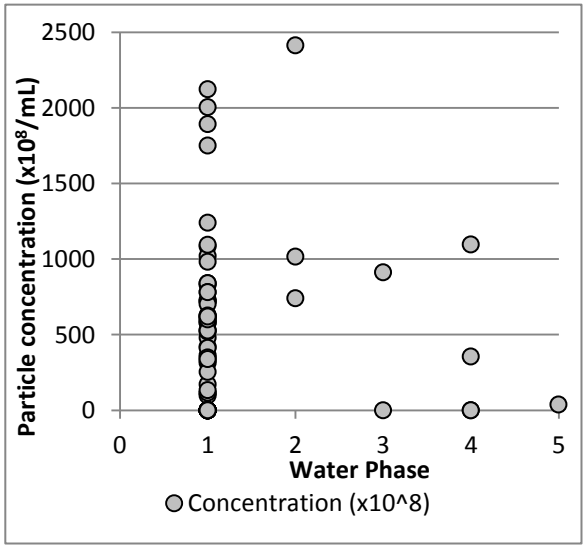

Figure 3-34: The particle concentration (per mL) against the water phase used in the respective anti-infective Pheroid vesicle formulations (obtained by CLSM analysis)

3.3.1.3.1.3 Preservatives and antioxidants

The preservatives and antioxidant used in the formulations are described in Tables 3-3 and 3-4 respectively. A wide range of particle concentrations (that corresponds with the trend observed in the reference formulations) are seen with the use of no preservatives or antioxidants and are to be expected (see Figures 3-35 and 3-36). The data suggests that the omission of antioxidants and/or preservatives did not influence the particle concentrations in the respective formulations with respect to the reference data. Limited data were obtained from anti-infective formulations that did contain preservatives and/or antioxidants. Antioxidant combination 2 was present in four formulations of which three had particle concentrations between 500 and 1000 $x10^8$ /mL while the other formulation had a concentration of ~2400$x10^8$ /mL.

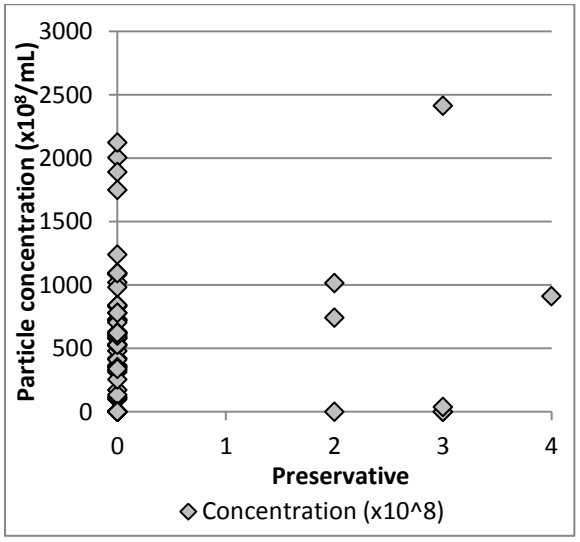

Figure 3-35: The particle concentration (per mL) against the preservative used in the respective anti-infective Pheroid vesicle formulations (obtained by CLSM analysis)

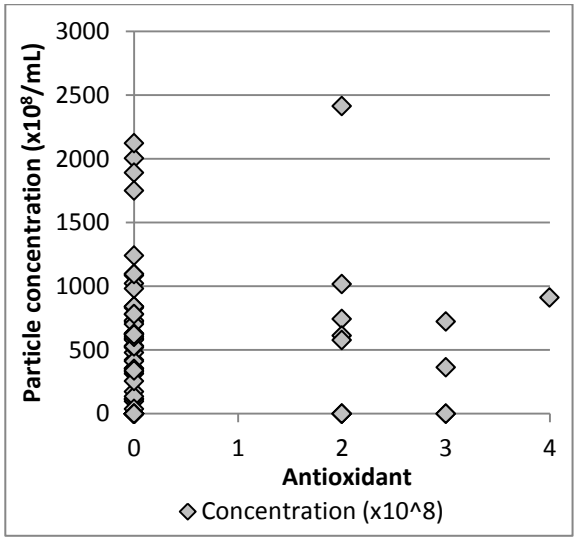

Figure 3-36: The particle concentration (per mL) against the antioxidant used in the respective anti-infective Pheroid vesicle formulations (obtained by CLSM analysis)

3.3.1.3.1.4 Other excipients

The other excipients are described in Table 3-5. Naturally, a lack of other excipients in the formulations would not have an influence on the particle concentration with regards to the concentration obtained with the reference formulations. Only combination 2 was present in more than one formulation that had the concomitant confocal reports (see Figure 3-37). The formulations, in which combination 2 was present, had particle concentrations that differed significantly from each other. This indicates that the combination did not affect the particle concentration. Sufficient data was not available on the other formulations to draw any conclusions.

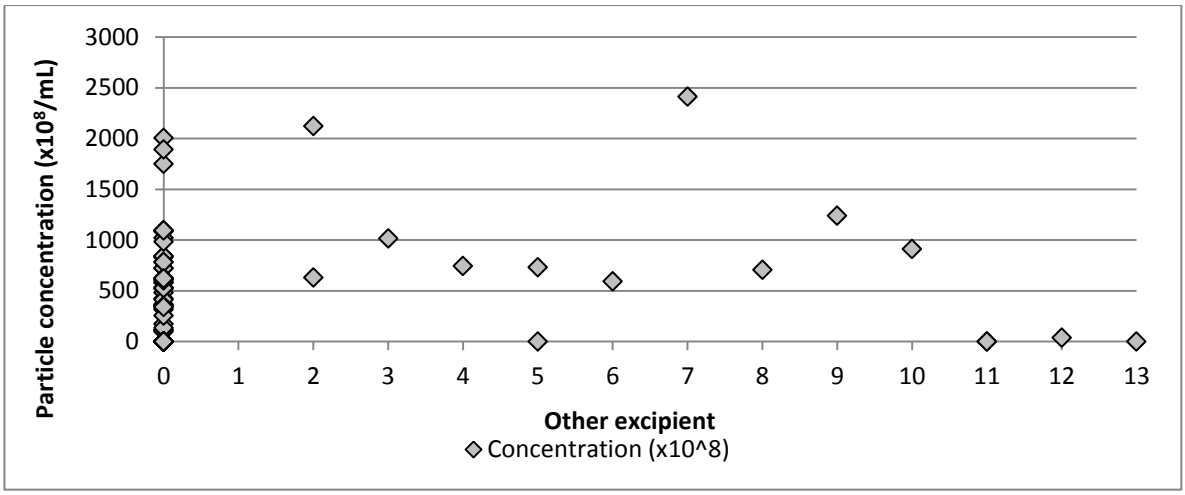

Figure 3-37: The particle concentration (per mL) against the other excipients used in the respective anti-infective Pheroid vesicle formulations (obtained by CLSM analysis)

3.3.1.3.1.5 Active ingredients

The anti-infective vesicle formulations that had the associated confocal analysis available are displayed with the corresponding active ingredients in Figure 3-38 (refer to Table 3-6 for a description of the active ingredients). Some Pheroid formulations that contained active ingredient 2, 4, 9, 15 and 46 were significantly more concentrated than the other formulations. On the other hand, several formulations contained very few particles. Active ingredients number 17, 18, 19, 22 and 37 had less than 150 x10^8 particles per mL.

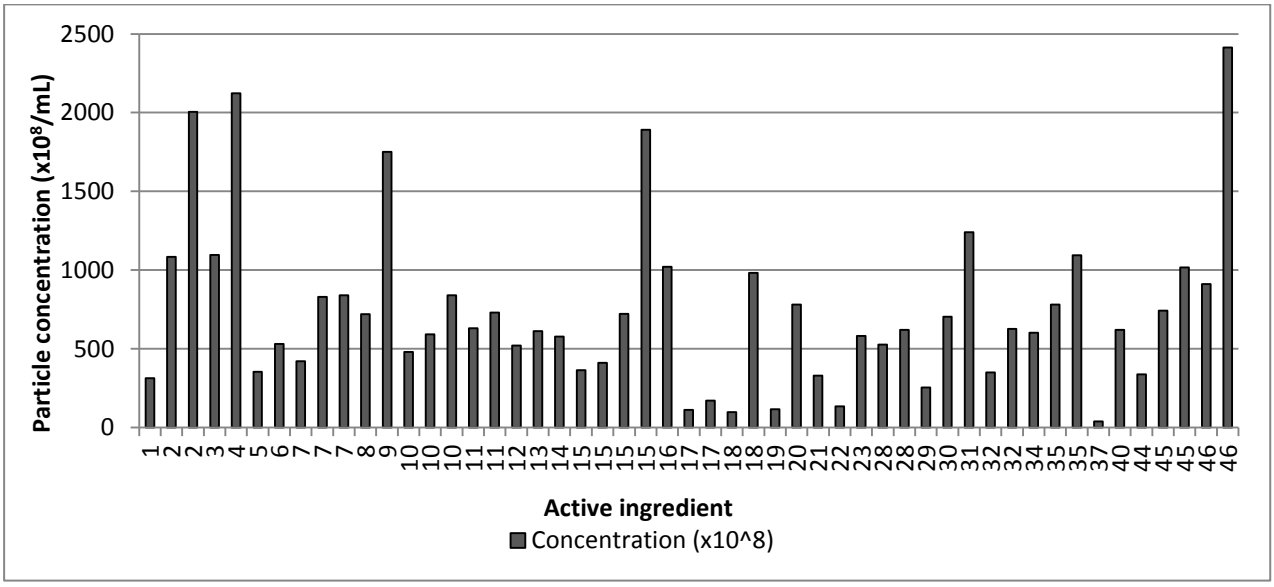

Figure 3-38: The particle concentration (per mL) against the active ingredient used in the anti-infective Pheroid vesicle formulations (obtained by CLSM analysis)

3.3.1.4 Zeta potential

3.3.1.4.1 Statistical description of reference formulations

Negative values were obtained for all the zeta potential measurements. The average zeta potential was -23.41 mV (±7.71). The measurements ranged from a minimum of -14.78 mV to a maximum of -35.90 mV and contained no outlying data (see Figure 3-39).

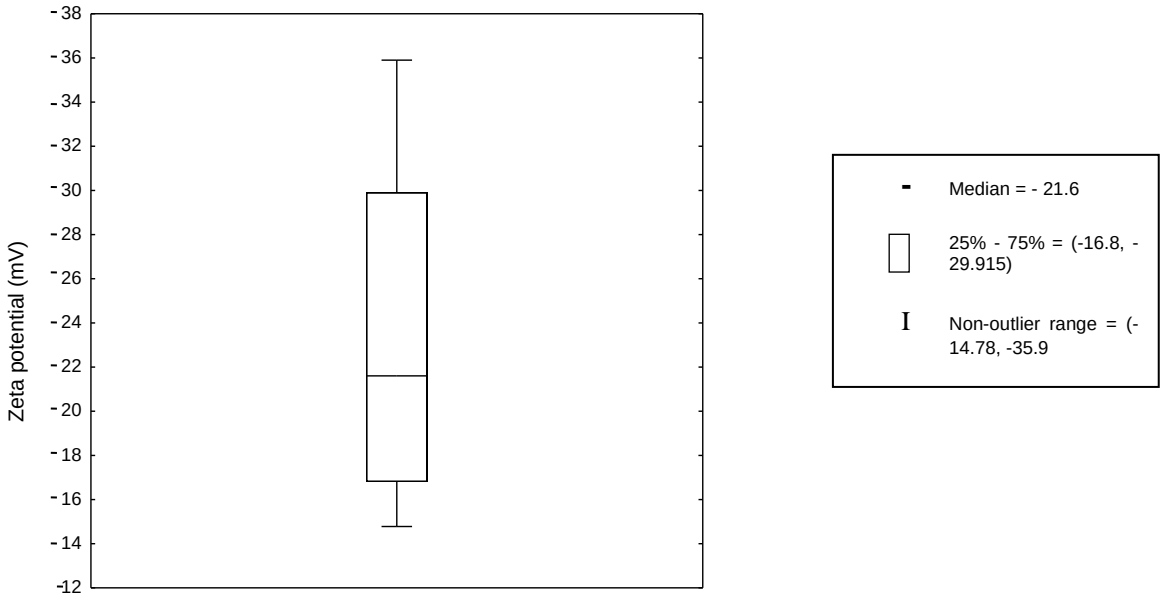

Figure 3-39: The average zeta potential (mV) in each Pheroid vesicle reference formulation

3.3.1.4.2 Anti-infective formulations

3.3.1.4.2.1 Oil phase

Oil phase 1 in both the reference and anti-infective formulations is parallel (see Figures 3-40 and 3-41). The wide distribution of the zeta potentials obtained from the different anti-infective formulations indicates that oil phase 1 did not likely influence the stability of the formulations.

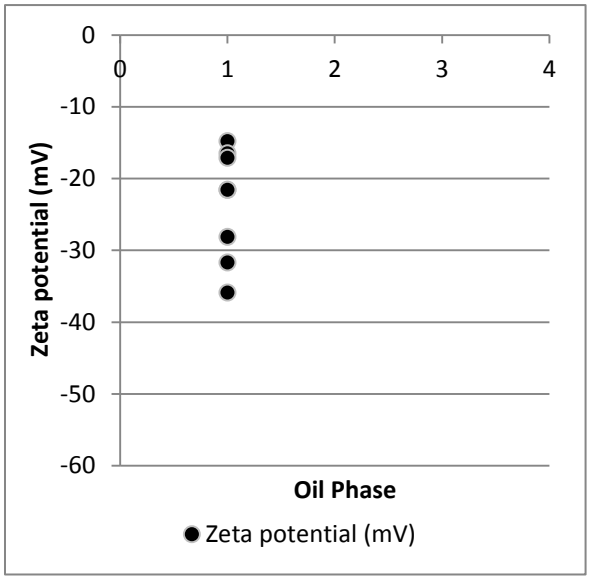

Figure 3-40: The different zeta potential measurements obtained against the oil phase used in the reference formulations (obtained by Zetasizer analysis)

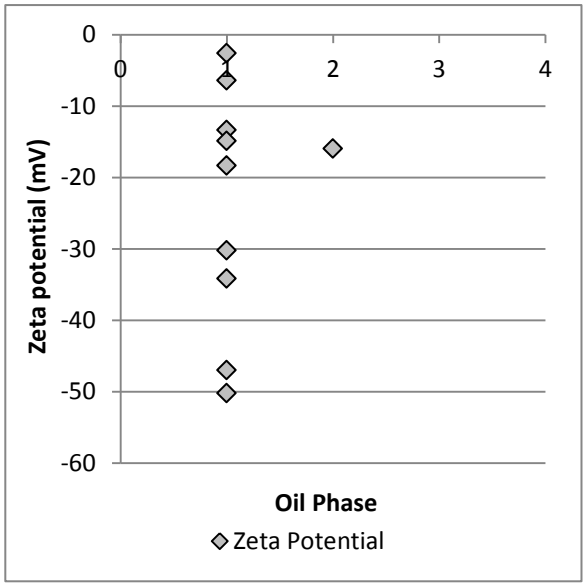

Figure 3-41: The different zeta potential measurements obtained against the various oil phases used in the anti-infective vesicle formulations (obtained by Zetasizer analysis)

3.3.1.4.2.2 Water phase

Unfortunately, only ten formulations had zeta potential measurements available of which all contained water phase number 1. Subsequently, the impact of the different water phases on stability could not be determined. However, because of the wide range of measurements displayed in Figure 3-42 and 3-43, it can be deduced that water phase 1, the typically used phase, did not likely influence the stability of the formulations.

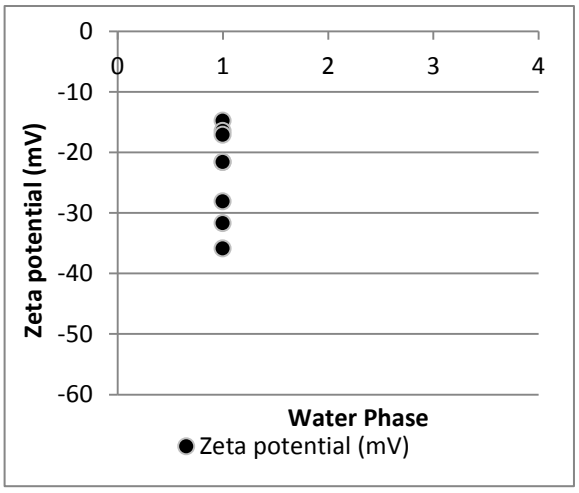

Figure 3-42: The different zeta potential measurements obtained against the water phase used in the reference formulations (obtained by Zetasizer analysis)

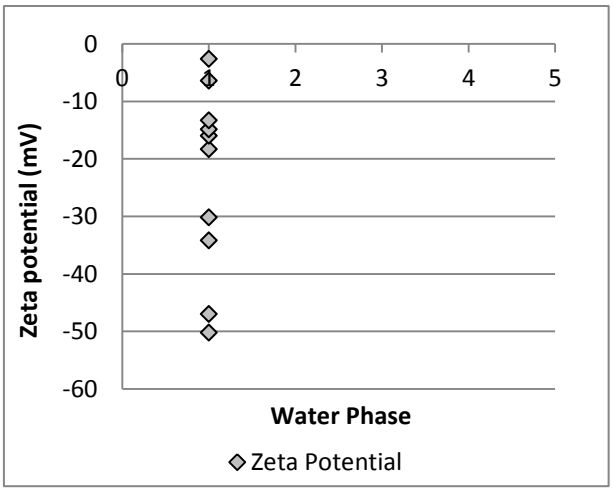

Figure 3-43: The different zeta potential measurements obtained for anti-infective Pheroid vesicle formulations in the presence of the water phases (obtained by Zetasizer analysis)

3.3.1.4.2.3 Preservatives and antioxidants

In comparison to the reference formulations, the formulations without preservatives or antioxidants had a much broader zeta potential distribution. However, the lack of these additives signifies that they did not likely influence the stability as they are absent in the reference formulations as well (see Figures 3-44 and 3-45). Three formulations contained antioxidant combination 3 (see Table 3-4 for a description). The wide distribution of measurements (ranging from a tendency to be unstable (0 to ±25 mV) to a tendency to be stable (> ±25 mV)) indicates that combination 3 did not likely influence the stability of the formulations either.

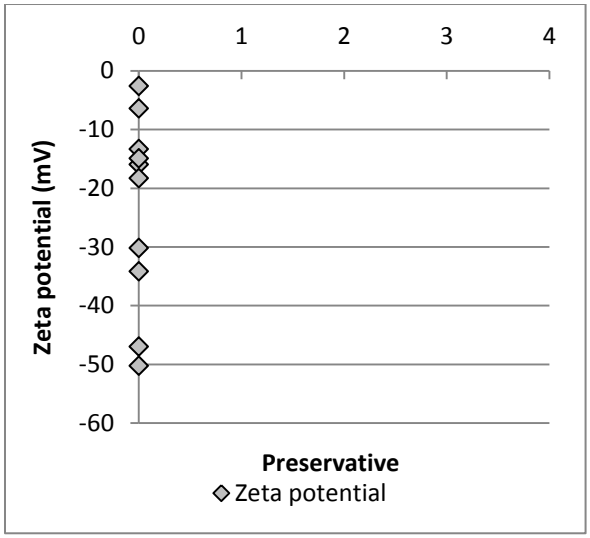

Figure 3-44: The different zeta potential measurements obtained for reference vesicle formulations in the presence of the preservatives (obtained by Zetasizer analysis)

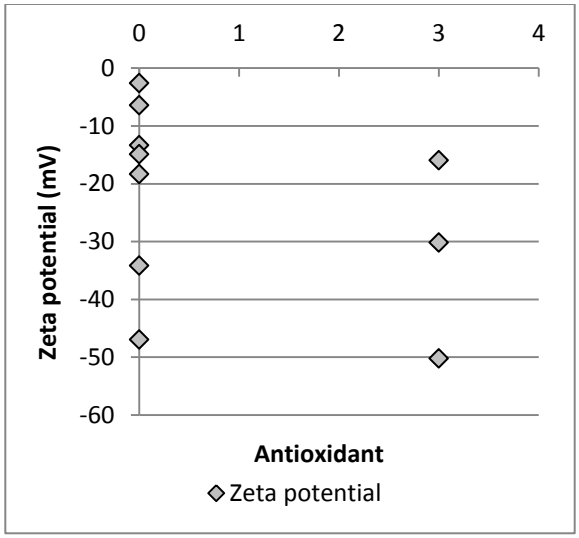

Figure 3-45: The different zeta potential measurements obtained for reference vesicle formulations in the presence of the antioxidants (obtained by Zetasizer analysis)

3.3.1.4.2.4 Other excipients

Very little data was available on the stability of the anti-infective formulations that included 'other excipients'. Consequently, the results on those formulations had no statistical significance and were not described (see Figure 3-46). Formulations that did not contain other excipients had wide-ranging zeta potentials and, as expected, did not influence the stability of the formulations with regards to the reference formulations. The ingredients used as the other excipients are described in Table 3-5).

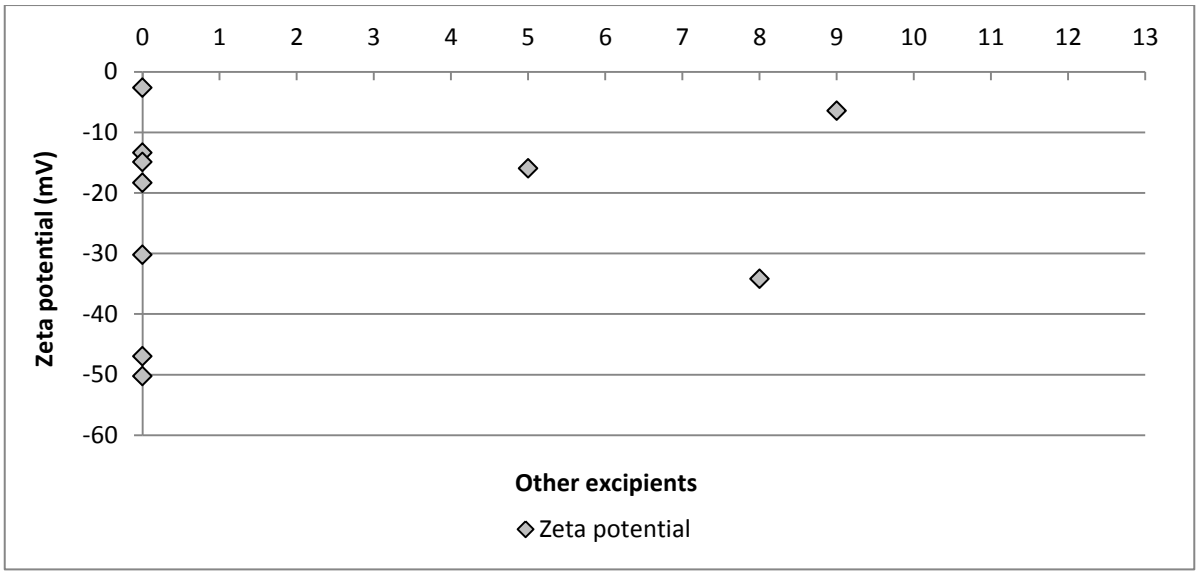

Figure 3-46: The different zeta potential measurements obtained for anti-infective Pheroid vesicle formulations in the presence of the other excipients (obtained by Zetasizer analysis)

3.3.1.4.2.5 Active ingredients

The formulations that included active ingredient 15, 29 and 30 had a tendency to be stable formulations with zeta potential measurements above -30 mV (see Figure 3-47). The other formulations had zeta potential measurements below -20 mV which indicates a tendency to be unstable formulations. A measurement that is close to zero indicates that the formulation tends to be more unstable and prone to aggregation. No correlation was visible between the particle size distribution and the zeta potential measurements that were obtained for the formulations. For instance, the two formulations with zeta potential measurements closest to zero had the most (53.63%) and the least (0.00%) amount of micron sized particles (see active ingredient number 28 and 31 in Figure 3-47). Refer to Table 3-6 for a description of the active ingredients.

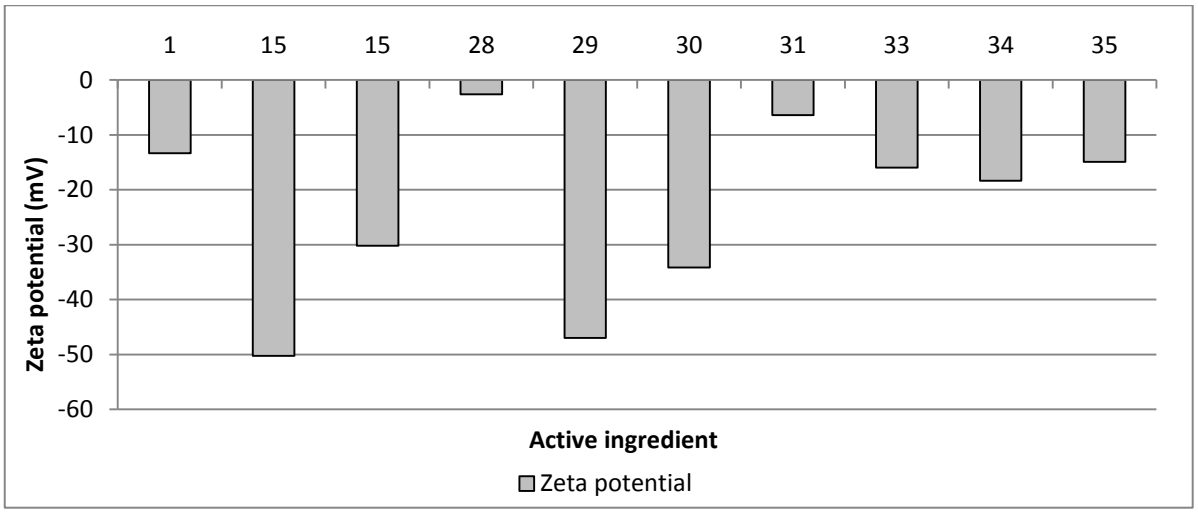

Figure 3-47: The different zeta potential measurements obtained for Pheroid vesicle formulations in the presence of the active ingredients (obtained by Zetasizer analysis)

3.3.1.5 Summary: Pheroid vesicles

A concise summary of the results obtained through the statistical analysis of the Pheroid vesicle formulations are presented in Table 3-7. Due to limited data available, definite conclusions could not be draw; however, it was observed that (refer to Table 3-7):

- the mastersize results were not likely influenced by the oil or water phases, possibly influenced by the preservatives and other excipients and most likely influenced by the antioxidants and active ingredients,
- the CLSM results were not likely influenced by the oil or water phases and were most likely influenced by the active ingredients. The antioxidants, preservatives and other excipients were not present in enough formulations to draw significant conclusions,
- the zeta potential results were not likely influenced by the oil and water phases or the antioxidants and were most likely influenced by the active ingredients. The preservatives and other excipients were not present in enough formulations to derive substantial conclusions.

Table 3-7: Table summarizing results from Pheroid vesicle statistical analysis

Pheroid vesicles				
	Mastersizer - particle size distribution	**CLSM - particle size**	**CLSM - particle concentration**	**Zeta Potential - stability**
Oil Phase	Results indicate that the oil phase did not likely influence the particle size distribution	Results indicate that the oil phase did not likely influence the average particle size	Results indicate that the oil phase did not likely influence the particle concentration	Results indicate that the oil phase did not likely influence the stability
Water Phase	Results indicate that the water phase did not likely influence the particle size distribution	Results indicate that the water phase did not likely influence the average particle size	Results indicate that the water phase did not likely influence the particle concentration (limited data was available for water phase 3 and 4 which tended to have a smaller particle concentration (see Figures 3-33 and 3-34))	Results indicate that the water phase did not likely influence the stability of the formulations

Table 3-7: Table summarizing results from Pheroid vesicle statistical analysis (continued)

	Mastersizer - particle size distribution	CLSM - particle size	CLSM - particle concentration	Zeta Potential - stability
Preservatives	Did not likely influence the particle size distribution; however, significant conclusions could not be drawn from the limited data	None of the formulations that included a preservative and/or antioxidant was over ~1500 nm in size (see Figures 3-27 and 3-28).	Significant conclusions could not be drawn from the limited data	No preservatives were present in the formulations and could thus not influence the stability in comparison to the reference formulations
Antioxidants	Formulations that contained Methyl Paraben and Propyl Paraben at different concentrations were inclined to specific particle size distributions (see 3.3.1.1.2.4)		Significant conclusions could not be drawn from the limited data	Results indicate that the antioxidants that were present in the formulations did not likely influence the stability
Other Excipients	Significant conclusions could not be drawn from the limited data although tendencies were observed (see Figures 3-15 and 3-16)	The other excipient combinations were only present in one formulation each; therefore no significant observations could be made	Only combination 2 was present in more than one formulation. The significantly different particle concentrations observed with those formulations indicates that the combination did not likely affect the particle concentration (see Figure 3-37)	Statistically significant conclusions could not be drawn due to the fact that very few formulations included other excipients
Active ingredient	Formulations with analogous actives mostly had similar particle size distribution in terms of percentage particles in the submicron and micron size ranges (see Figure 3-17)	Most of the active ingredients that were present in more than one formulation had varying average particle sizes (contradictory to Mastersize results)	Tendencies were observed with several active ingredients (see Figure 3-38)	Tendencies were observed with several active ingredients (see Figure 3-47)

3.3.2 Pheroid micro-sponges

The Pheroid micro-sponge reference formulations consisted of twenty-one formulations of which all had complete particle size distribution analysis data. The zeta potential measurements of ten formulations were available but, unfortunately, no confocal reports were obtainable for any of the formulations (except for the size span of two formulations which were too little to have statistical significance). Consequently, the CLSM analysis could not be compared with that of the anti-infective formulations. A total of nineteen Pheroid micro-sponge formulations were obtained for the anti-infective data analysis. Fourteen, thirteen and eight formulations had respectively Mastersize, CLSM and zeta potential reports available.

3.3.2.1 Particle size and particle size distribution

3.3.2.1.1 Statistical description of reference formulations

On average, the reference formulations comprised of 27.28% submicron sized particles. The section of submicron sized particles varied from 11.40 to 49.31% between the different formulations. No outlying or extreme values were present in the dataset (see Figure 3-48).

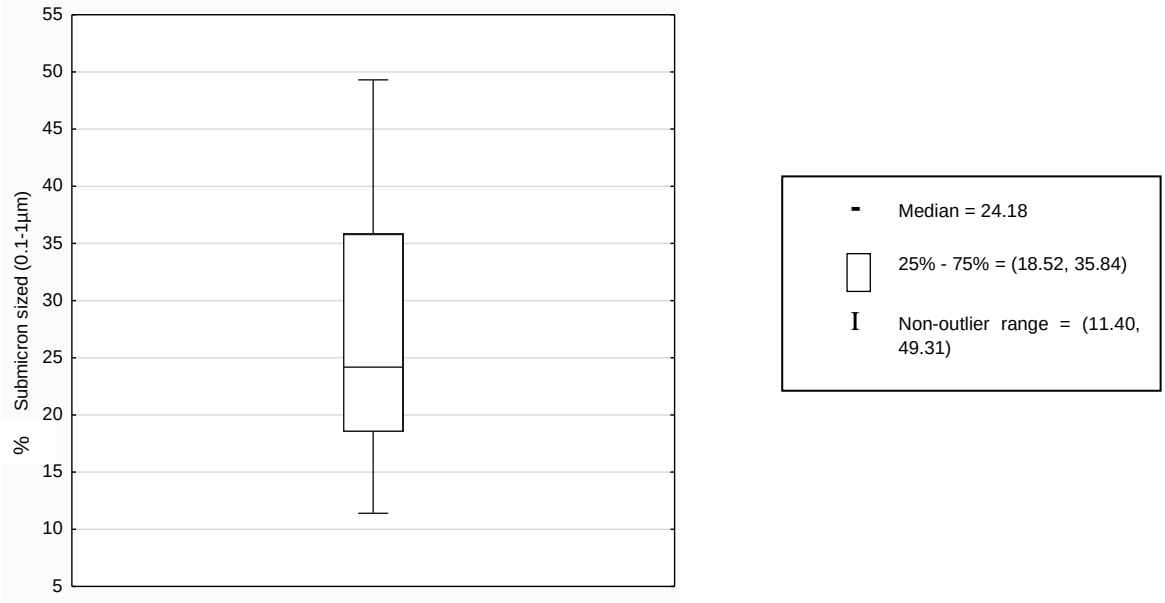

Figure 3-48: Percentage submicron sized particles (0.1 to 1 µm) in the Pheroid micro-sponge reference formulations (obtained from Mastersize analysis)

The formulations comprised of 32.88 to 58.92% micron sized particles (portrayed in Figure 3-49). 50.00% of the formulations had less than 52.90% micron sized particles. No outliers or extreme values were found.

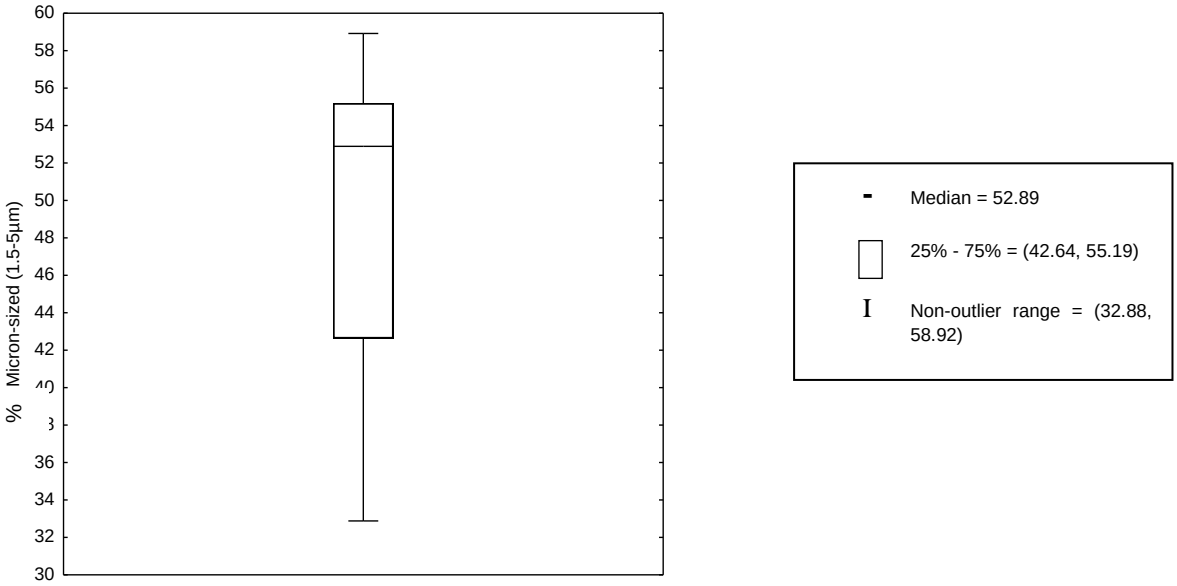

Figure 3-49: Percentage micron sized particles (1.5 to 5 µm) in the Pheroid micro-sponge reference formulations (obtained from Mastersize analysis)

3.3.2.1.2 Anti-infective micro-sponge formulations

Figures 3-50 and 3-51 are visual representations of the particle size distribution in the various reference and anti-infective micro-sponge formulations respectively. At first glance, no trend is noticeable for the percentage submicron and micron sized particles in both the reference and the anti-infective formulations. However, after calculation of the average percentage particles in the submicron and micron sized range, a slight difference is observed. On average, the reference formulations had fewer submicron (27.28% against 31.91%) and more micron sized particles than the anti-infective micro-sponge formulations (49.18% compared to 43.30%). The contrary was expected due to the additional ingredients found in the anti-infective formulations. The rest of the particles in the formulations are mainly above the micron sized range. The various ingredients in the anti-infective formulations are subsequently discussed to determine which ingredient(s) most likely affected the particles sizes. A summary of the ingredients added to each anti-infective micro-sponge formulation (identified by means of the batch number (e.g. S08008)) is presented in Table 3-8 (see Table 3-9 to 3-13 for a description of the ingredients). The reference formulations are not described in a similar manner since they exclusively contain Pheroid ingredients in the patented ratios.

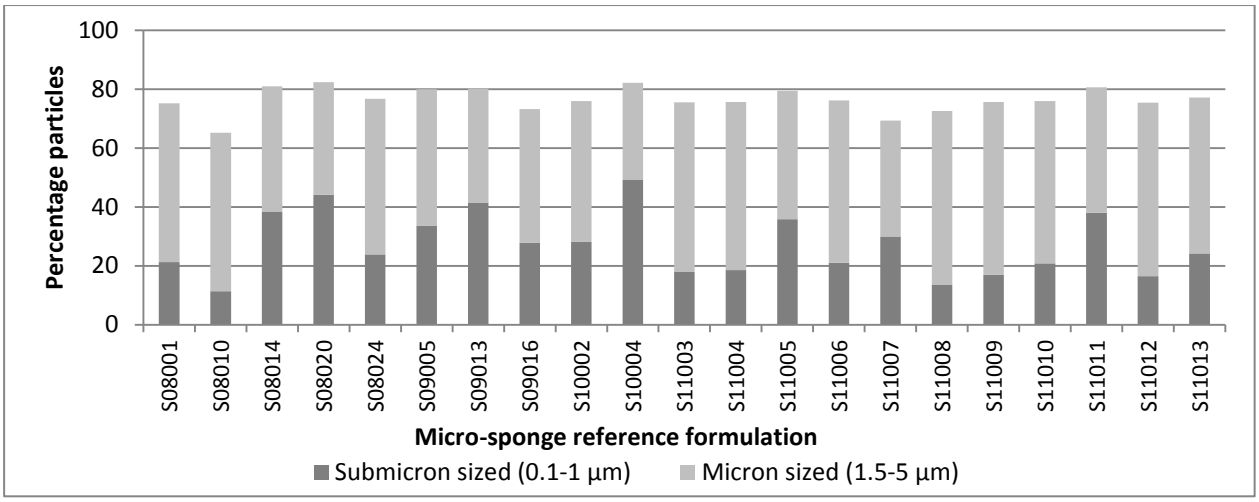

Figure 3-50: Percentage submicron and micron sized particles in the respective Pheroid micro-sponge reference formulations (obtained by Mastersize analysis)

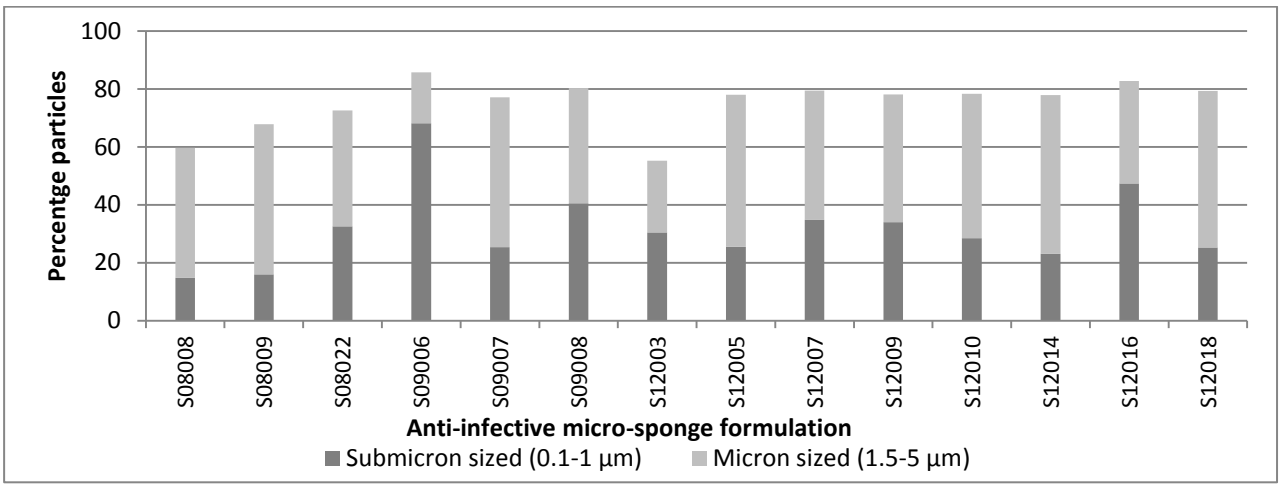

Figure 3-51: Percentage submicron and micron sized particles in the respective anti-infective Pheroid micro-sponge formulations (obtained by Mastersize analysis)

Table 3-8: Outline of the ingredients in the respective anti-infective Pheroid micro-sponge formulations

BATCH NO..	OIL PHASE	WATER PHASE	ACTIVE INGREDIENT	PRESERVATIVE	ANTIOXIDANT	OTHER EXCIPIENT
S08006	2	1	1	1	1	1
S08008	1	1	2	0	0	1
S08009	1	1	3	0	0	1
S08021	1	2	4	0	2	2
S08022	1	1	5	0	0	1
S09002	1	1	6	0	0	1
S09006	1	1	2	0	0	1
S09007	1	1	7	0	0	1
S09008	1	1	3	0	0	1
S09011	3	2	8	2	0	3
S09012	3	2	9	3	0	4
S12003	1	1	10	0	0	1
S12005	1	1	11	0	0	1
S12007	1	1	11	0	0	5
S12009	1	1	12	0	0	5
S12010	1	1	13	0	0	1
S12014	1	1	11	0	0	6
S12016	1	1	14	0	0	7
S12018	4	1	14	0	0	7

3.3.2.1.2.1 Oil phase

The percentage submicron and micron sized particles in the anti-infective formulations that utilized oil phase 1 are presented in Figures 3-52 and 3-53. The distributions of the particles are similar to that of the reference formulation (see Figure 3-54) and widely spread, signifying that the oil phase did not influence the particle sizes.

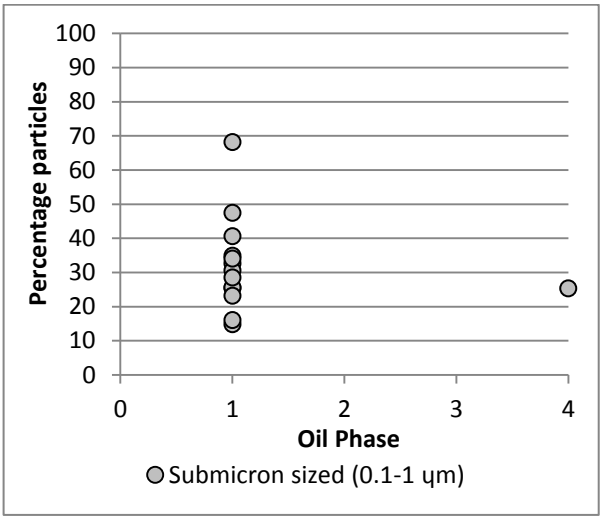

Figure 3-52: Percentage submicron sized particles against the oil phase used in the respective anti-infective Pheroid micro-sponge formulations (obtained by Mastersize analysis)

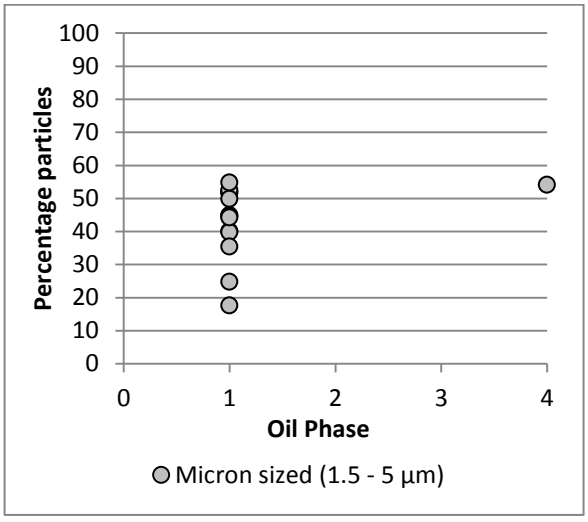

Figure 3-53: Percentage micron sized particles against the oil phase used in the respective anti-infective Pheroid micro-sponge formulations (obtained by Mastersize analysis)

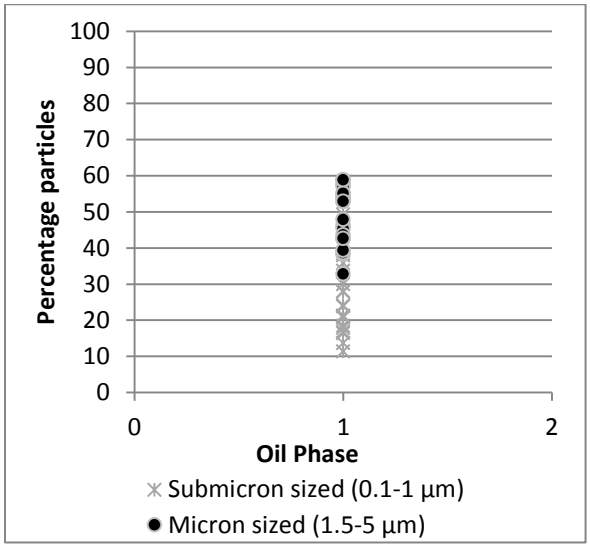

Figure 3-54: Percentage submicron and micron sized particles against the oil phase used in the Pheroid micro-sponge reference formulations (obtained by Mastersize analysis)

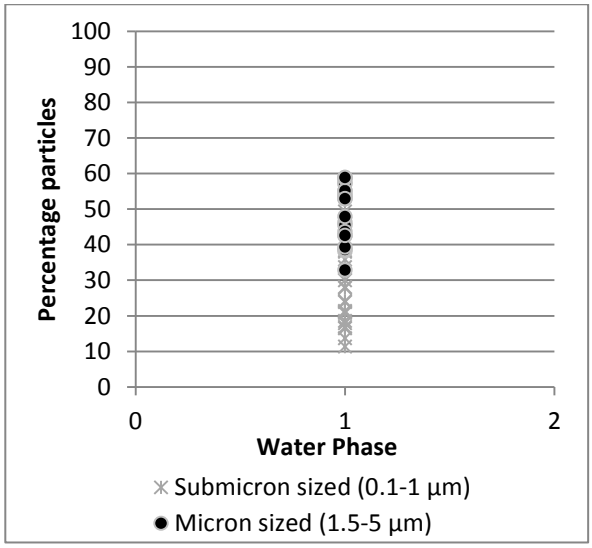

Figure 3-55: Percentage submicron and micron sized particles against the water phase used in the Pheroid micro-sponge reference formulations (obtained by Mastersize analysis)

3.3.2.1.2.2 Water phase

Table 3-9: Description of the various water phases used in the anti-infective Pheroid micro-sponge formulations

Nr.	Percentage of formulation	Description	Frequency
1	80-95	$N_2O{\cdot}H_2O$	16
2	35-50	$N_2O{\cdot}H_2O$	3

Only two different water phases were present in the anti-infective formulations (described in Table 3-9) but no size distribution analysis reports were available for the three formulations that contained water phase 2. The water phase used in the reference formulations (Figure 3-55) was comparable to water phase 1 in the anti-infective micro-sponge formulations (see Figures 3-56 and 3-57). The fraction submicron and micron sized particles in the anti-infective formulations were widely distributed, which showed that the water phase did not influence the particle sizes in the formulation.

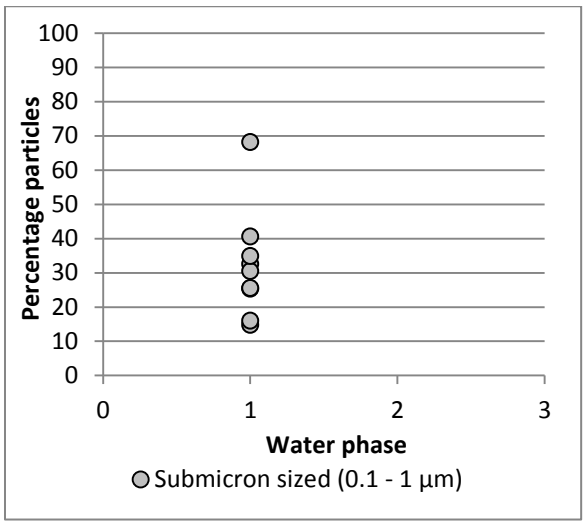

Figure 3-56: Percentage submicron sized particles against the water phase used in the respective anti-infective Pheroid micro-sponge formulations (obtained by Mastersize analysis)

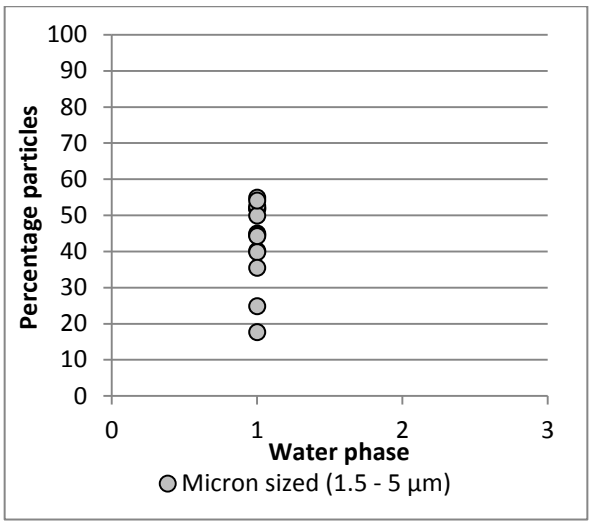

Figure 3-57: Percentage micron sized particles against the water phase used in the respective anti-infective Pheroid micro-sponge formulations (obtained by Mastersize analysis)

3.3.2.1.2.3 Preservatives

Three anti-infective formulations made use of preservatives (described in Table 3-10). However, the Mastersize analysis for these formulations could not be obtained. The data that could be acquired were for formulations that did not contain any preservatives. In other words, in their absence, preservatives could not have influenced the particle size and size distribution (emphasized by the broad particle distribution displayed in Figures 3-58 and 3-59). As a result of the insufficient data, the effect of preservatives on micro-sponge particle sizes could not be determined.

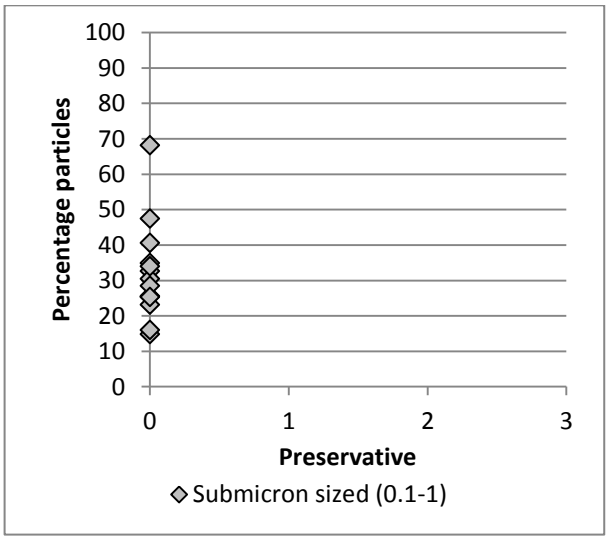

Figure 3-58: Percentage submicron sized particles against the preservatives used in the respective anti-infective Pheroid micro-sponge formulations (obtained by Mastersize analysis)

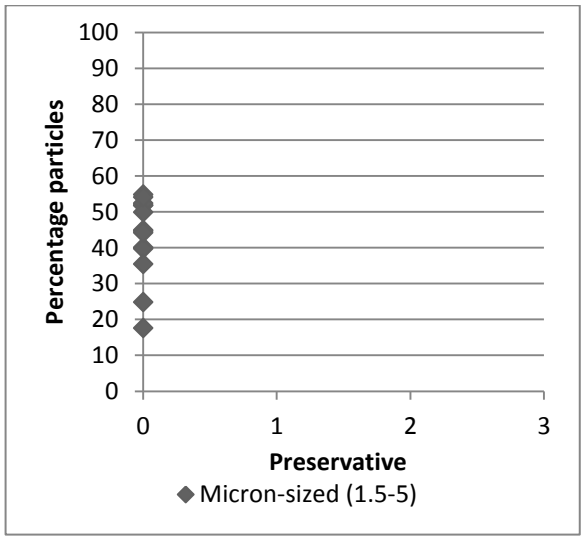

Figure 3-59: Percentage micron sized particles against the preservatives used in the respective anti-infective Pheroid micro-sponge formulations (obtained by Mastersize analysis)

Table 3-10: Various preservative combinations used in the anti-infective Pheroid micro-sponge formulations

No.	Ingredient	%	Frequency
1	Methyl Paraben Propyl Paraben	0.50 0.02	1
2	Methyl Paraben Propyl Paraben	0.40 0.08	1
3	Methyl Paraben	0.10	1

Table 3-11: Various antioxidant combinations used in the anti-infective Pheroid micro-sponge formulations

No.	Ingredient	%	Frequency
1	BHA BHT TBHQ	0.02 0.01 0.10	1
2	Ascorbyl Palmitate	0.20	1

3.3.2.1.2.4 Antioxidants

As was the case with the preservatives, no Mastersize data could be obtained for the formulations that included antioxidants (described in Table 3-11). Figures 3-60 and 3-61 display particle size distributions that correlate with the spread observed in the reference formulations. Due to the lack of data, no conclusions could be drawn on the effects of antioxidants on the size distribution of particles in the formulation. The omission of antioxidants in the anti-infective micro-sponge formulations did not cause the particle size distribution to differ from that of the reference formulations.

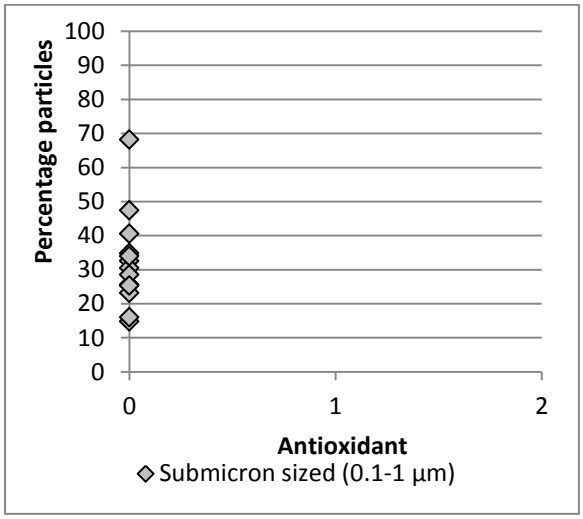

Figure 3-60: Percentage submicron sized particles against the antioxidant used in the respective anti-infective Pheroid micro-sponge formulations (obtained by Mastersize analysis)

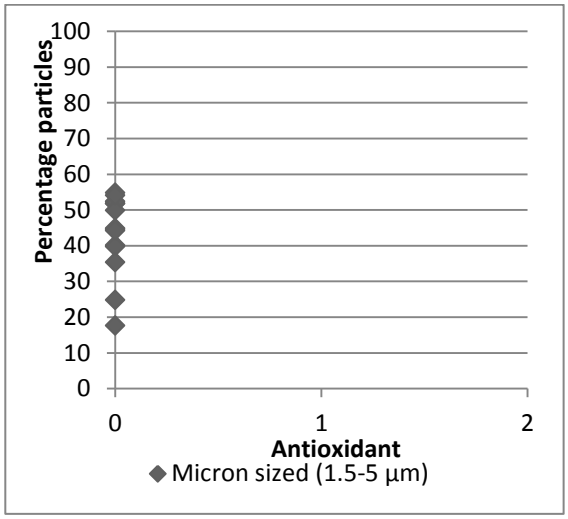

Figure 3-61: Percentage micron sized particles against the antioxidant used in the respective anti-infective Pheroid micro-sponge formulations (obtained by Mastersize analysis)

3.3.2.1.2.5 Other excipients

Table 3-12 Various other excipients used in the anti-infective Pheroid micro-sponge formulations

Nr.	Excipient	%	Frequency
1	Incromega E7010 Incromega E3322	0.20 0.20	11
2	Incromega E7010 Incromega E3322	2.50 2.50	1
3	Incromega E7010 Incromega E3322 PEG 400 Tween 80 Span 60 Xanthan gum	0.25 0.25 20.0 4.50 0.50 1.50	1
4	Incromega E7010 Incromega E3322 PEG 400 Cetyl alcohol	0.25 0.25 20.0 15.0	1
5	Incromega E7010 Incromega E3322 DCM	0.25 0.25 0.20	2
6	Incromega E7010 Incromega E3322 PEG 400	0.25 0.25 2.00	1
7	Incromega E7010 Incromega E3322 NH_4OH (1M)	0.22 0.22 9.94	2

The other excipients included in various anti-infective micro-sponge formulations are described in Table 3-12. Numbers 2, 3 and 4 as well as two formulations that made use of combination 1 had no Mastersize data available (see Figures 3-62 and 3-63). The formulations that included number 1, in accordance with the reference formulations, displayed a wide particle distribution which indicated that the specific excipient combination did not manipulate the particle sizes. The percentage submicron and micron sized particles observed in the two formulations that included number 5 had very similar size distributions. Number 7 had between 20.00 and 60.00% particles in the submicron and micron size range. A tendency could not be distinguished for formulations that made use of excipients 2 to 7 due to the lack of data.

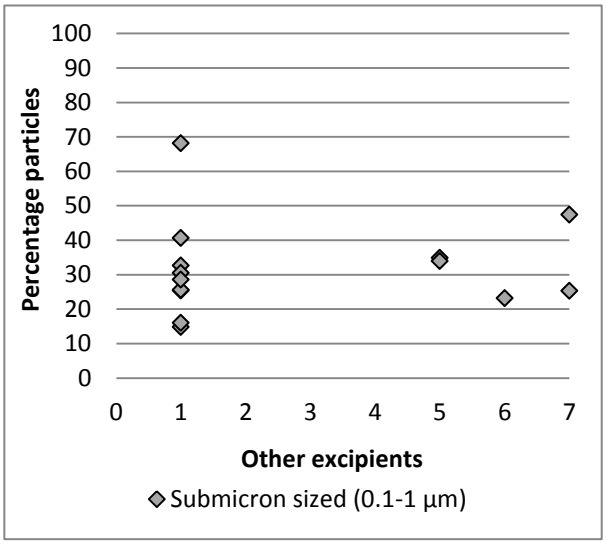

Figure 3-62: Percentage submicron sized particles against the other excipients used in the respective anti-infective Pheroid micro-sponge formulations (obtained by Mastersize analysis)

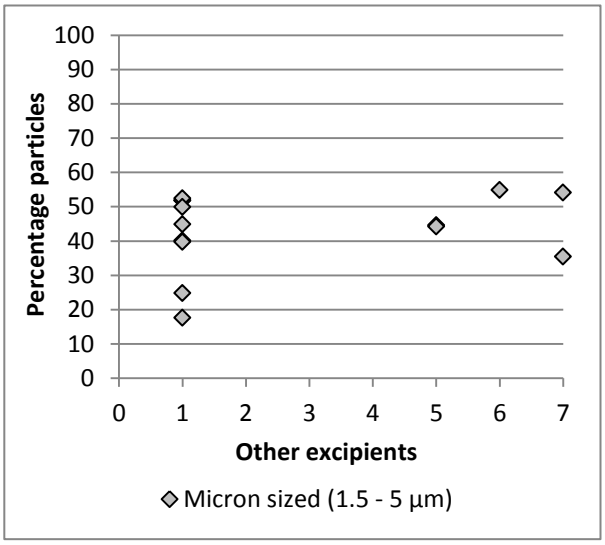

Figure 3-63: Percentage micron sized particles against the other excipients used in the respective anti-infective Pheroid micro-sponge formulations (obtained by Mastersize analysis)

Table 3-13: Various active ingredients used in the anti-infective Pheroid micro-sponge formulations

Nr.	Active ingredient	%	Frequency
1	Mefloquine	2.50	1
2	Mefloquine	0.12	2
3	Artesunate	0.05	2
4	Rifampicin Isoniazid	10.00 5.00	1
5	Mefloquine	0.02	1
6	Artemether	0.07	1
7	Artemether	0.04	1
8	Azelaic acid Nicotinamide	15.00 4.00	1
9	Azelaic acid Nicotinamide	20.00 4.00	1
10	Clofazimine	2.00	1
11	Clofazimine	0.50	3
12	Clofazimine	0.20	1
13	Artemisone	0.05	1
14	Cephalexin monohydrate Kanamycin monosulphate	0.06 0.04	2

3.3.2.1.2.6 Active ingredients

The two formulations that included active ingredient 2, displayed inverse values for the percentage submicron and micron sized particles (illustrated in Figure 3-64). The formulations that contained number 2 had almost double a number of submicron particles in the one formulation with respect to the other. The formulation that contained active ingredient 5 had much less mefloquine than the formulations that contained active ingredient 2 but the rest of the ingredients were parallel. The formulation that included active ingredient 5 had a particle size distribution that was between the size distributions of the active ingredient 2 formulations. Active ingredient number 11 was present in three formulations which had similar particle size distribution. The formulations that included number 14 had significantly different amounts of submicron particles. Refer to Table 3-13 for a description of the various active ingredients.

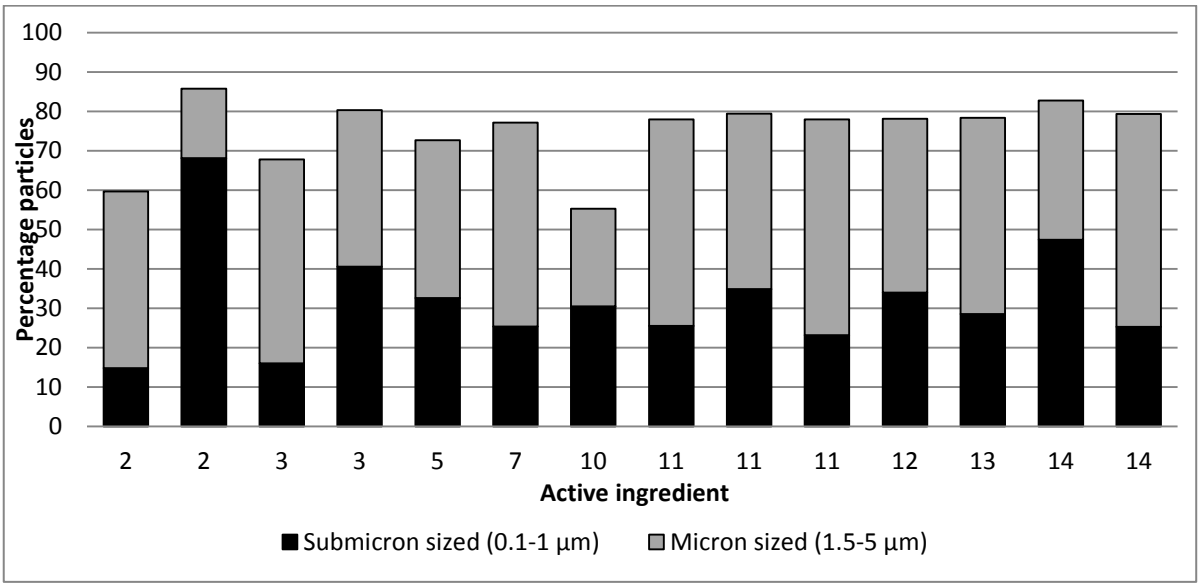

Figure 3-64: Percentage submicron and micron sized particles against the active ingredient used in the Pheroid micro-sponge reference formulations (obtained by Mastersize analysis)

3.3.2.2 CLSM analysis: largest particle in each formulation

3.3.2.2.1 Statistical description of reference formulations

The only available CLSM data on the reference formulations were the size span of two formulations. Both had particles smaller than 1 µm with the largest particle being 8 and 10 µm respectively. However, due to the fact that only two measurements were available, this data had no significance and a statistical description of the reference formulations could not be compiled.

3.3.2.2.2 Anti-infective formulations

Figure 3-65 reflects the largest particle in each of the respective anti-infective formulations. Since all the formulations contain submicron particles (see Figure 3-64), the size span of each formulation would range from <1 µm to the value stipulated in the figure below. The only confocal data available on the reference formulations were two values indicating the largest particle in each formulation (8 µm and 10 µm respectively). The latter sizes exceed most of the values presented for the anti-infective micro-sponge formulations in Figure 3-65 but could not be considered as significant as only two reference values were obtained. Moreover, no other confocal data (e.g. average size or particle concentration) were available to serve as confirmation of the findings.

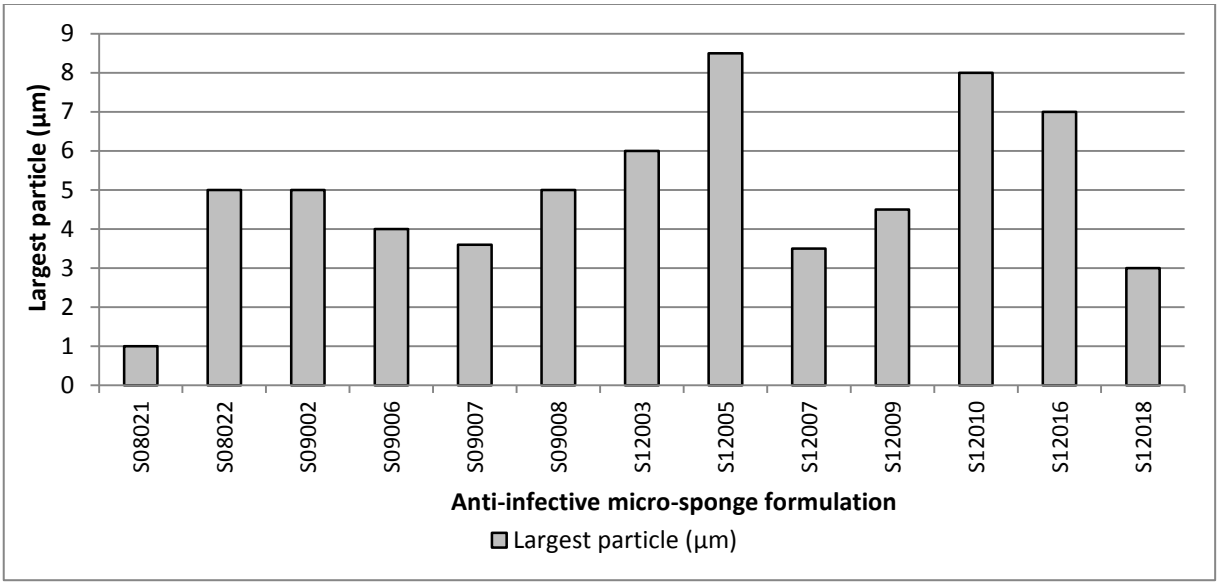

Figure 3-65: The largest particle in every anti-infective Pheroid micro-sponge formulation (obtained by CLSM analysis)

3.3.2.2.2.1 Oil and water phases

The wide spread of data obtained from the formulations that contained oil and water phase 1 respectively, signified that those factors did not influence the average particle sizes (reflected in Figures 3-66 and 3-67). The ratio of ingredients in oil phase 4 was more equally divided than oil phase 1 (which comprised of the standard ratio) but was only present in one formulation thus no

concrete deductions could be formed. Water phase 1 and 2 differed only in volume (present in formulation) and not in ingredients (see Table 3-9). Water phase 2 was only present in one formulation and the average particle size of the formulation was similar to that found with the water phase 1 formulations.

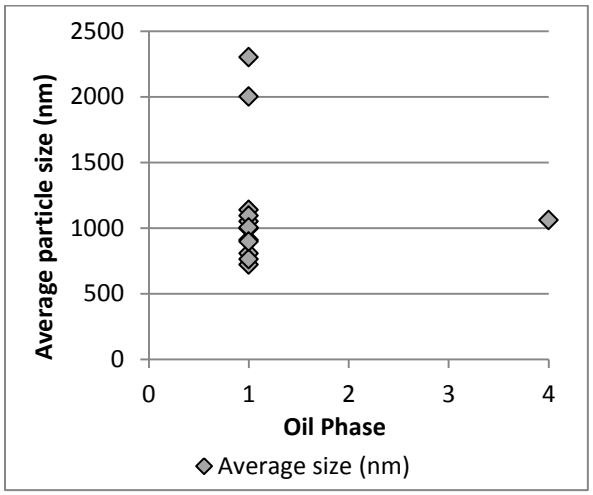

Figure 3-66: The average particle size in every anti-infective Pheroid micro-sponge formulation against the oil phase used (obtained by CLSM analysis)

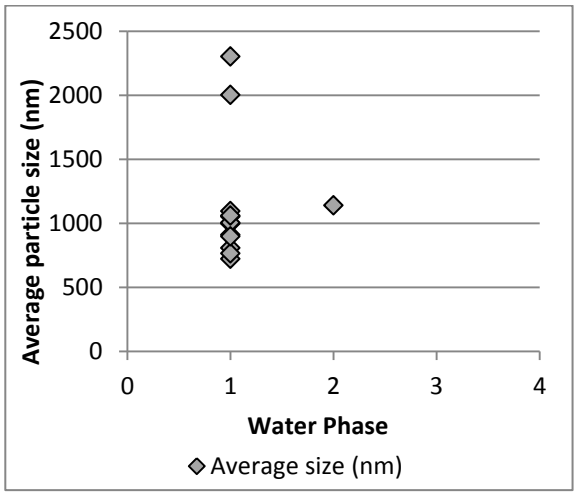

Figure 3-67: The average particle size in every anti-infective Pheroid micro-sponge formulation against the water phase used (obtained by CLSM analysis)

3.3.2.2.2.2 Preservatives and antioxidants

Confocal reports were unavailable for the anti-infective formulations that included preservatives or antioxidants with one exception (presented in Figures 3-68 and 3-69). Antioxidant combination 2 (described in Table 3-11) was present in one formulation and had an average particle size that was in line with the formulations that did not contain any antioxidants.

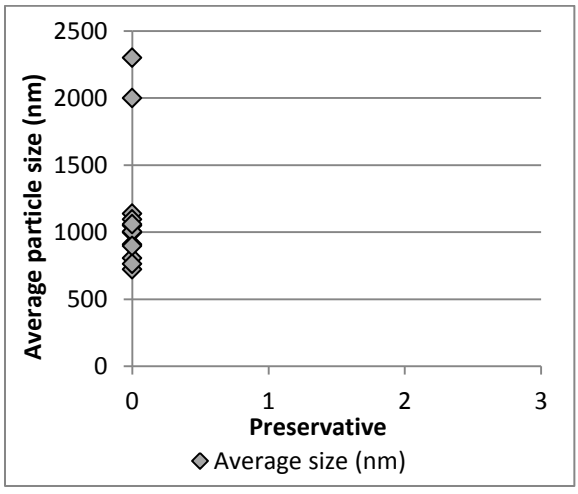

Figure 3-68: The average particle size in every anti-infective Pheroid micro-sponge formulation against the preservative used (obtained by CLSM analysis)

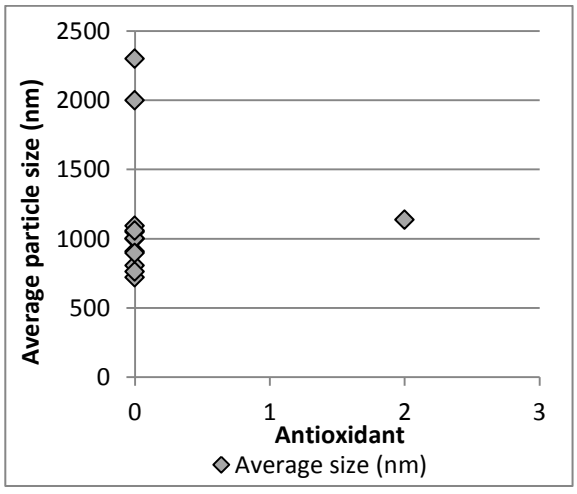

Figure 3-69: The average particle size in every anti-infective Pheroid micro-sponge formulation against the antioxidant used (obtained by CLSM analysis)

3.3.2.2.2.3 Other excipients

Details on the other excipient combinations are expressed in Table 3-12. Combination one was added to eleven formulations of which eight had data on confocal analysis. The presence of the excipients in formulations that have wide-ranging particle sizes suggests that the combination did not influence the sizes (see Figure 3-70). However, due to a lack of reference and anti-infective data, this could not be confirmed. Both excipients 5 and 7 were present in two formulations which had average particle sizes that were in accordance with the average particle sized throughout the various formulations.

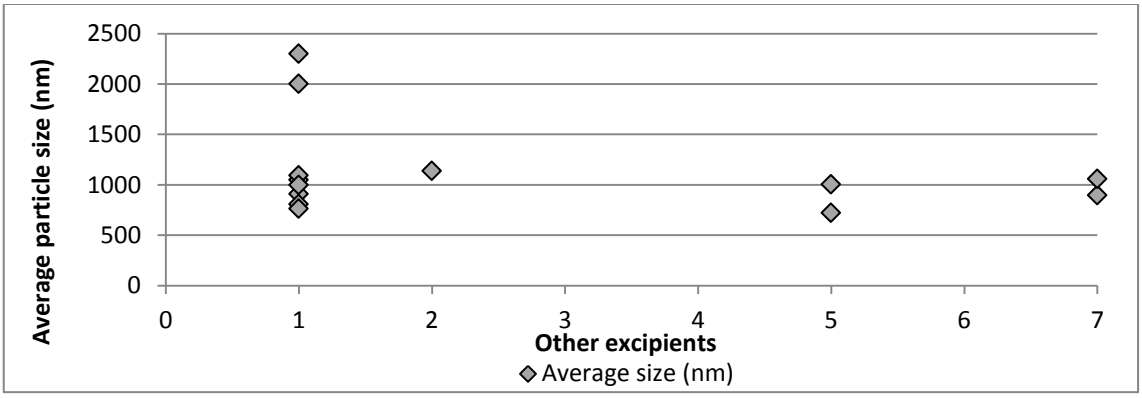

Figure 3-70: Graph that displays the average particle size in every anti-infective Pheroid micro-sponge formulation against the other excipients used (obtained by CLSM analysis)

3.3.2.2.2.4 Active ingredients

Active ingredients 11 and 14 (outlined in Table 3-13) were the only actives that were present in more than one formulation that had the concomitant confocal reports. Both active ingredients were present in formulations that had average particle size values that were close to each other (displayed in Figure 3-71). Unfortunately, limited data hampered the formation of significant trends.

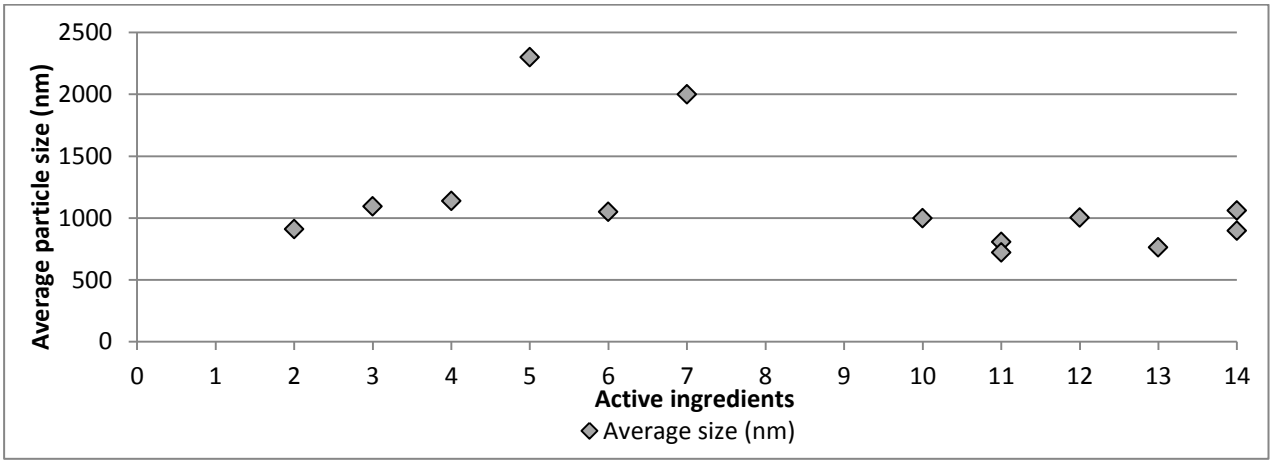

Figure 3-71: The average particle size in every anti-infective Pheroid micro-sponge formulation against the other excipients used (obtained by CLSM analysis)

3.3.2.3 CLSM analysis: particle concentration

3.3.2.3.1.1 Oil and water phases

The particle concentrations that were obtained with the addition of various oil and water phases are displayed in Figure 3-72 and 3-73 respectively. The formulations that contained oil phase 1 had concentrations ranging from 70 x10^8/ mL to an extreme value of 5372 x10^8/ mL. The formulation, with which the latter value was encountered, also included water phase 2 (described in Table 3-9). However, in consideration of the previous results, the oil and water phases would not likely have had such a radical impact on the particle concentration.

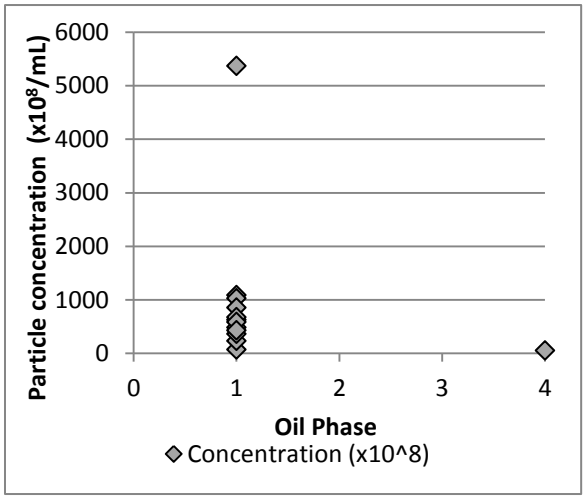

Figure 3-72: Graph that displays the particle concentration (per mL) of every anti-infective Pheroid micro-sponge formulation against the oil phase used (obtained by CLSM analysis)

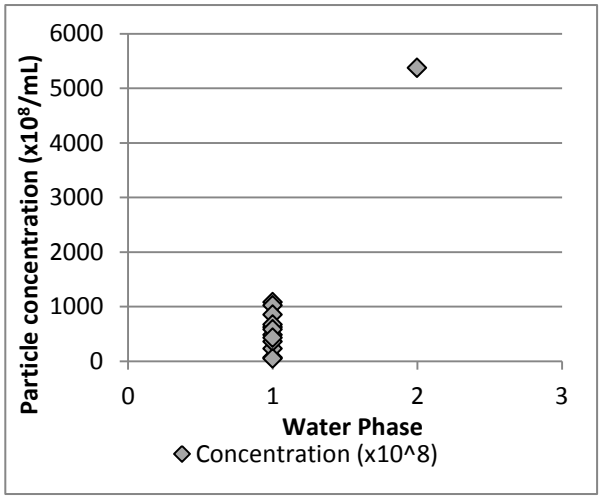

Figure 3-73: Graph that displays the particle concentration (per mL) of every anti-infective Pheroid micro-sponge formulation against the water phase used (obtained by CLSM analysis)

3.3.2.3.1.2 Preservatives and antioxidants

None of the anti-infective micro-sponge formulations contained any preservatives; consequently, the latter could not have an influence on the particle concentration with regard to the values obtained with the reference formulations (see Figure 3-74). Only one formulation that had a confocal report available contained an antioxidant (see Figure 3-75). This formulation, which included ascorbyl palmitate, had a significantly higher particle concentration than any of the other formulations. This observation cannot be attributed to the specific antioxidant as other factors were also present and sufficient data was lacking.

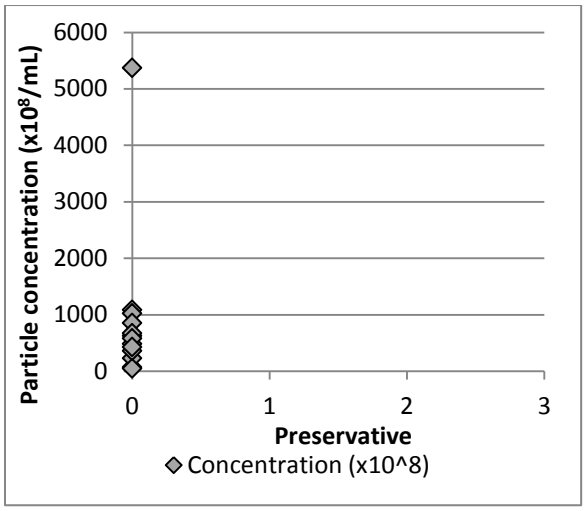

Figure 3-74: The particle concentration (per mL) of every anti-infective Pheroid micro-sponge formulation against the preservative used (obtained by CLSM analysis)

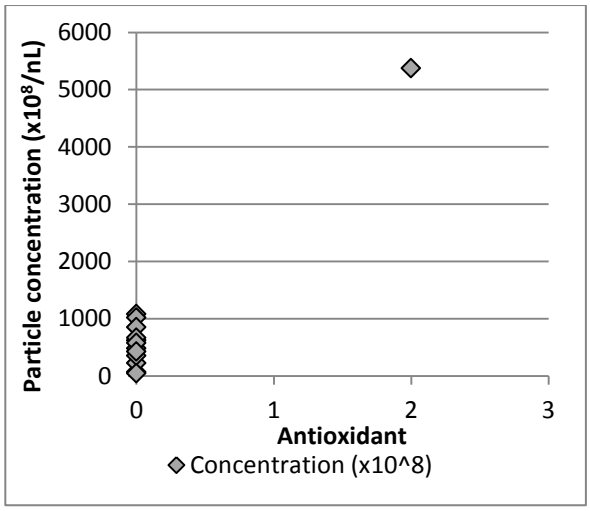

Figure 3-75: The particle concentration (per mL) of every anti-infective Pheroid micro-sponge formulation against the antioxidant used (obtained by CLSM analysis)

3.3.2.3.1.3 Other excipients

All the other excipients were present in formulations that had less than 1100 x10^8 particles per mL - except for combination 2 (described in Table 3-12). The latter was present in the formulation that had a concentration of more than 5000 x10^8 particles per mL (presented in Figure 3-76). Unfortunately, this combination was not included in any other formulation so that results could be compared.

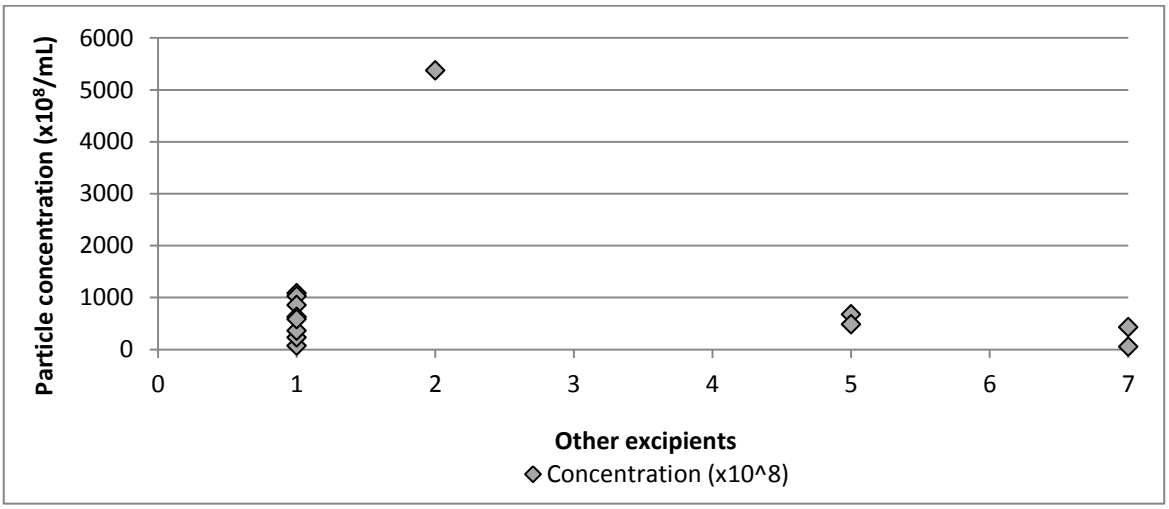

Figure 3-76: The particle concentration (per mL) of every anti-infective Pheroid micro-sponge formulation against the other excipients used (obtained by CLSM analysis)

3.3.2.3.1.4 Active ingredients

Figure 3-77 reflects the particle concentration per mL that corresponds with the active ingredient(s) used in the formulation (see Table 3-13). Formulations of which confocal analysis

was not available were excluded from the graph to promote ease of reading. Two formulations that contained active ingredient 5 and 14 respectively had very low concentrations (70 and 38 $x10^8$/ mL respectively). The formulation in which active ingredient 4 was present had an abnormally high amount of particles while the other formulations had concentrations that extended to 1100 $x10^8$ per mL.

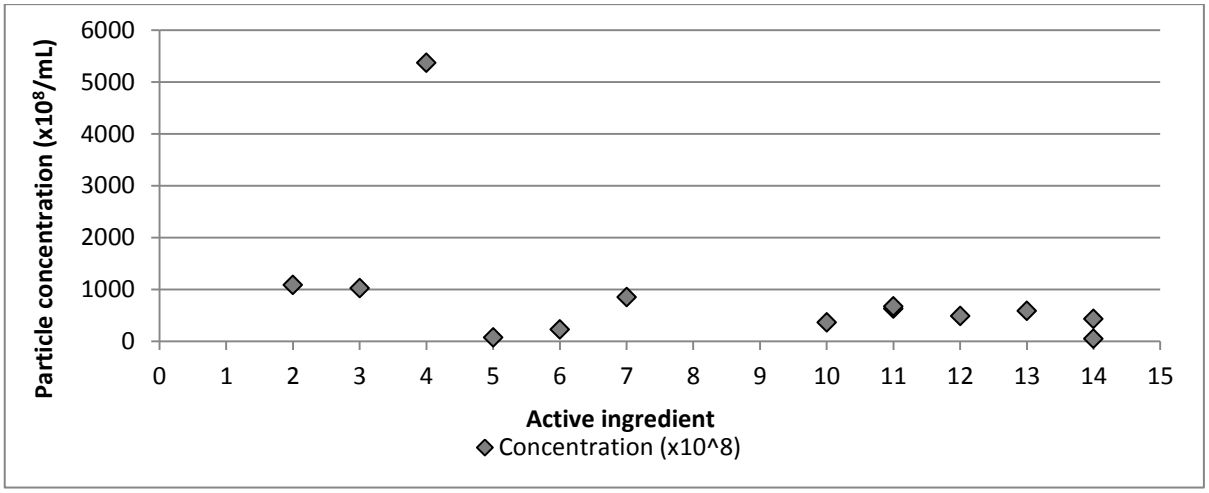

Figure 3-77: The particle concentration (per mL) of every anti-infective Pheroid micro-sponge formulation against the active ingredient used (obtained by CLSM analysis)

3.3.2.4 Zeta potential measurement

3.3.2.4.1 Statistical description of reference formulations

The zeta potential of the reference formulations averaged -36.2 mV which indicates a tendency to stability. An abnormal value of -18 mV fell outside the general range of measurements which spanned from -35.8 to -44.1 mV (presented in Figure 3-78). The average zeta potential obtained with the micro-sponge reference formulations pointed towards more stable formulations compared to the averages obtained for pro-Pheroid (-31.8 mV) and vesicle (-23.41 mV) reference formulations.

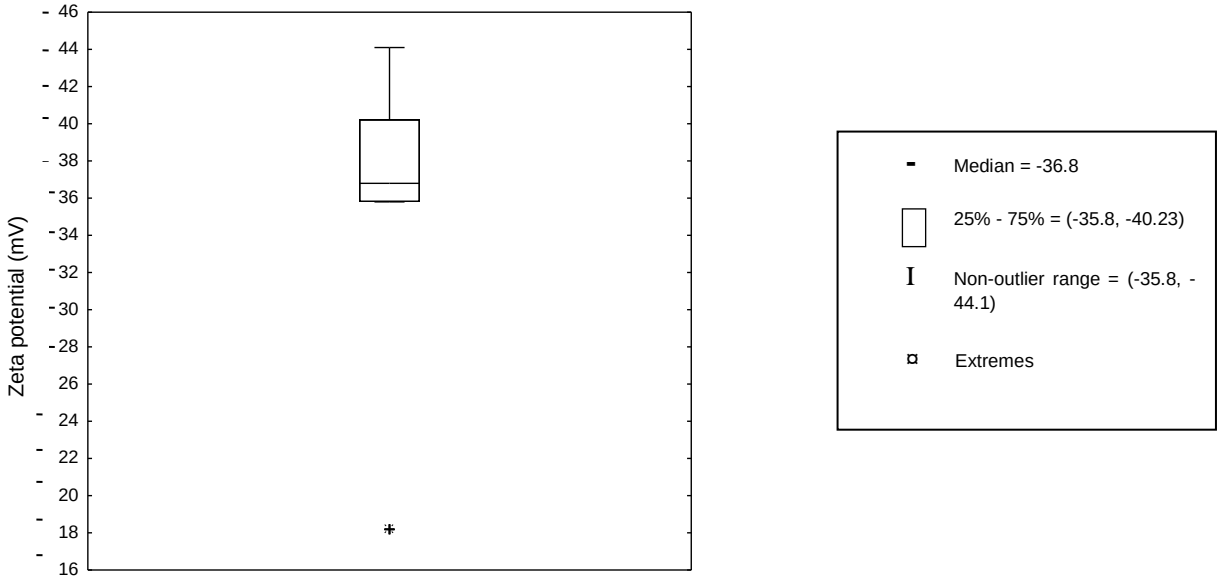

Figure 3-78: The average zeta potential (mV) in each Pheroid micro-sponge reference formulation

3.3.2.4.2 Anti-infective micro-sponge formulations

3.3.2.4.2.1 Oil phase

The wide range of zeta potential measurements obtained with the anti-infective micro–sponge formulations that utilized oil phase 1, ranging from a tendency to be unstable (0 to ±25 mV) to a tendency to be stable (> ±25 mV), correlates with the reference range of measurements (see Figures 3-79 and 3-80). This signifies that the oil phase did not likely influence the stability of the formulations.

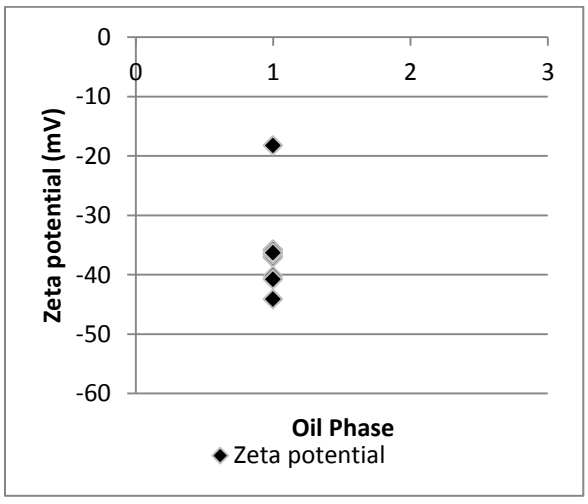

Figure 3-79: The different zeta potential measurements obtained against the oil phase used in the reference formulations (obtained by Zetasizer analysis)

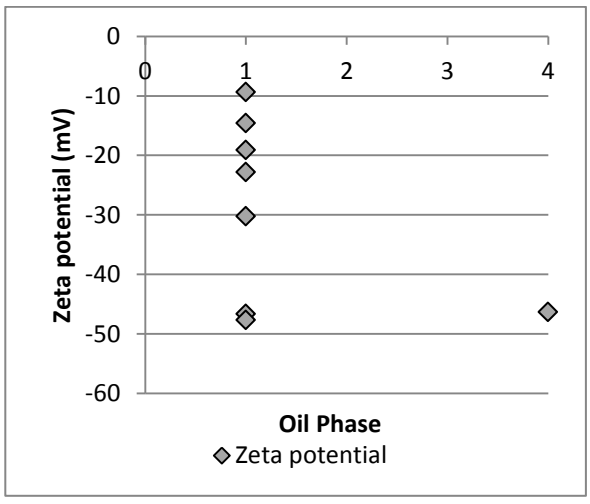

Figure 3-80: The different zeta potential measurements obtained against the oil phase used in the anti-infective micro-sponge formulations (obtained by Zetasizer analysis)

3.3.2.4.2.2 Water phase

Water phase 1 was consistently used in all the formulations that resulted in broadly distributed zeta potential measurements (refer to Figure 3-82). Moreover, the distribution of measurements was similar to that obtained for the reference formulations and was therefore unlikely to be responsible for any deviations in the stability of the formulations (reflected in Figure 3-81).

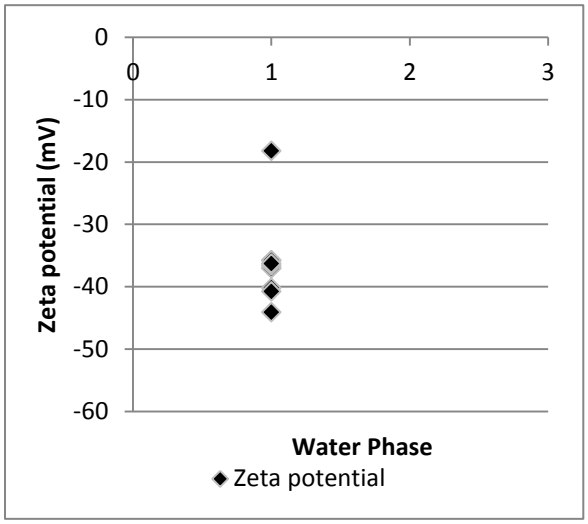

Figure 3-81: The different zeta potential measurements obtained against the water phase used in the reference formulations (obtained by Zetasizer analysis)

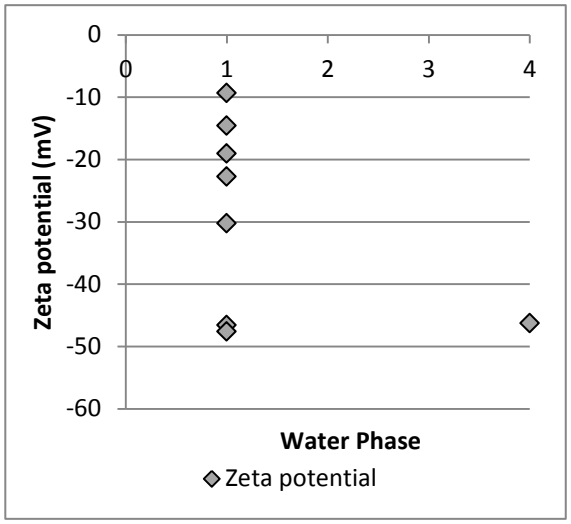

Figure 3-82: The different zeta potential measurements obtained against the water phase used in in the anti-infective micro-sponge formulations (obtained by Zetasizer analysis)

3.3.2.4.2.3 Preservatives or antioxidants

None of the formulations that contained a preservative or antioxidant had the concomitant zeta potential reports. Therefore, the impact of these ingredients on the stability of Pheroid micro-sponges could not be evaluated. The stability of the formulations that was manufactured without preservatives or antioxidants is illustrated in Figures 3-83 and 3-84.

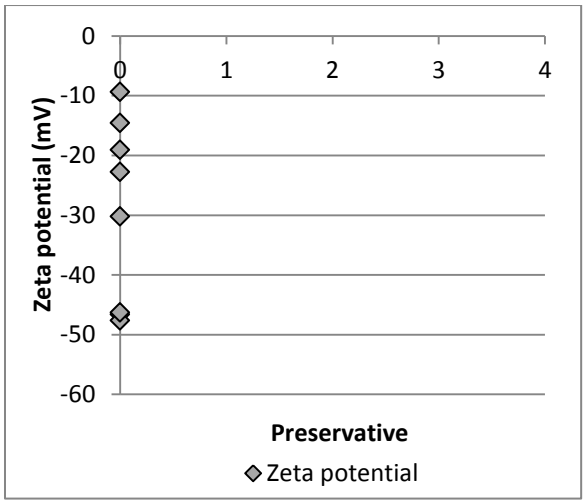

Figure 3-83: The different zeta potential measurements obtained against the preservative used in in the anti-infective micro-sponge formulations (obtained by Zetasizer analysis)

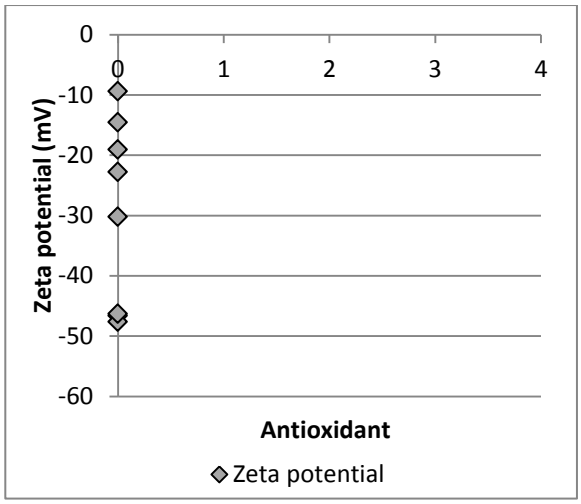

Figure 3-84: The different zeta potential measurements obtained against the antioxidant used in in the anti-infective micro-sponge formulations (obtained by Zetasizer analysis)

3.3.2.4.2.4 Other excipients

The distribution found for the formulations that included excipient combination 1 may signify that stability was not influenced by its presence. Combination 5 was present in two formulations with zeta potential measurements of -9.37 and -22.77 mV respectively (see Table 3-12 for a description of the excipients). The latter measurements indicate that the formulation is likely to be unstable and will tend to aggregate. A single formulation utilized excipient 6 and had a stable zeta potential measurement of -30.23 mV. The two formulations that contained excipient combination 7 tended to be very stable and their measurement values were grouped at around -50 mV (see Figure 3-85). However, due to data limitation, it could not be assumed that the latter combination of excipients enhanced the formulation's stability.

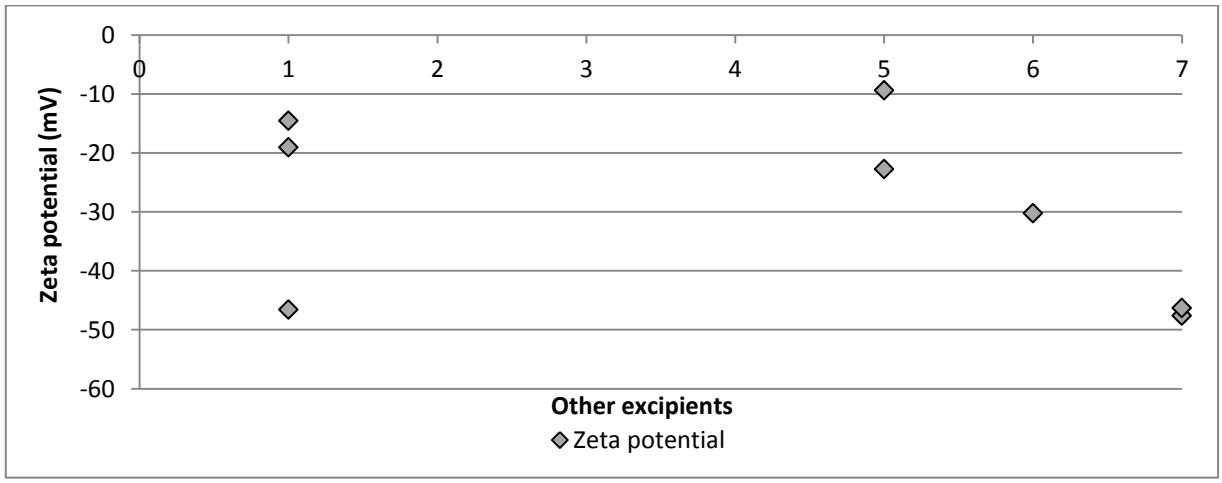

Figure 3-85: The different zeta potential measurements obtained against the other excipients used in in the anti-infective micro-sponge formulations (obtained by Zetasizer analysis)

3.3.2.4.2.5 Active ingredients

The available zeta potential measurements along with the corresponding active ingredient in the respective micro-sponge formulations are portrayed in Figure 3-86. In general, the formulations that contained active ingredient 10, 11 and 12 had zeta potentials below -25 mV. This indicates unstable formulations that are inclined to aggregate (refer to Table 3-13 where the active ingredients are defined). Active ingredient 11 was present in another formulation that had a zeta potential of -30 mV (stable formulation) and was therefore not likely responsible for the fluctuation of stability between the formulations. The particle sizes observed in the formulations that contained active ingredient 11 did not correlate with the zeta potential measurements that were obtained (see Figure 3-50 and 3-51). The formulation with the largest particles was more stable than the other formulation. Zeta potential measurements closer to zero are usually obtained with unstable formulations that tend to aggregate. Both formulations to which active ingredient 14 was added to, had a very high zeta potential measurement. This translates to a tendency to be very stable although the active's involvement in the stability of the formulation could not be established.

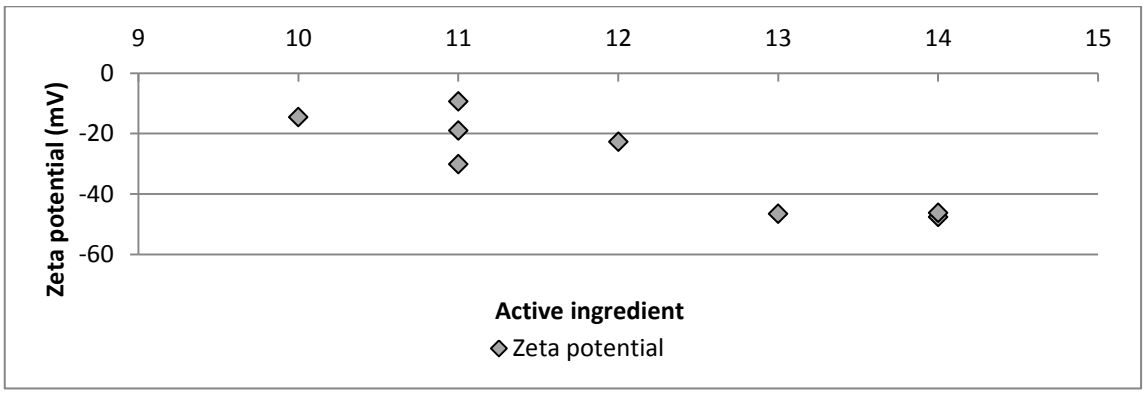

Figure 3-86: The different zeta potential measurements obtained against the active ingredient used in in the anti-infective micro-sponge formulations (obtained by Zetasizer analysis)

3.3.2.5 Summary: Pheroid micro-sponges

A concise summary of the results obtained through the statistical analysis of the Pheroid micro-sponge formulations are presented in Table 3-14. Due to limited data available, definite conclusions could not be draw; however, it was observed that (refer to Table 3-14):

- the mastersize results were not likely influenced by the oil or water phases, possibly influenced by the antioxidants and preservatives and most likely influenced by the other excipients and active ingredients,

- the CLSM results were not likely influenced by the oil or water phases and were most likely influenced by the other excipients and the active ingredients. The antioxidants, preservatives were not present in enough formulations to draw significant conclusions,

- the zeta potential results were not likely influenced by the oil or water phases and were most likely influenced by the other excipients and the active ingredients. The antioxidants and preservatives were not present in enough formulations to derive substantial conclusions.

Table 3-14: Table summarizing the results from the Pheroid micro-sponge statistical analysis

Pheroid micro-sponges				
	Mastersizer - particle size distribution	**CLSM - particle size**	**CLSM - particle concentration**	**Zeta Potential - stability**
Oil Phase	Results indicate that the oil phase did not likely influence the particle size distribution	Results indicate that the oil phase did not likely influence the average particle size	Results indicate that the oil phase did not likely influence the particle concentration	Results indicate that the oil phase did not likely influence the particle concentration
Water Phase	Results indicate that the water phase did not likely influence the particle size distribution	Results indicate that the water phase did not likely influence the average particle size	Results indicate that the water phase did not likely influence the particle concentration	Results indicate that the water phase did not likely influence the particle concentration
Preservatives	Significant conclusions could not be drawn from the data since no preservatives were used in any of the formulations	Significant conclusions could not be drawn from the data since no preservatives were used in any of the formulations	Significant conclusions could not be drawn from the data since no preservatives were used in any of the formulations	Significant conclusions could not be drawn from the data since no preservatives were used in any of the formulations
Antioxidants	Significant conclusions could not be drawn from the data since no antioxidants were used in any of the formulations	Only one formulation contained an antioxidant and had an average particle size that was in line with the formulations that did not contain any antioxidants. This indicates that the latter antioxidant did not likely influence the particle size (see Figure 3-69)	Only one formulation contained an antioxidant and had a significantly larger particle concentration than any of the other formulations. Since the data was limited and other factors were present the observation could not be attributed to the antioxidant (see Figure 3-75)	Significant conclusions could not be drawn from the data since no antioxidants were used in any of the formulations

Table 3-14: Table summarizing the results from the Pheroid micro-sponge statistical analysis (continued)

	Mastersizer - particle size distribution	CLSM - particle size	CLSM - particle concentration	Zeta Potential - stability
Other Excipients	Significant conclusions could not be drawn from the limited data although tendencies were observed in the particle size distributions (see Figures 3-62 and 3-63)	Tendencies in the particle sizes were observed although significant conclusions could not be drawn from the limited data (see Figure 3-70)	Tendencies in the particle sizes were observed although significant conclusions could not be drawn from the limited data (presented in Figure 3-76)	Tendencies were observed with certain 'other excipient' combinations whereas no tendencies could be established with other combinations (see Figure 3-85)
Active ingredient	Tendencies were observed with certain active ingredients whereas no tendencies could be established with other active ingredients (illustrated in Figure 3-64)	Tendencies in the particle sizes were observed although significant conclusions could not be drawn from the limited data (displayed in Figure 3-71)	Tendencies in the particle sizes were observed although significant conclusions could not be drawn from the limited data (reflected in Figure 3-77)	Tendencies were observed with certain active ingredients whereas no tendencies could be established with other active ingredients (see Figure 3-86)

3.3.3 Pro-Pheroid

17 pro-Pheroid formulations were analysed as reference formulations. The complete Mastersize and CLSM analysis reports were available for all of the formulations but only four included a zeta potential measurement. Therefore, the results obtained from the particle size distribution and CLSM analysis could be considered as statistically significant, but not the zeta potential. A total of 40 anti-infective pro-Pheroid formulations were processed. 39 of the formulation contained particle size distribution analysis, 35 had confocal reports and eight were analysed for stability.

3.3.3.1 Particle size and particle size distribution

3.3.3.1.1 Statistical description of reference formulations

An average of 45.44% particles in the reference formulations was 0.1 to 1 µm in size. The % submicron size particles in the various formulations ranged from 2.88 to 100.00% (see Figure 3.87). The interquartile range was wide and spread from 14.99 to 71.97%.

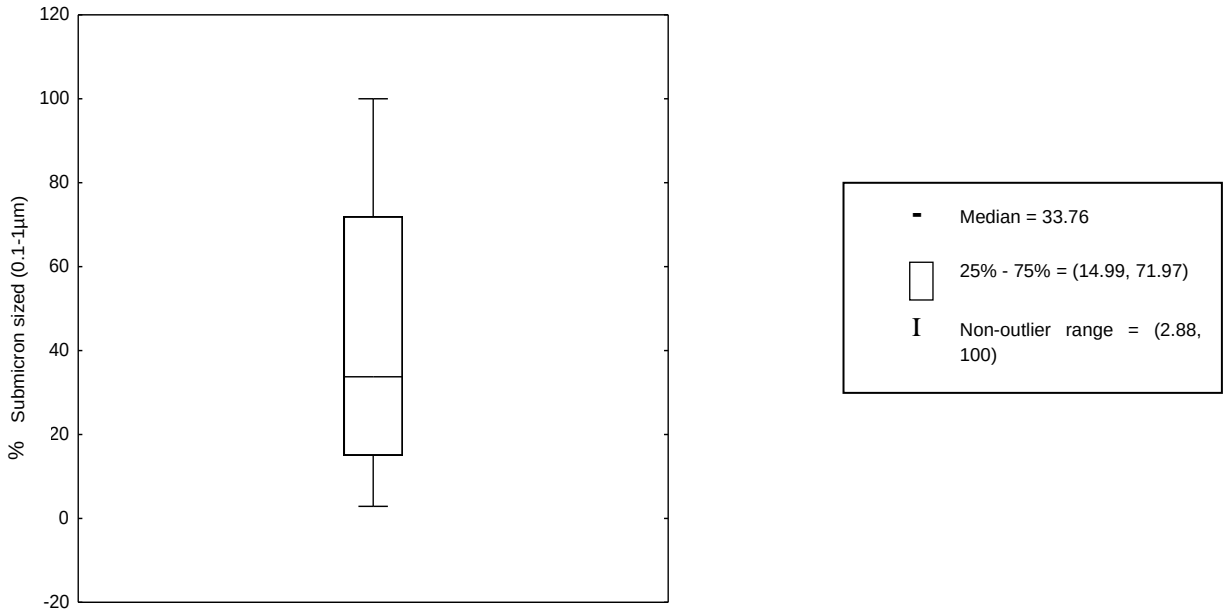

Figure 3-87: Percentage submicron sized particles (0.1 to 1 µm) in the pro-Pheroid reference formulations (obtained from Mastersize analysis)

The fractions of micron sized particles that make up the respective formulations are visually reflected in Figure 3-88. Fifty per cent of the formulations had less than 15.02% micron-sized particles and, on average, 21.03% of particles in the reference formulations were micron-sized. Minimum and maximum values of respectively 0 and 45.70% were obtained. No outlying or extreme values were present.

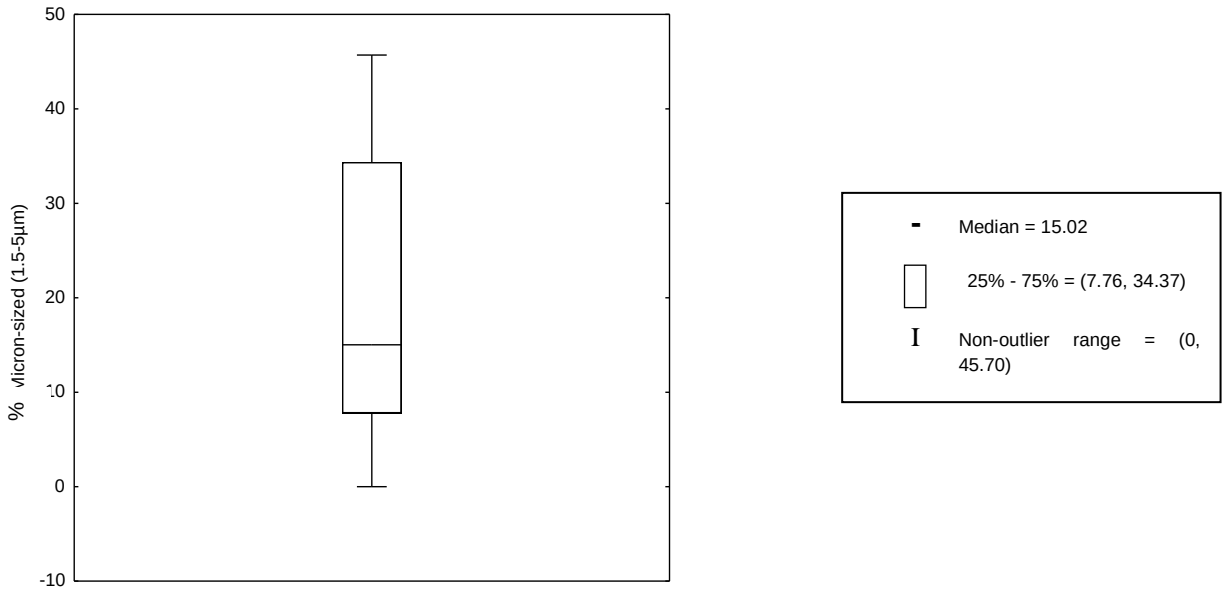

Figure 3-88: Percentage micron sized particles (1.5 to 5 µm) in the pro–Pheroid reference formulations (obtained from Mastersize analysis)

3.3.3.1.2 Anti-infective pro-Pheroid formulations

Both the reference and the anti-infective formulations had a higher percentage submicron than micron sized particles (see Figures 3-89 and 3-90). The anti-infective formulations generally had

more submicron sized particles and much less micron sized particles than the reference formulations. However, those ranges do not account for particles larger than 5 µm and may lead to an inaccurate perception. For instance, the anti-infective formulation, P14006 (which signifies the formulation's unique batch number), is displayed in Figure 3-90 as a zero value. This formulation had a $d_{0.9}$ of 316.5 µm which means that 90.00% of the particles were ≤316.5 µm in size. Thus, exclusively focusing on the percentage submicron and micron sized particles can be misleading (with the exception of 100.00% values).

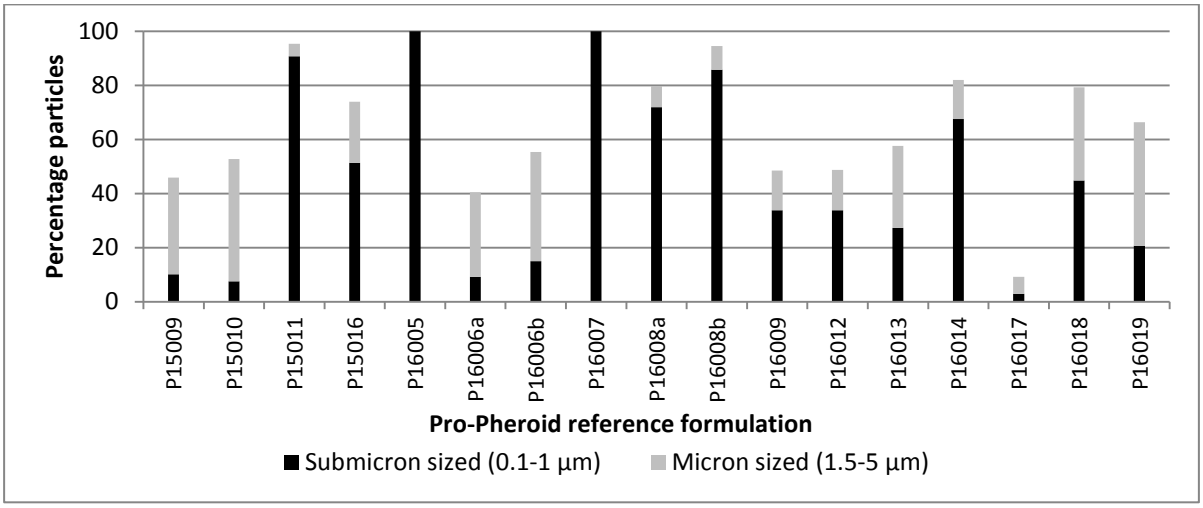

Figure 3-89: Percentage submicron and micron sized particles in the respective pro-Pheroid reference formulations (obtained by Mastersize analysis)

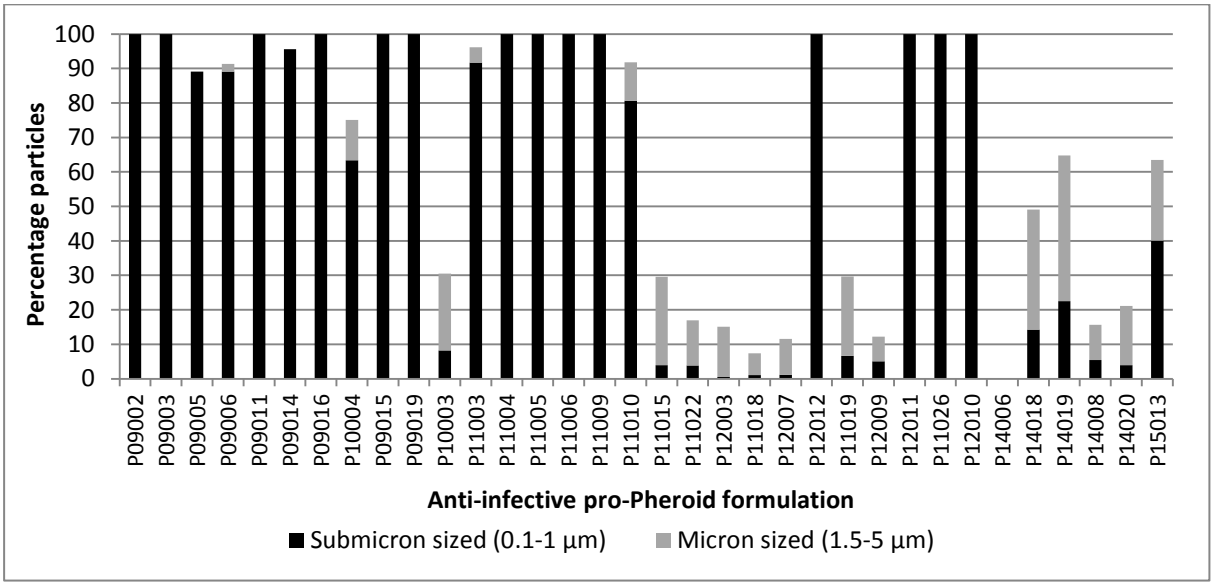

Figure 3-90: Percentage submicron and micron sized particles in the respective anti-infective pro-Pheroid formulations (obtained by Mastersize analysis)

An outline of the ingredients included in each anti-infective pro-Pheroid formulation (identified by means of the batch number (e.g. P09002)) is presented in Table 3-15 (refer to Table 3-16 to 3-19 for descriptions of the ingredients). The reference formulations are not described since they exclusively contain Pheroid ingredients in the patented ratios.

Table 3-15: Outline of the ingredients present in the anti-infective pro-Pheroid formulations

BATCH NO.	OIL PHASE	ACTIVE INGREDIENT	PRESERVATIVE	ANTIOXIDANT	OTHER EXCIPIENT
P09002	1	1	2	1	0
P09003	1	1	0	1	0
P09005	1	2	0	3	2
P09006	1	3	0	2	2
P09011	1	5	1	2	0
P09014	1	6	0	4	3
P09015	1	7	0	1	3
P09016	1	6	0	4	3
P09019	1	7	0	1	3
P09020	3	16	0	4	3
P10003	1	7	0	1	4
P10004	1	6	0	5	3
P11003	1	8	0	0	0
P11004	1	8	0	0	5
P11005	1	8	0	0	0
P11006	1	8	0	0	5
P11009	1	8	0	0	0

Table 3-8: Outline of the ingredients present in the anti-infective pro-Pheroid formulations (continued)

BATCH NO.	OIL PHASE	ACTIVE INGREDIENT	PRESERVATIVE	ANTIOXIDANT	OTHER EXCIPIENT
P11010	1	8	0	0	0
P11015	1	9	0	6	1
P11018	1	10	0	6	1
P11019	1	11	0	6	5
P11022	1	9	0	6	1
P11026	1	12	0	6	5
P12003	1	9	0	6	1
P12007	1	10	0	6	1
P12009	1	11	0	6	1
P12010	1	12	0	6	5
P12011	1	11	0	6	5
P12012	1	10	0	6	5
P14006	3	15	3	0	7
P14008	1	17	3	6	5
P14018	1	16	0	0	0
P14019	1	16	0	0	0
P14020	1	17	0	0	0
P15013	4	17	4	0	0

A large number of the anti-infective formulations with a very low percentage of particles in the submicron and micron sized range are anti-malarials, in particular artemisone (presented in Figure 3-90) and might be the result of solubility issues since more favourable particle size distributions were obtained with the active in Pheroid vesicle formulations (see Figure 4-17). Active ingredient 10 and 11 were present in formulations that contained similar ingredients. One formulation of each active contained excipient 5 and had 100.00% submicron sized particles whereas the other formulation contained excipient 1 and had ~10% particles in the submicron and micron size range combined. The formulations are arranged according to active ingredients in Figure 3-101 and might simplify the evaluation of data in terms of active ingredients.

3.3.3.1.2.1 Oil Phase

The pro-Pheroid reference formulations experienced a wide distribution of particles. The presence of submicron sized particles in the various formulations ranged from ~2.00% to 100.00% while the percentage micron sized particles did not surpass 50.00% (see Figures 3-91 and 3-92).

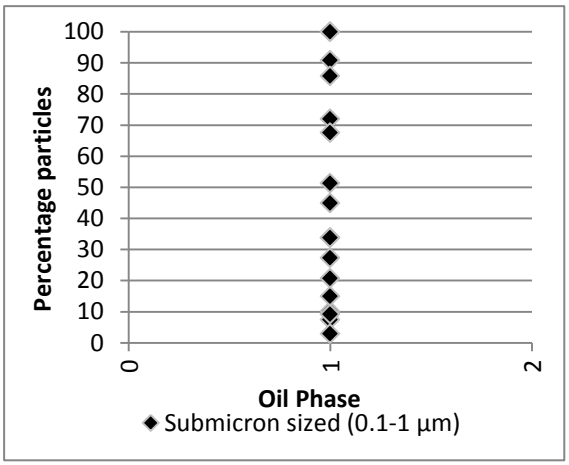

Figure 3-91: Percentage submicron sized particles against the oil phase used in the respective pro-Pheroid reference formulations (obtained by Mastersize analysis)

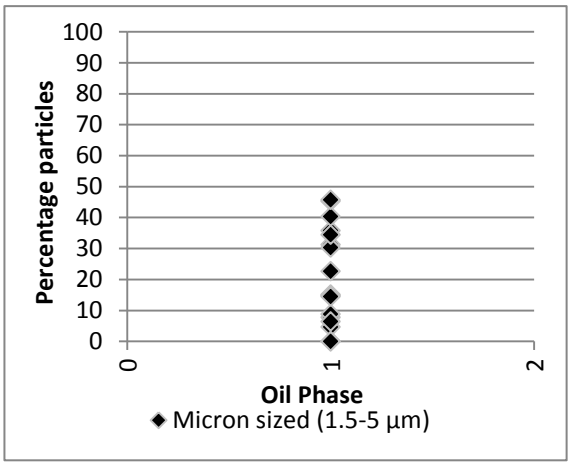

Figure 3-92: Percentage micron sized particles against the oil phase used in the respective pro-Pheroid reference formulations (obtained by Mastersize analysis)

Oil phase 1 used in the anti-infective formulations is similar to that of the reference formulations. This is also noticeable by merging the distribution of particle sizes displayed in Figures 3-93 and 3-94. Oil phases 2, 3 and 4 were only present in one formulation each. The formulation in which oil phase 3 was present had extremely large particles that did not fall into the submicron or micron size ranges. However, this oil phase comprised of a large amount of Kolliphor™ EL and did not include the other oil phase ingredients. The latter formulation was produced as an exception and is by no means the norm.

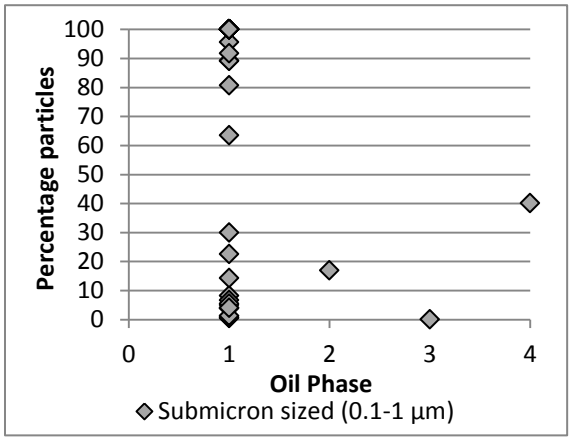

Figure 3-93: Percentage submicron sized particles against the oil phase used in the anti-infective pro-Pheroid formulations (obtained by Mastersize analysis)

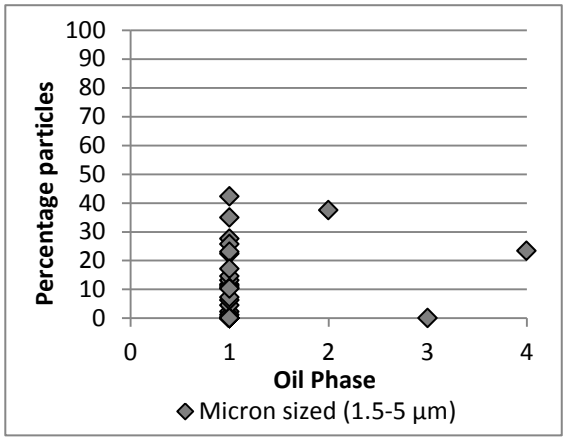

Figure 3-94: Percentage micron sized particles against the oil phase used in the anti-infective pro-Pheroid formulations (obtained by Mastersize analysis)

3.3.3.1.2.2 Preservatives

The particle size distribution obtained with the addition of preservatives is reflected in Figures 3-95 and 3-96. Formulations that did not include any preservatives had a distribution similar to the reference formulations. Preservative 1 and 2 were included in formulations that had 100.00% submicron sized particles while the formulations that preservative 3 and 4 were added to, had very large particles (most not within the submicron and micron size ranges). Unfortunately, the obtainment of more definite trends was hampered by the limited availability of data. The various preservatives are described in Table 3-16.

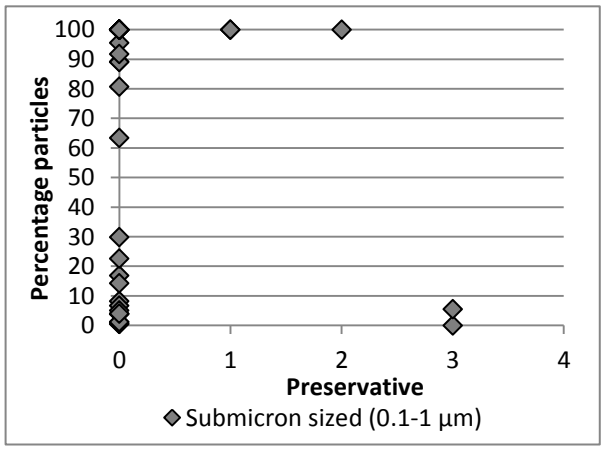

Figure 3-95: Percentage submicron sized particles against the preservative used in the anti-infective pro-Pheroid formulations (obtained by Mastersize analysis)

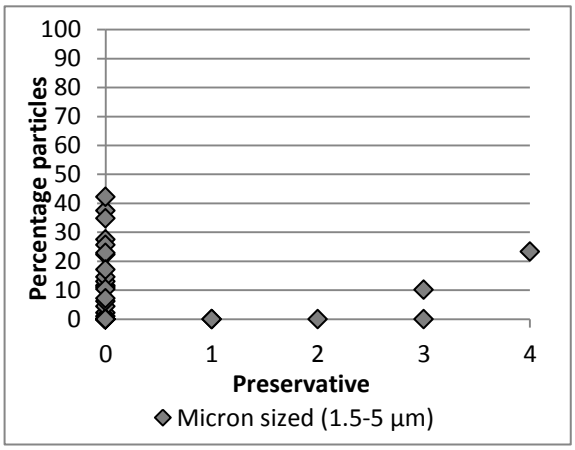

Figure 3-96: Percentage micron sized particles against the preservative used in the anti-infective pro-Pheroid formulations (obtained by Mastersize analysis)

Table 3-16: Various preservative combinations used in the anti-infective pro-Pheroid formulations

Nr.	Preservative	%	Frequency
1	Propylparaben	0.02	1
2	Butylparaben	0.03	1
3	Benzyl alcohol	1.07	2
4	Benzyl alcohol	24.85	1

Table 3-17: Various antioxidant combinations used in the anti-infective pro-Pheroid formulations

Nr.	Antioxidant	%	Frequency
1	BHA BHT	0.05 0.27	5
2	BHA BHT	0.01 0.10	2
3	BHA BHT Ascorbyl Palmnitate	0.01 0.12 0.27	1
4	BHA BHT Ascorbyl Palmnitate	0.02 0.20 0.08	3
5	BHA BHT Ascorbyl Palmnitate	0.20 2.00 0.08	1
6	BHA BHT	0.01 0.01	12

3.3.3.1.2.3 Antioxidants

The omission of antioxidants in the anti-infective pro-Pheroid formulations could not cause the particle size distribution to vary from that obtained with the reference formulations. Combination 2, 3 and 4 were present in formulations that consisted of a very high percentage (up to 100.00%) submicron sized particles (see Figure 3-97 and 3-98). Combination 1 and 6 was present in formulations that covered a broad range of particle sizes and was therefore not likely responsible for the obtained particle sizes. A description of the assorted antioxidant is presented in Table 3-17.

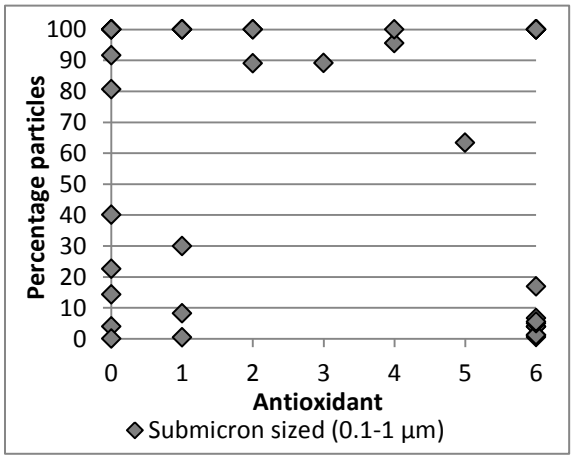

Figure 3-97: Percentage submicron sized particles against the antioxidant used in the anti-infective pro-Pheroid formulations (obtained by Mastersize analysis)

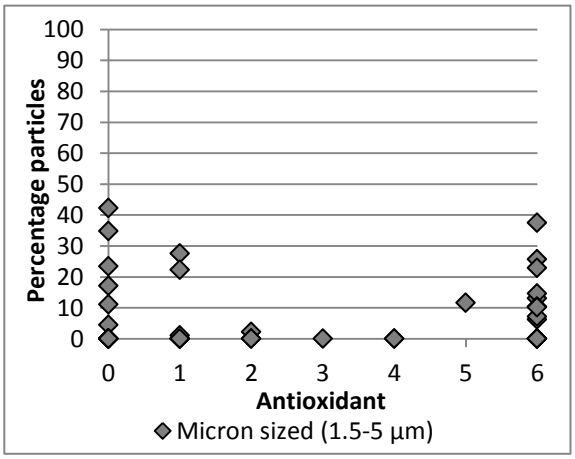

Figure 3-98: Percentage micron sized particles against the antioxidant used in the anti-infective pro-Pheroid formulations (obtained by Mastersize analysis)

3.3.3.1.2.4 Other excipients

Table 3-18: Various other excipients used in the anti-infective pro-Pheroid formulations

Nr.	Other excipient	%	Frequency
1	PEG 400	19.42	6
2	PEG 400	2.70	2
3	PEG 400	4.00	6
4	Beeswax	2.00	1
5	PEG 400	5.00	8
6	PEG 400 Tween 80	4.90 2.27	1
7	Propylene glycol PEG 400	18.93 54.00	1

Formulations that contained other excipient combination 1 had a low percentage submicron and micron sized particles (displayed in Figure 3-99 and 3-100). The formulations to which combination 2 and 3 were added generally comprised of particles in the submicron range (see Table 3-18 for a description of the excipients). Combination 4 was present in formulations that comprised of 0.00 to 30.00% submicron and micron sized particles. Combination 7 was present in a formulation with abnormally large particles.

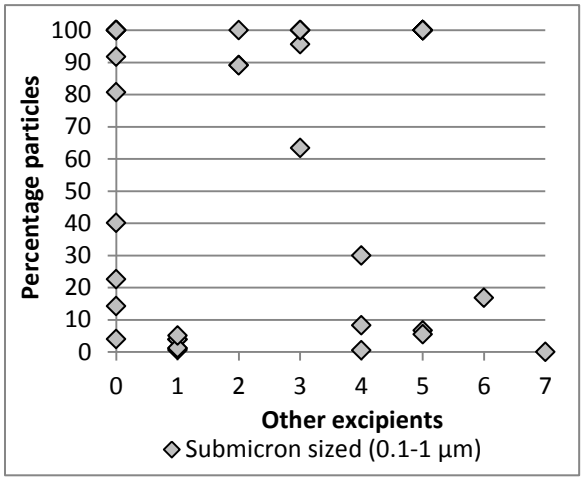

Figure 3-99: Percentage submicron sized particles against the other excipients used in the anti-infective pro-Pheroid formulations (obtained by Mastersize analysis)

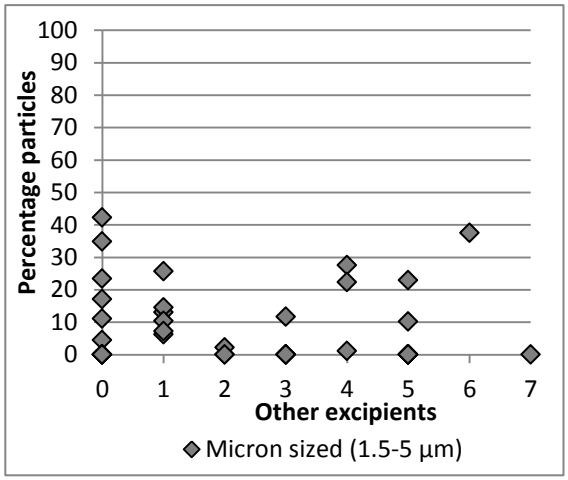

Figure 3-100: Percentage micron sized particles against the other excipients used in the anti-infective pro-Pheroid formulations (obtained by Mastersize analysis)

3.3.3.1.2.5 Active ingredients

Refer to Table 3-19 for a description of the various active ingredients used in the anti-infective pro-Pheroid formulations. The percentage submicron and micron sized particles in these formulations along with the concomitant active ingredient is presented in Figure 3-101. Several of the formulations comprised 100.00% of submicron sized particles. However, a few active ingredients were present in formulations that contained a great deal of large particles that did not fall within the submicron and micron size ranges. Active ingredient 7, 9, 10, 15 and 17 were present in such formulations. Active ingredient 10, for instance, was also present in a

formulation that consisted completely of submicron particles and would therefore not be the causative factor of the large particles in the other formulations.

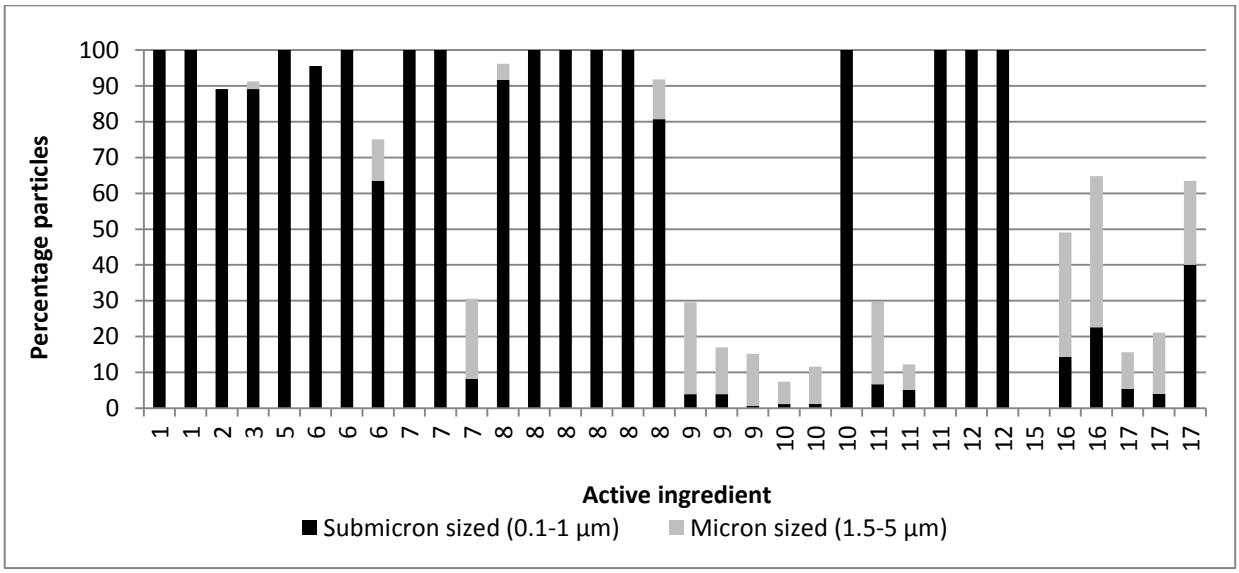

Figure 3-101:Percentage submicron sized particles against the active ingredient used in the anti-infective pro-Pheroid formulations (obtained by Mastersize analysis)

Table 3-19: Various active ingredients used in the anti-infective pro-Pheroid formulations

Nr.	Active ingredient	%	Frequency
1	Nevirapine	0.024	2
2	Isoniazid Ethambutol	7.50 27.50	1
3	Rifampicin Pyrazinamide	25.45 25.45	1
4	Pyrazinamide	40.00	1
5	Mefloquine-HCl	0.20	1
6	Rifampicin Pyrazinamide	11.79 44.20	3
7	Isoniazid Ethambutol	10.31 32.42	3
8	Propolis	10.00	6
9	Artemisone	18.00	3
10	Artemisone	0.04	3
11	Amodiaquine	0.047	3
12	Amodiaquine	24.90	2
13	MMV 034055	1.98	1
14	Abamectin 98% Tc	2.00	1
15	Dicyclanil	5.00	1
16	Glufosinate ammonium	20.00	3
17	Chlorpyrifos	48.00	3

3.3.3.2 CLSM analysis: largest particle in each formulation

3.3.3.2.1 Statistical description of reference formulations

On average, particles that were visualized with the confocal microscope were ≤11.47 µm in size. The largest particle in the formulations ranged from 2 to 20 µm in size although observations were concentrated in the first (2 to 8 µm) and third (10 to 16 µm) quartile groups (see Figure 3-102). During analysis, no crystals were observed in any of the formulations.

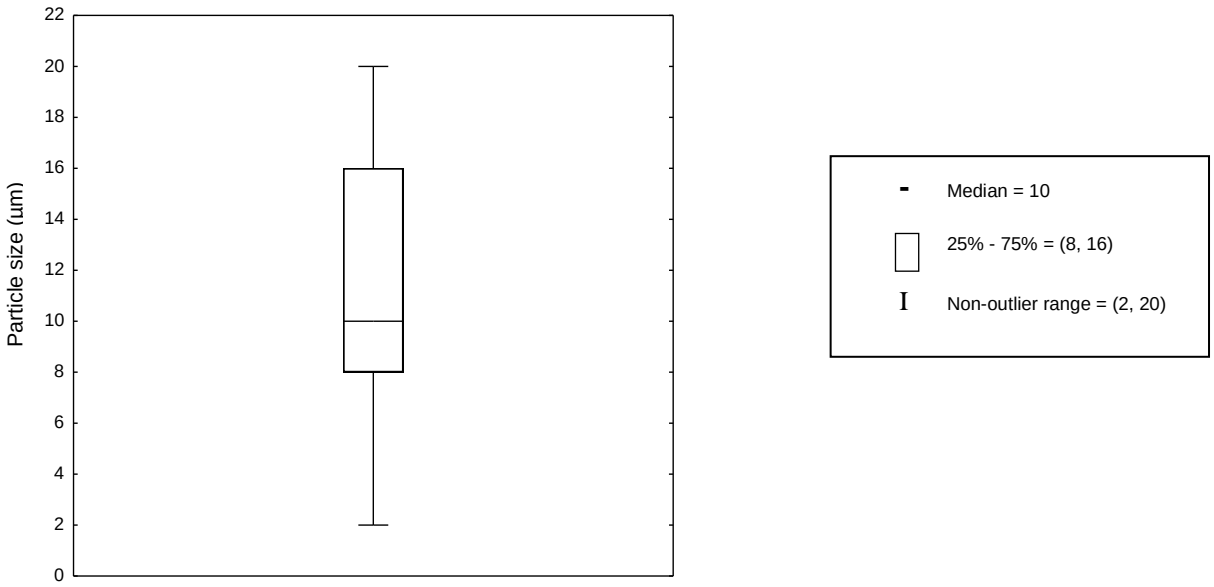

Figure 3-102: The largest particle in each pro–Pheroid reference formulation (obtained from CLSM analysis)

The reference formulations had particle concentrations ranging from 45.57 x10^8 to 2396 x10^8 and averaged at 1299.42 x10^8 particles per mL. 75.00% of the formulations had concentrations below 1486.4 x10^8 per mL (reflected in Figure 3-103). One formulation had an abnormally high particle concentration (7106 x10^8 /mL).

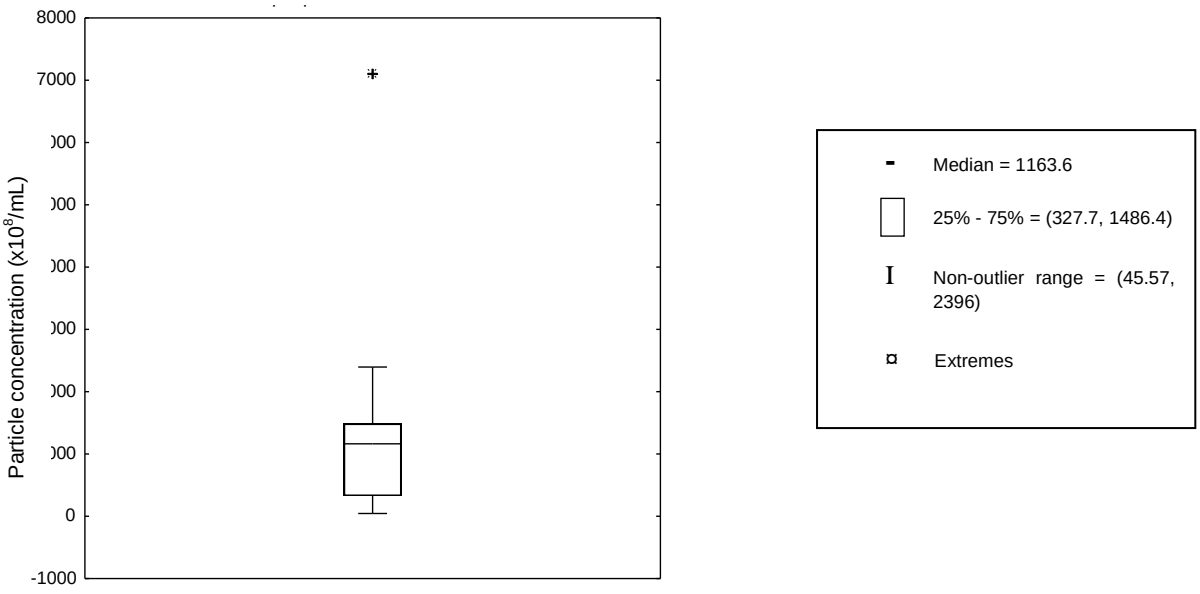

Figure 3-103: The Pheroid concentration (x10^8 per mL) in each pro–Pheroid reference formulation (obtained from CLSM analysis)

The pro-Pheroid reference formulations had an average size of 722.82 nm within the 200 to 5000 nm diameter range that was visible with the wavelengths used for the analysis. One outlier value was present (1123 nm) whereas the average particle size in other formulations ranged between 537 and 992 nm. 75.00% of the data were concentrated in the first three quartile groups (537 to 779 nm) and the other 25.00% ranged between 780 and 992 nm in size (see Figure 3-104).

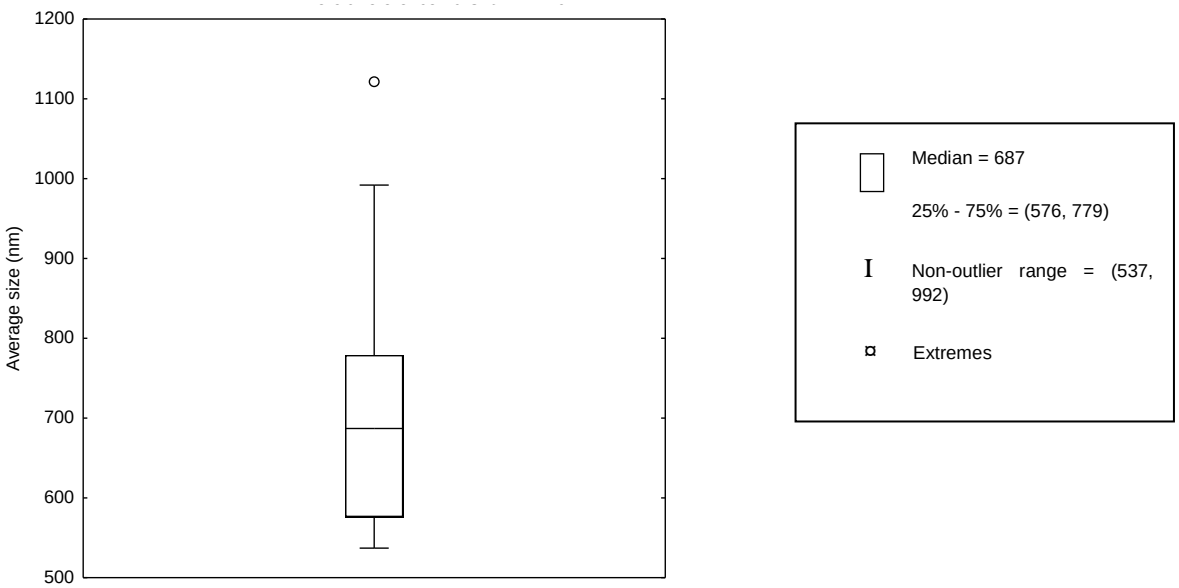

Figure 3-104:The average Pheroid size (nm) in each pro–Pheroid reference formulation (obtained from CLSM analysis)

3.3.3.2.2 Anti-infective pro-Pheroid formulations

3.3.3.2.2.1 Oil Phase

The average particle sizes in anti-infective formulations were much larger than those in the reference formulations (see Figures 3-105 and 3-106). The size distribution of oil phase 1 in the anti-infective formulations indicates that the oil was not the determining factor of the particle sizes but that the other additives (not present in the reference formulations) most likely were.

3.3.3.2.2.2 Preservatives and antioxidants

The only preservative (with confocal data) that was present in more than one formulation is combination 3 (see Figure 3-107). The latter's formulations differed widely in average particle sizes which signifies that the preservative did not influence the particle sizes. The formulations that included antioxidant 1 and 2 had values that were moderately collected. Formulations that utilized combination 4 had average particle sizes that were more distributed than combination 1 and 2 but not nearly as widely spread as combination 6 (see Figure 3-108). Regardless of antioxidant 6's presence in the respective formulations, various particle size averages were

obtained suggesting that the excipient did not influence the particle sizes. Table 3-16 and 3-17 described the respective additives.

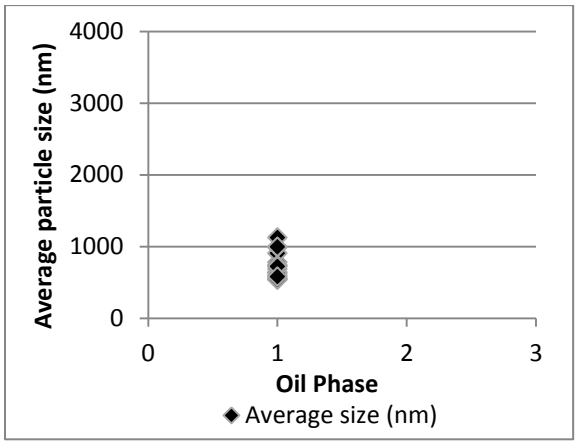

Figure 3-105:The average particle size in the pro-Pheroid reference formulations against the oil phase used (obtained by CLSM analysis

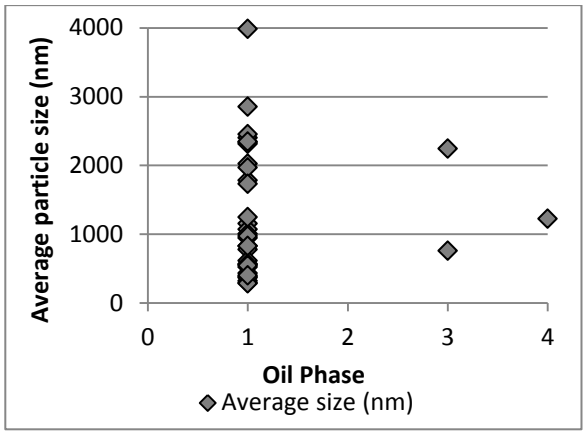

Figure 3-106:The average particle size in the anti-infective pro-Pheroid formulations against the oil phase used (obtained by CLSM analysis)

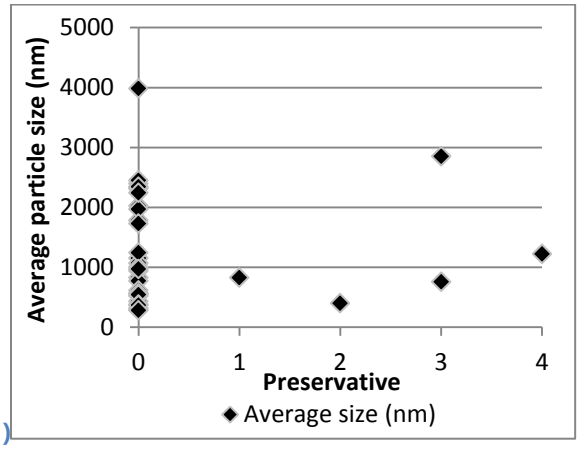

Figure 3-107:The average particle size in the anti-infective pro-Pheroid formulations against the preservative used (obtained by CLSM analysis)

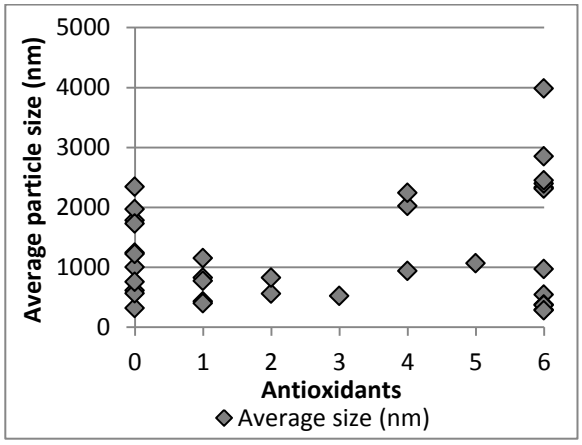

Figure 3-108:The average particle size in the anti-infective pro-Pheroid formulations against the antioxidant used (obtained by CLSM analysis)

3.3.3.2.2.3 Other excipients

The formulations that contained excipients number 1, 3 and 5 had average particle size distributions that were broadly distributed (Figure 3-109). Therefore, it is unlikely that these excipients had an impact on the average particle sizes. The two formulations that included excipient 2 had similar average particle sizes (between 520 and 560 nm). An outline of the excipients is presented in Table 3-18.

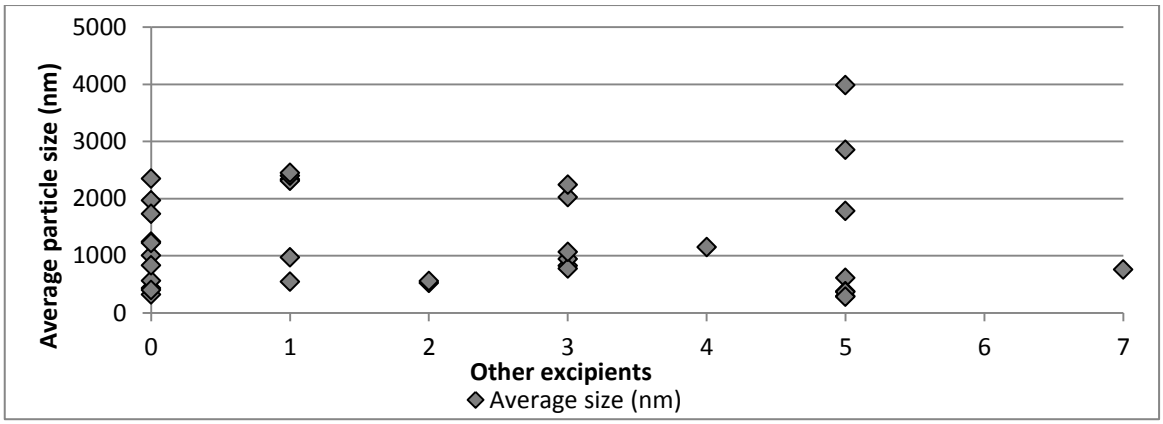

Figure 3-109:The average particle size in the anti-infective pro-Pheroid formulations against the other excipients used (obtained by CLSM analysis)

3.3.3.3 CLSM analysis: particle concentration

3.3.3.3.1.1 Oil phase

One reference formulation had an extreme value (>7000 x10^8/mL) while the others had concentrations ranging from ~40 x10^8 to ~2400 x10^8 per mL(presented in Figure 3-110). The anti-infective formulations that included oil phase 1 had similar particle concentrations than the non-outlier range in the reference formulations suggesting that the oil phase did not influence the particle concentrations of the various formulations (displayed in Figure 3-111). The formulation that contained oil phase 3 had a very low concentration. The latter oil phase did not comprise of the usual oil phase ingredients but a high amount of only one ingredient. Oil phase 4 contained the usual ingredients but their ratios were inverted. Unfortunately, oil phase 4 was only added to one formulation and its effect on the particle concentration could not be determined.

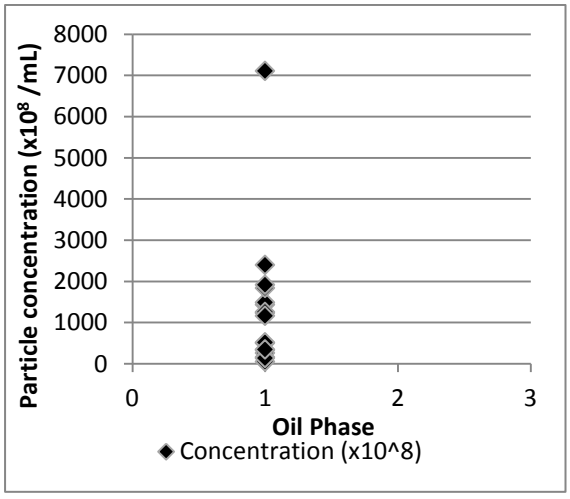

Figure 3-110:The particle concentration (per mL) of pro-Pheroid reference formulations against the oil phase used (obtained by CLSM analysis)

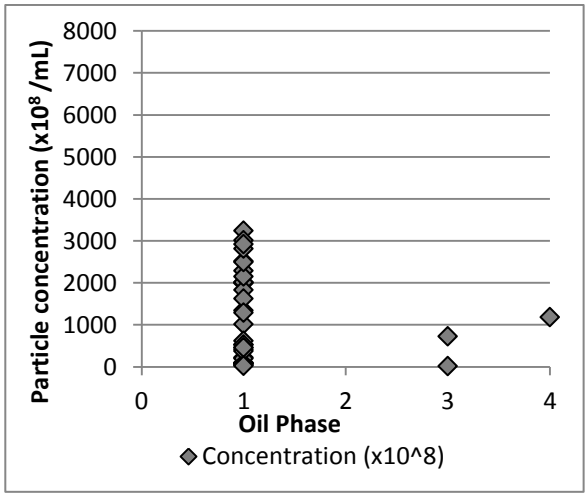

Figure 3-111:The particle concentration (per mL) of anti-infective pro-Pheroid formulation against the oil phase used (obtained by CLSM analysis)

3.3.3.3.1.2 Preservatives and antioxidants

The various preservatives were only available in one formulation each (that had particle concentration data). No significant observations could be drawn from such limited data (refer to Figure 3-112). Antioxidant combination 1, 4 and 6 were present in formulations that had an assortment of particle concentrations (see Figure 3-113). The data indicates that these additives did not influence the concentrations. Antioxidant 2 was only present in two formulations and both had high particle concentrations (2816 x10^8 and 3014 x10^8 per mL respectively). Needless to say, the formulation that did not include any preservatives or antioxidants had similar concentration distributions than the reference formulations. The preservatives and antioxidants which were present in the various formulations are described in Table 3-16 and 3-17.

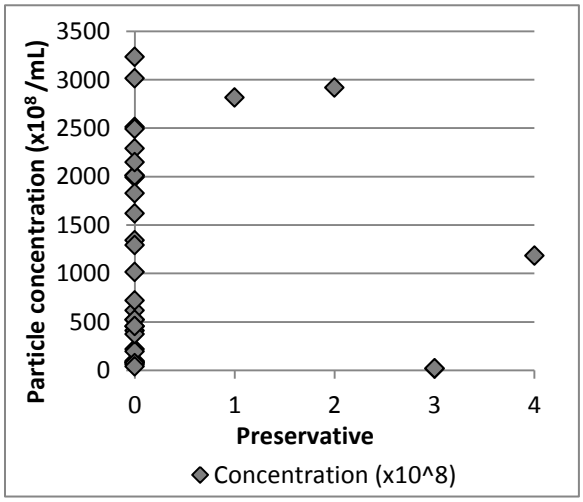

Figure 3-112:The particle concentration (per mL) of anti-infective pro-Pheroid formulation against the preservative used (obtained by CLSM analysis)

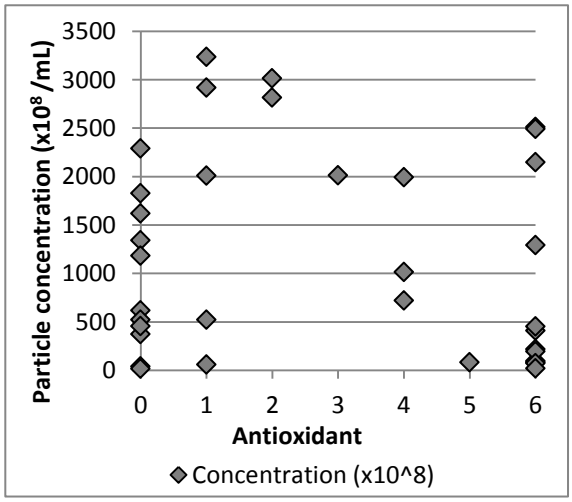

Figure 3-113:The particle concentration (per mL) of anti-infective pro-Pheroid formulation against the antioxidant used (obtained by CLSM analysis)

3.3.3.3.1.3 Other excipients

The ingredients of the various excipient combinations are described in Table 3-18. The formulations that made use of excipient combination 1 had concentrations below 500 x10^8 per mL whereas those that contained combination 2 had concentrations upward of 2000 x10^8 per mL (displayed in Figure 3-114). Combination 3 and 5 were present in formulations with widely distributed particle concentrations.

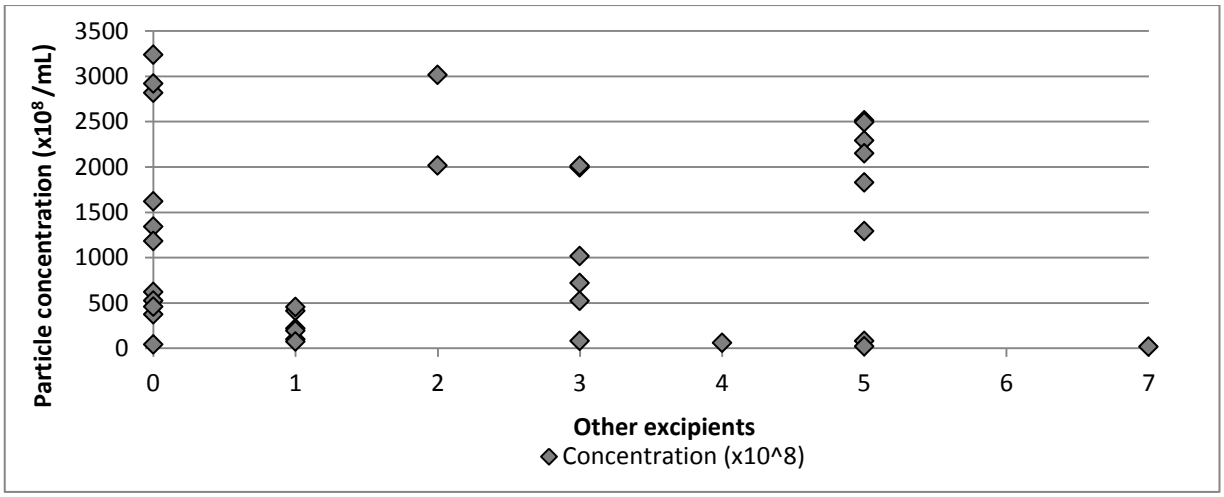

Figure 3-114:The particle concentration (per mL) of anti-infective pro-Pheroid formulation against the other excipients used (obtained by CLSM analysis)

3.3.3.3.1.4 Active ingredients

Both formulations that contained active ingredient 1 were highly concentrated (around 3000 x10^8 particles per mL). Active ingredient 6, 7, 8 and 11 were present in formulations that had a wide range of particle concentrations (refer to Figure 3-115). These active ingredients were therefore not likely to have had an influence on the particle concentration in the respective formulations. The formulations that contained active ingredient 9, 12 or 16 had particle concentrations that were grouped in a specific range. Refer to Table 3-19 for an outline of the active ingredients utilized in the respective anti-infective pro-Pheroid formulations.

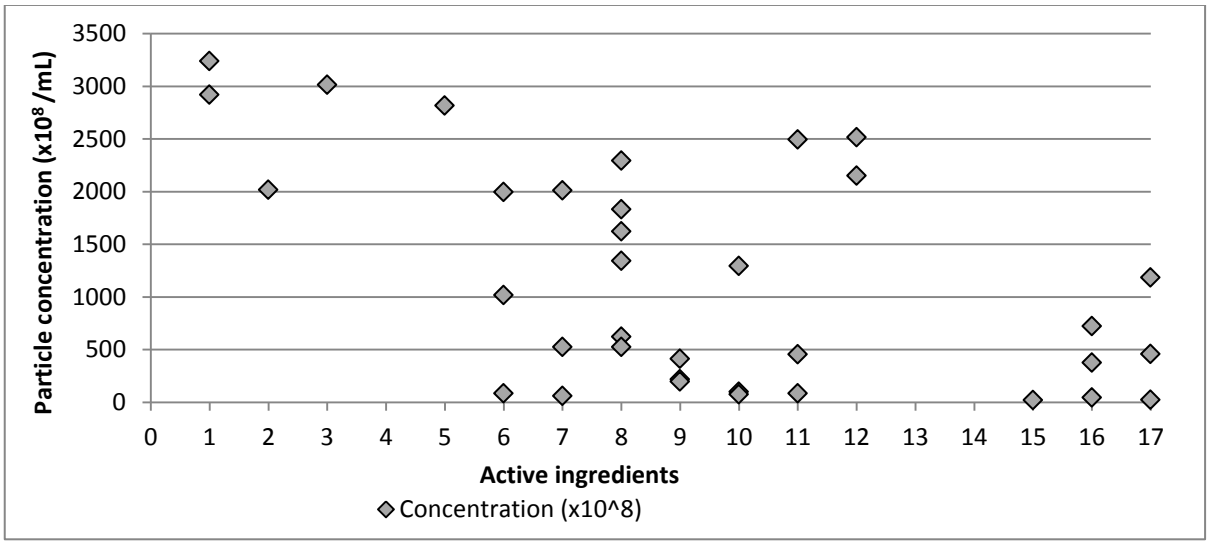

Figure 3-115:The particle concentration (per mL) of anti-infective pro-Pheroid formulation against the active ingredient used (obtained by CLSM analysis)

3.3.3.4 Zeta potential

3.3.3.4.1 Statistical description of reference formulations

The zeta potential for the reference formulations ranged between -15.3 and -50 mV and averaged -31.8 mV. The mean and median (-30.95 mV) values for the dataset were similar, which indicated a relatively symmetrical data distribution. No outlier or extreme values were found (see Figure 3-116).

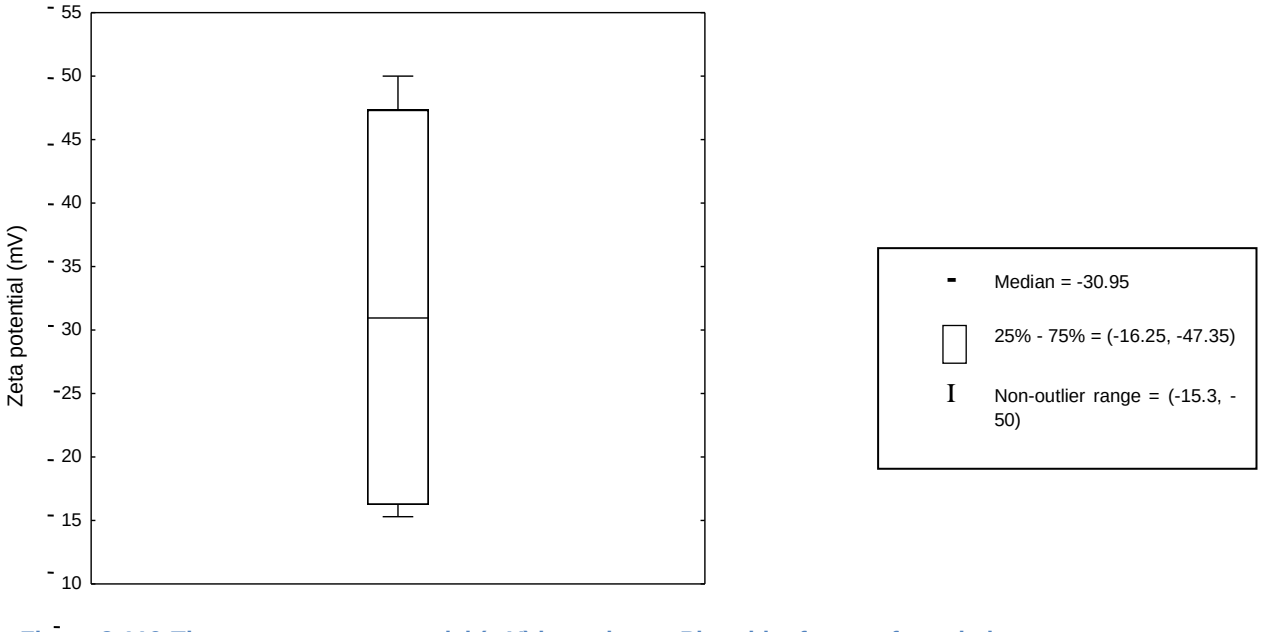

Figure 3-116:The average zeta potential (mV) in each pro–Pheroid reference formulation

3.3.3.4.2 Anti-infective formulations

3.3.3.4.2.1 Oil phase

Very little data was available on the stability of the reference and anti-infective pro-Pheroid formulations (four and eight measurements respectively). While a few reference values were widely spread (see Figure 3-117), that of the anti-infective formulations were clustered together between 0 and -10 mV (presented in Figure 3-118). The zeta potential of the anti-infective formulations signifies unstable formulations that are inclined to aggregate. The anti-infective pro-Pheroid formulations that had zeta potential reports available, only differed in active ingredient and other excipients present (in terms of ingredients).

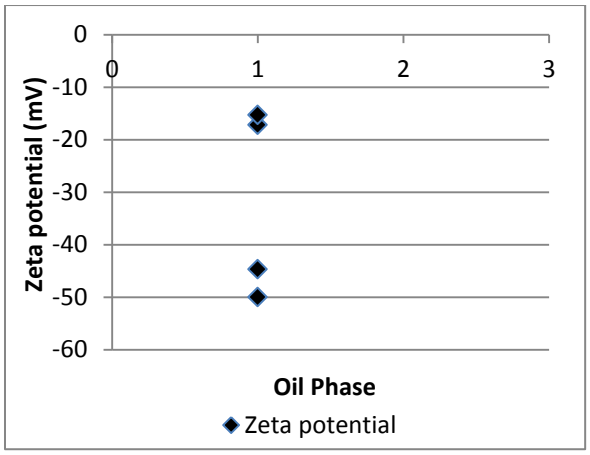

Figure 3-117:The different zeta potential measurements obtained against the oil phase used in the reference pro-Pheroid formulations (obtained by Zetasizer analysis)

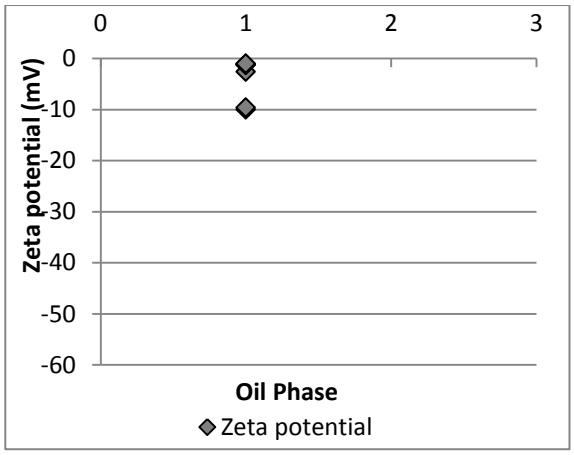

Figure 3-118:The different zeta potential measurements obtained against the oil phase used in the anti-infective pro-Pheroid formulations (obtained by Zetasizer analysis)

3.3.3.4.2.2 Preservatives and antioxidants

No preservatives were present in the formulations that had zeta potential available and could therefore not have influenced the measurements with regards to the reference values (see Figure 3-119). All eight formulations contained antioxidant 6 which comprised of BHA and BHT. Unstable zeta potential measurements between -1 and -10 mV were obtained; however, the reference formulations had values reaching -50 mV and were therefore much more stable (see Figure 3-120). Some formulations contained the same antioxidant and active ingredient but had ranging zeta potential measurements; thus, no significant conclusions could be drawn.

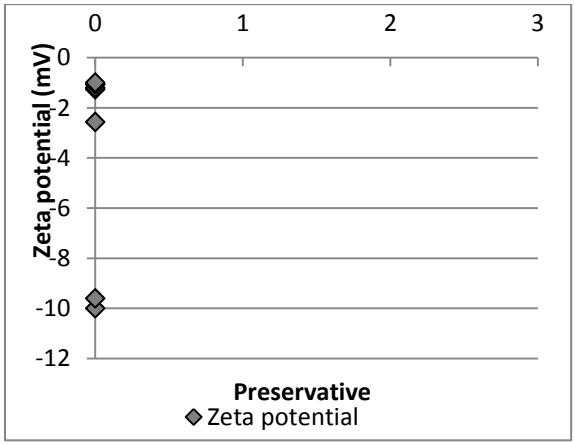

Figure 3-119:The different zeta potential measurements obtained against the preservative used in the anti-infective pro-Pheroid formulations (obtained by Zetasizer analysis)

Figure 3-120:The different zeta potential measurements obtained against the antioxidant used in the anti-infective pro-Pheroid formulations (obtained by Zetasizer analysis)

3.3.3.4.2.3 Other excipients

An outline of the other excipients and the active ingredients are given in Table 3-18 and 3-19. Only formulations that contained excipient combination 1 and 5 had zeta potential measurements available (presented in Figure 3-121). The two formulations that included

combination 1 had measurements that were clustered close to zero. Combination 5 was included in formulations that, at best, reached a zeta potential of -10 mV. These measurements indicate that the formulations were unstable and had a tendency to aggregate (from 0 to ± 25 mV). Excipient combination 5 was present in two formulations that contained active ingredient 11 and 12. With both active ingredients, the one formulation had a much better zeta potential measurement than the other, parallel formulation.

3.3.3.4.2.4 Active ingredients

Active ingredient 10 was present in three formulations which had zeta potential measurements that were grouped close to -1 mV (reflected in Figure 3-122). The formulation that included number 11 had two measurements that were close to -1 mV and one value around -10 mV. Number 12 was present in two formulations that differed by ~7 mV. The data indicates that all of these formulations were unstable.

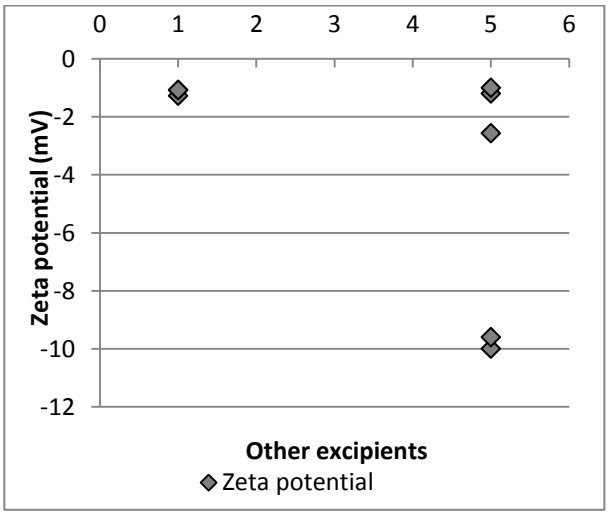

Figure 3-121: The different zeta potential measurements obtained against the other excipients used in the anti-infective pro-Pheroid formulations (obtained by Zetasizer analysis)

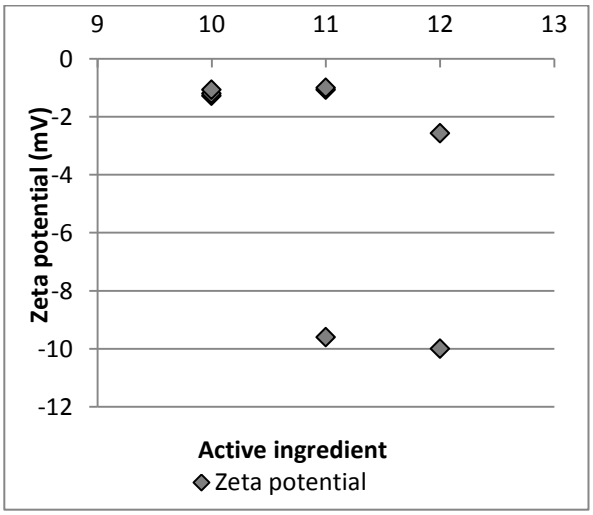

Figure 3-122: The different zeta potential measurements obtained against the active ingredient used in the anti-infective pro-Pheroid formulations (obtained by Zetasizer analysis)

3.3.3.5 Summary: pro-Pheroid

A concise summary of the results obtained through the statistical analysis of the pro-Pheroid formulations are presented in Table 3-20. Due to limited data available, definite conclusions could not be draw; however, it was observed that (refer to Table 3-20):

- the mastersize results were not likely influenced by the oil phase, possibly influenced by the antioxidants and preservatives and other excipients and most likely influenced by the active ingredients,

- the CLSM results were not likely influenced by the oil phase or preservatives, possibly influenced by the antioxidants and most likely influenced by the other excipients and the active ingredients. The antioxidants, preservatives and other excipients were not present in enough formulations to draw significant conclusions,

- the zeta potential results were not likely influenced by the oil phase and were most likely influenced by the antioxidants. The preservatives, other excipients and active ingredients were not present in enough formulations to derive concrete conclusions.

Table 3-20: Table summarizing the results from the pro-Pheroid statistical analysis

Pro-Pheroid				
	Mastersizer - particle size distribution	**CLSM - particle size**	**CLSM - particle concentration**	**Zeta Potential - stability**
Oil Phase	Results indicate that oil phase 1 (the norm) did not influence the particle size distribution. Extremely large particles were observed with formulations that contained only a high concentration of Kolliphor™ EL as oil phase (see Figures 3-93 and 3-94)	Results indicate that the oil phase did not likely influence the average particle size	Results indicate that variations in the oil phase could influence the particle concentration	Significant conclusions could not be drawn from the limited data
Preservatives	Significant conclusions could not be drawn from the limited data although inclinations in the particle size distributions were observed (see Figures 3-95 and 3-96)	Results indicate that the preservatives did not likely influence the average particle size	Significant conclusions could not be drawn from the limited data	No preservatives were present in the formulations and could thus not influence the stability in comparison to the reference formulations

Table 3-20: Table summarizing the results from the pro-Pheroid statistical analysis (continued)

	Mastersizer - particle size distribution	CLSM - particle size	CLSM - particle concentration	Zeta Potential - stability
Antioxidants	Significant conclusions could not be drawn from the limited data although tendencies were observed in the particle size distributions (see Figure 3-97 and 3-98)	Tendencies in the particle sizes were observed although significant conclusions could not be drawn from the limited data (see Figure 3-108)	Tendencies in the particle sizes were observed although significant conclusions could not be drawn from the limited data (see Figure 3-113)	All the formulations contained the same antioxidant combination and were less stable than the reference formulations (see Figure 3-120)
Other Excipients	Significant conclusions could not be drawn from the limited data although inclinations in the particle size distributions were observed (displayed in Figure 3-99 and 3-100)	Tendencies in the particle sizes were observed although significant conclusions could not be drawn from the limited data (see Figure 3-109)	Tendencies were observed with different 'other excipient' combinations (displayed in Figure 3-114)	Tendencies in the stability were observed although significant conclusions could not be drawn from the limited data (presented in Figure 3-121)
Active ingredient	Tendencies were observed with certain active ingredients (refer to Figure 3-101)	Tendencies were observed with certain active ingredients	Tendencies were observed with certain active ingredients (refer to Figure 3-115)	Significant conclusions could not be drawn from the limited data

3.3.4 Discussion: Comparative analysis of the variables with a focus on specific active ingredients

A comparative discussion of various formulations with corresponding active ingredients is given in the following section. This serves to give an overall idea of the effects that were encountered on the characterization results of the Pheroid formulations due to the variables that were present. Only selected formulations are discussed as certain actives were only present in one formulation and could therefore not be compared. Additionally, results from formulation characterization were not available for all the formulations since the instruments were not operational or available at all times or the results could not be obtained since it was not filed properly.

3.3.4.1 Anti-infective Pheroid vesicle formulations

Azithromycin (4.71%) was present in two formulations, V09017a and V09017b. Both had very few particles in the submicron and micron sized ranges (respective averages of 10.65% and

14.98% between the two formulations). For the most part, the particles were extremely large and did not fall within those ranges. The $d_{0.9}$ of a formulation indicates that 90.00% of the particles in the formulation are smaller than the stated value. V09017a had a $d_{0.9}$ of 120.3 µm while the $d_{0.9}$ of V09017b was 573 µm. The formulations had the exact same method of manufacture but the laser obscuration obtained during the Mastersize analysis differed. Obscuration is the degree to which the light from the laser beam is obscured from the particles being measured and should be between 10.00 and 20.00% to obtain reliable results. During the analysis of V09017a, the obscuration range was adhered to (14.77%) whereas an obscuration of 24.95% was obtained with V09017b. Particles can encounter secondary refraction if the obscuration is above 20.00% because there are too many particles present. This is most likely why formulation V09017b had a much higher $d_{0.9}$ than V09017a. The two formulations contained the same oil and water phases and did not contain any antioxidants or preservatives. The only physical difference between the formulations was the amount of other excipients that were added. 0.20% PEG 400 was added to V09017a while V09017b contained 0.40%. According to the confocal report, the formulations did not have particles that were larger than 5 µm in size and had average particle sizes of 2490 and 1363 nm respectively. During the confocal analysis, Nile red is dissolved in DMSO which would not likely cause crystals. V09017a and V09017b had particle concentrations (730 $x10^8$ and 630 $x10^8$ per mL respectively) that were similar to the average particle concentration for the anti-infective vesicle formulations (738.3 $x10^8$ /mL). The fact that the CLSM report stated that V09017a contained more particles which were on average larger than V09017b could only be attributed to the difference in excipient concentration between the two formulations since no other variables were present. No zeta potential measurements could be obtained for either formulation.

Formulation V10004 and V10023 contained 0.50% mefloquine. The formulations contained the same oil and water phases and no additives were added to either formulation. The same parameters were applied in the manufacture of both formulations; however, the mefloquine was combined at different stages of the manufacturing processes. In formulation V10004, the active was combined with $N_2O•H_2O$, added to the prepared oil phase and then homogenized. To manufacture formulation V10023 the mefloquine was mixed with one of the oil phase ingredients, combined with the rest of the oil phase, added to the $N_2O•H_2O$ and homogenized. 100.00% of the particles in V10023 were submicron sized whereas V10004 contained 87.41% and 5.09% particles in the submicron and micron sized range respectively. Unfortunately, no zeta potential measurements were available for either formulation and only V10023 had a confocal report so the data could not be compared. Another formulation, V09004, contained 0.10% mefloquine and adhered to the same manufacturing parameters as the former formulations. The method of manufacture was similar to that of formulation V10023 but the active was added to another ingredient in the oil phase before the rest of the components were

combined (as described for V10023). Like formulation V10004 and V10023, V09004 did not contain any other additives and were made with the same oil and water phases. The latter formulation comprised of 76.28% submicron and 13.15% micron sized particles. Confocal microscopy revealed that, on average, its particles were larger than formulation V10023's particles (499 nm against 320 nm). Additionally, it contained more particles (532 $x10^8$) per mL than V10023 (117 $x10^8$). This incidence (more, larger particles) would, in theory, be a result of a larger oil phase contained in formulation V09004 (compared to V10023) although this was not the case. The latter could therefore also indicate the presence of crystals or non-Pheroid particles, human error or that the characterization equipment might have required maintenance at the time. The results signify that the manufacturing process might have a major impact on the solubility of an active ingredient and subsequently influence the formulation characteristics.

Formulation V08028 contained 0.077% artesunate while ~0.03% less was added to formulation V09008. The formulations were equal in oil and water phases as well as the lack of any other additives. During the manufacture of the two formulations, the actives were added to different oil phase ingredients before all the ingredients were combined and homogenization followed. The formulations were manufactured under the exact same parameters. During the particle size analysis, V08028 had a laser obscuration of 41.28% which signify that the particle size distribution results were unreliable although the values did not differ much from those obtained with V09008 (obscuration of 19.12%). The confocal report indicated that V09008 contained more particles which were overall larger than that of V08028. Since the particles are formed by the oil content but V09008 had the same amount of oil as V08028, the particles that were observed could have been crystals and not Pheroid. Thus, the results were most likely influenced by solubility issues due to the difference in manufacturing methods. It is possible that the 0.03% difference in active ingredient played a role as well.

Quinine (1.25%) was present in three formulations, V09016, V09025, and V09031. The first two formulations were very similar and only differed with regards to the stage when the active was combined. In V09016 the active was added to $N_2O{\bullet}H_2O$ and in V09025 the active was combined with some of the oil phase ingredients. In formulation V09031, the active was added to the solvent (glacial acetic acid) before it was combined with the oil phase. The rest of the manufacturing methods were parallel. During the CLSM analysis, crystals were observed in formulation V09016 and V09025. The different manufacturing techniques may have caused the former formulation to have larger crystals than the latter. No crystals were observed in formulation V09031 and were most likely due to the solvent that was used. V09016 formed the central of the three formulations in terms of particle concentration and average particle size values. V09025 had less concentrated, large particles, while V09031 had much smaller particles than both formulations but contained only a few more particles per mL than V09025. No zeta

potential measurements were available for any of the formulations. The results obtained from these formulations highlight the influence of solubility on the formulation characterization yet again.

Formulations V09036 and V09042 contained 0.046% and 0.03% amodiaquine respectively and were manufactured exactly the same. No preservatives, antioxidants or other excipients were used. For both formulations, more than 40.00% of the particles were in the submicron size range. During the Mastersize analysis, a laser obscuration of 27.27% was obtained for V09036 which hampered the credibility of its particle size distribution; as a result, the values were not compared. The average particle size of V09042 was double that of V09036 and much fewer particles were present (412 $x10^8$ set against 1892 $x10^8$). Although the formulations were practically equivalent, the confocal results differed extensively. The latter could be a result of the 0.016% difference in active ingredients or human error during manufacturing or characterization. Formulation V11038 and V11039 (0.046% amodiaquine each) was similar to the previous two formulations but had BHA (0.01%) and BHT (0.01%) in addition. The antioxidants were added to some of the oil phase ingredients, but the general outline of the manufacturing method was the same as with formulation V09036 and V09042. The formulations that included the antioxidants had mostly particles in the micron sized range whereas the other two formulations had more submicron sized particles. During the manufacturing process, the oil phase of formulation V11038 was accidentally heated 20°C higher than with formulation V11039. This may have caused formulation V11038 to have larger, less concentrated particles (1321 nm, 365 $x10^8$ particles per mL) than V11039 (417 nm, 722 $x10^8$ particles per mL). Additionally, V11038 was less stable than the other formulation (-30.2 mV compared to -50.23 mV). The Mastersize report suggests that the addition of the antioxidants resulted in a higher percentage micron sized particles. The confocal results were too diverse to draw any significant conclusions.

Two parallel formulations were manufactured with doxycycline (2.56%). Their particle sizes and particle size distributions were in line, but contradictory data were obtained by the confocal analysis. V09040 and V09048 had average particle sizes of 782 and 1987 nm respectively. Moreover, V09040 had ten times more particles per mL than V09048 (983 $x10^8$ compared to 98 $x10^8$ /mL). However, CLSM is suited for qualitative analysis and enables, at most, a semi-quantitative analysis (Testa, 2001:112).

Formulation V09002 and V09003 were similar formulations that contained rifampicin (1.00%) and isoniazid (0.50%) as active ingredients. However, they contained different combinations of other excipients (number 3 and 4 respectively, see Table 3-4). The formulation that contained excipient combination 3 had smaller, more concentrated particles whereas the opposite was

seen in the formulations that contained combination 4. No Mastersize or stability data were available.

Formulation V13013 contained 0.10% doxycycline while formulation V13045 contained 0.10% of the hyclate salt form of doxycycline. The other ingredients and the manufacturing method were the same. The $d_{0.9}$ of V13013 was 205.75 µm in comparison with a value of 2.774 µm for V13045. Since no other variables were present and the laser obscuration was in the proposed ranges during the analysis, the increased particle sized had to be caused by the different chemical compositions of the active ingredients which subsequently influenced the solubility of the active ingredients. The zeta potential measurements and CLSM results were not available for analysis.

3.3.4.2 Anti-infective pro-Pheroid formulations

Two nevirapine (0.024%) formulations, P09002 and P09003, were analysed; the former formulation contained a preservative (0.03% butylparaben). Both had 100.00% submicron sized particles but the measurements took place at a laser obscuration below 1.70% and were therefore not reliable values. Both formulations contained round about 3000 $x10^8$ particles per mL. P09003 had slightly larger particles on average compared to the other formulation (432 and 397 nm respectively). The reduced particle concentration and sizes observed in P09002 was likely due to the addition of the preservative.

P09016 and P10004 contained the same active ingredients, (rifampicin and pyrazinamide), excipients and antioxidants although the latter formulation contained considerably more BHA and BHT. P09016 comprised of 100.00% submicron sized particles whereas the other formulation contained 63.42%. Moreover, P09016 had a much higher particle concentration than P10004 (1994 $x10^8$ and 82 $x10^8$ particles per mL respectively) which is to be expected since particle size should be inversely proportional to the amount of particles. The average particle sizes of the two formulations did not vary by much. The particle sizes were most likely influenced by the antioxidant quantities, the sequence in which the ingredients were combined (i.e. solubility), or both.

Two identical formulations which contained isoniazid and ethambutol had similar average particle sizes, but significantly different particle concentrations. P09015 contained 523 $x10^8$ particles per mL in comparison with P09019 that had 2011 $x10^8$. Both formulations comprised 100.00% of submicron sized particles although the measurements were carried out at a laser obscuration of ~5%. P10003 was made with the same active ingredients and oil phase as the latter formulations but 2.00% beeswax was used as an excipient instead of 4.00% PEG 400. The formulation was also manufactured in a different order than the others. The formulation resulted in much larger particles than the similar formulations and contained only 8.22%

submicron sized particles. Only 60 $x10^8$ particles were present per mL. The difference in particle size and concentration is most probably because of its composition, beeswax would increase the oil phase content whereas PEG 400 would not. Another formulation, P09005, also contained isoniazid and ethambutol. Less BHA and BHT were added compared to the previous formulations and an additional antioxidant, ascorbyl palmitate (2.70%) was utilized. Furthermore, less PEG 400 was added (2.70%), but the same procedure was followed as P09015 and P09019 to manufacture the formulation (beeswax was not added). It had 89.13% submicron and 0.00% micron sized particles (and 10.89% particles above 5 µm in size), 2015 $x10^8$ particles were present per mL and the particles were generally 827 nm in size.

Formulation P11018 and P12007 were made with artemisone (0.04% and 0.02% respectively). Both were manufactured in the same manner and included the same ingredients. Both had the greatest fraction of particles in the micron sized range although this was only 6.26 and 10.42% in turn. The formulations contained between 70 and 95 $x10^8$ particles per mL but P11018 displayed larger particles (2311 nm) on average than the other formulation (542 nm). The former two formulations contained 19.42% PEG 400 whereas formulation P12012 (0.02% artemisone) only utilized 5.00%. The rest of the conditions were parallel. However, 100.00% of its particles fell within the submicron range. It had a significantly higher particle concentration than the previous formulations (1292 $x10^8$ /mL) and a mean particle size of 284 nm. The results signify that the lower percentage of PEG 400 used in P12012 was more suitable for the formulation and that the high PEG 400 concentration resulted in lesser and larger particles within the formulations (i.e. characterization results were influenced by solubility). All three formulations had unstable zeta potential measurements that ranged between -1.07 and -1.27 mV and indicates a tendency to aggregation (i.e. larger particles). The Mastersize and zeta potential measurements were carried out on the same days (between 3 and 5 days after the formulations were manufactured).

Amodiaquine was present in two pro-Pheroid formulations, P11019 and P11026, in a concentration of 0.046 and 24.9% in turn. Whilst P11019 had 6.69% particles in the submicron size range and 22.95 in the micron size range, P11026 comprised completely of submicron sized particles. Both formulations were unstable and had zeta potential measurements close to -10mV. P11019 had 82 $x10^8$ particles per mL against a particle concentration of 2150 $x10^8$ per mL obtained by the other formulation. The average particle size found in P11019 (3984 nm) differed significantly from the value observed in P11026 (292 nm). The only difference in the production of the formulations was the amount of active ingredient. In this case, the increased amount of active ingredient seemed to be favourable to the formulation's characteristics.

3.3.4.3 Anti-infective Pheroid micro-sponge formulations

Formulation S08008 and S09006 were the same in all aspects except for the fact that the active ingredient, mefloquine was added at different stages of the production. S09006 had significantly more submicron sized particles (68.14%) than the other formulation (14.80%). Since Incromega E7010 and E3322 were present in the same concentrations in both formulations, the particle size variance was most likely caused by the different sequences in which the ingredients were added. The ingredients and manufacturing method of S08022 were analogous to that of S09006 but contained less mefloquine. The average particle size was 2300 nm whereas S09006 had a mean particle size of 909 nm in addition to being more concentrated (1083 $x10^8$ against 70 $x10^8$ particles per mL). S09006 did not have a Mastersize analysis report to serve as comparison.

Clofazimine (5 mg/mL) was present in three formulations that comprised of the same oil and water phases, but their excipients differed. With formulation S12005, the active was first combined with the oil phase ingredients and then mixed with the other ingredients in the general order. For the manufacture of S12007, the active was first dissolved in DCM while PEG 400 was used to dissolve the active in formulation S12014. Next, the rest of the ingredients were combined in the same manner as the S12005. The excipients comprised of Incromega E7010, Incromega E3322, and small quantities of the solvents. The Incromegas (0.25%) were present in S12014 and S12007 while S12005 contained 0.20%. S12007 was the only formulation that had more submicron than micron sized particles. S12014, with a zeta potential measurement of -30.23 mV, was much more stable than S12005 and S12007 with values of -19.07 and -9.37 mV respectively. The latter two formulations had particle concentrations and average particle sized that were alike. No confocal data was available for formulation S12014. The formulation that contained PEG 400 was the most stable formulation. The formulation that contained DCM had the smallest particles according to the Mastersize analysis but was very unstable.

Artemether was present in formulation S09007 and S09002 (0.04 and 0.07% respectively). The method of preparation and the rest of the ingredients were the same. The formulation with the lowest amount of active had the highest particle concentration and had larger mean particle size. The latter incident could only be attributed to an erroneous report since the values are inversely proportional to each other. The Mastersize analysis report and the zeta potential measurements could not be acquired.

3.3.4.4 Summary: Comparative analysis of the variables with a focus on specific active ingredients

- differences in results obtained from parallel formulations was most likely indicative of variables in manufacture process (e.g. solubility),

- formulations that were equivalent in ingredients but differed with regards to the mixing stages usually had markedly different analysis results,
- depending on the excipient(s) and the percentage(s) that were present in the formulations, certain tendencies were observed,
- solubility had a substantial influence on the analysis results,
- different analysis results were observed in formulations that had similar ingredients which differed only in the chemical composition of a single ingredient,
- the laser obscuration should be kept between 10.00 and 20.00% during mastersize analysis to obtain reliable results,
- some results that were obtained could be attributed to human error, therefore, detailed SOP's should be in place and should be adhered to by all applicable personnel, and
- CLSM is suited for qualitative analysis and enables, at most, a semi-quantitative analysis.

3.3.5 Conclusion: Statistical analysis of data

The oil and water phases did not have a detectable influence on the data. This is mainly because the standard oil and water phases are applied to the practically all the formulations. The preservatives, antioxidants, other excipients, and/or active ingredients appeared to have influenced the characterization data of several formulations. Although no definitive observations could be made due to insufficient data.

The method of manufacturing (i.e. order in which the ingredients are mixed, the stage of the manufacturing in which the active is added and parameters such as temperature) had a substantial effect on the particle sizes, confocal reports and the stability of the formulations. This emphasizes the effect that solubility has on the formulation characteristics since it forms the basis of formulation production.

With regards to the Mastersize analysis, it became clear during the study that the laser obscuration did not always range between 10.00% and 20.00% and might influence the particle size distribution report. In future analyses, care should be taken to abide by the stated range to ensure that reliable results are obtained. CLSM analysis is semi-quantitative and should rather be utilized to analyse the qualitative properties (e.g. internal structure of the particles) as opposed to the particle sizes and concentration. (Testa, 2001:112).

It should however be noted that, some formulations were identical, prepared by the same person and in the same time slot and still had radically different characterization results which could be attributed to variations in solubility (i.e. mixing the one formulation more thoroughly than it's equal, thus resulting in formulations with different characteristics). In fact, solubility most likely had an impact on the majority of the incidents that were discussed in this chapter. Therefore, during every stage of formulation, from the research to the characterization thereof

and especially while manufacturing, tremendous focus should be on the solubility aspects. This would likely reduce the need for more extreme measures to ensure the desired outcome.

In conclusion to this chapter: it is of grave importance that future studies are documented with the concomitant characterization reports so that this study can be recommenced with success at a later stage in time. Furthermore, it is essential that a wide range of formulations are manufactured, one variable at a time, and with various characteristics so that the gaps in the dataset can be filled because due to insufficient data and the erratic behaviour observed in numerous formulations the aim of establishing standardized specifications, was hampered.

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CHAPTER 4 FORMULATION AND EVALUATION OF A PHEROID–BASED AZOXYSTROBIN FORMULATION

4.1 Introduction

This chapter embodies an overview of the experimental procedures followed to prepare a Pheroid-incorporated azoxystrobin formulation as well as the design of a field study in the form of trial plot studies (the ®–symbol indicating a registered trademark for the Pheroid® is omitted for ease of reading).

4.2 Feasibility evaluation of formalized specifications

The results obtained from the data processing and the statistical analysis thereof was intended to provide insight on the manufacture of the azoxystrobin/pro-Pheroid formulation. In other words, it was envisioned that the formulations would be based on conclusions drawn from examining the impact that variations in the formulation parameters had on previous formulation outcomes. However, the statistical data analysis did not result in the formalization of Pheroid specification ranges - primarily due to insufficient data. Subsequently, the azoxystrobin/pro-Pheroid formulation could not be manufactured according to described ranges as aimed. Nevertheless, it was decided to continue with the formulation despite the fact that it could not be applied as a source of verification for the specifications.

4.3 Establishment of an azoxystrobin/pro-Pheroid formulation

The analysis performed during the preceding studies highlighted the importance of the solubility of active ingredients in formulations of Pheroid. Therefore, in order to establish which ingredients to include in the formulation, solubility studies were carried out. The experimental study was aimed at effectively delivering a 25.00% ($^{w}/_{w}$) azoxystrobin/pro-Pheroid formulation for comparison against the commercial product, Amistar 250 SC in relatively fatty safe solvents. During solubility studies, however, it proved difficult to deliver such a high concentration due to crystallization of the formulation concentrates or precipitation upon dilution with water (routinely done in the field prior to application on crops). Ultimately, it was decided to manufacture the formulation at a lower strength and adapt the dilution factor accordingly to deliver a strength parallel to that of the 25.00% formulation after dilution. Eventually, two slightly different formulations (in terms of ingredients) were manufactured with concentrations of 6.25% and 12.50% ($^{w}/_{w}$) respectively. To promote ease of reading, all trademark symbols (e.g. Kolliphor™) are omitted.

4.3.1 Solubility studies

Former solubility studies carried out at the DST/NWU PCDDP indicated that azoxystrobin did not dissolve sufficiently in H_2O, Pheroid, propylene glycol, ethanol, vitamin F ethyl ester, Kolliphor EL, HCl, NH_4OH, PEG 400 or Tween 80 (unpublished results, D. Wilken). The latter tests, performed by PCDDP researchers, were carried out in accordance with the necessary SOP's and GLP measures in place and were therefore not repeated during this study.

The solvents that were utilized could not be toxic or harmful to the environment since the formulation was intended for agricultural application. Furthermore, the solvents and excipients had to be economically viable (especially in comparison to the comparator).

Literature indicates that azoxystrobin has the highest solubility in organic solvents and studies were initiated accordingly (MacBean, 2010). The most effective azoxystrobin solvent, DCM, has a solubility of 400mg/L at 20°C (Paranjape *et al.*, 2014:38). The density of DCM is 1.325g/mL (Bisen & Sharma, 2012:293), meaning that 0.4g active can be dissolved in 1325g solvent (i.e. 0.03% ($^{w}/_{w}$) formulation).

The crux of this study was the incorporation of Pheroid technology in an azoxystrobin formulation with a targeted strength of 25.00% ($^{w}/_{w}$). Consequently, several of the solubility studies were carried out with a combination of solvents with the aim of obtaining increased solubility. Nevertheless, to observe the effect of the individual solvents on the formulation, the solubility studies also generally included formulations containing a single solvent. While numerous solubility experiments were carried out during the study, only a selected few are presented in the following pages due to space constraints. All formulations were kept at laboratory room temperature.

A summary of the first solubility tests is given in Table 4-1. Efforts were made to dissolve the active in several solvents (Pheroid oil phase, Dimethyl sulfoxide (DMSO), acetonitrile (ACN), DCM, benzyl alcohol (BnOH), and combinations thereof). The preparation of the pro-Pheroid (i.e. Pheroid oil phase) based on the method described by Grobler (2009), and was altered as required.

Table 4-1: Azoxystrobin solubility studies with organic solvents

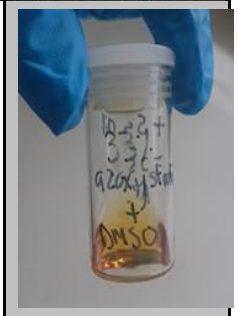
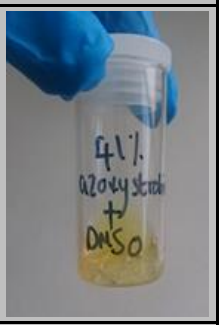
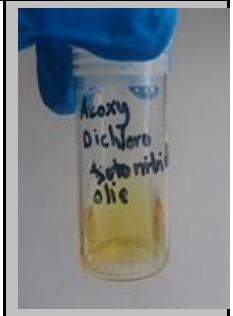
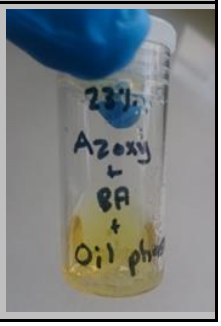
Solvent	Azoxystrobin concentration	Method	Outcome	
Vitamin F Ethyl Esther and Kolliphor RH40 (ratio 30:70)	25.00%($^{w}/_{w}$)	1. Weigh off compound and add to solvent. 2. Vortex for a few seconds. 3. Heat until boiling point or dissolved.	The active ingredient was dissolved but crystal formation started within 72 hours	
DMSO	33.00%($^{w}/_{w}$)		Dissolved	
DMSO	41.00%($^{w}/_{w}$)		Unsuccessful; crystallization after 24-48h	
DMSO + H_2O	25.00%($^{w}/_{w}$)		Unsuccessful; crystallization after 24-48h	
DMSO + Tween 80	26.00%($^{w}/_{w}$)		Unsuccessful; crystallization after 24-48h	

Table 4-1 Azoxystrobin solubility studies with organic solvents (continued)

Solvent	Azoxystrobin concentration % ($^{w}/_{w}$)	Method	Outcome	
ACN	25.00%($^{w}/_{w}$)	1. Weigh off compound and add to solvent. 2. Vortex for a few seconds. 3. Heat until boiling point or dissolved.	Unsuccessful; crystallization after 24-48h	
DCM + ACN	25.00%($^{w}/_{w}$)		Unsuccessful; crystallization after 24-48h	
DCM	25.00%($^{w}/_{w}$)		Did not dissolve	
DCM + DMSO	25.00%($^{w}/_{w}$)		Unsuccessful; crystallization after 24-48h	
BnOH/pro-Pheroid	23.00%($^{w}/_{w}$)		Unsuccessful; crystallization after 24-48h	

The formulations presented in Table 4-1 were mainly unsuccessful. Either the active did not dissolve or crystal formation was noted within 48 hours. Only one formulation (with DMSO as solvent) remained stable after 36 hours. A larger volume of the formulation was manufactured and gassed, but it was immediately disregarded upon dilution with water (for application on crops) as precipitation occurred (see Figure 4-1). Shaking did not improve the results.

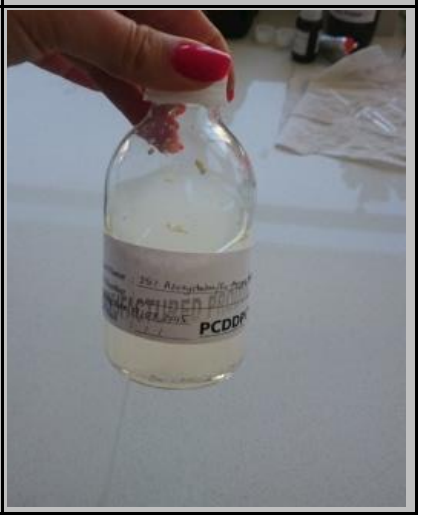
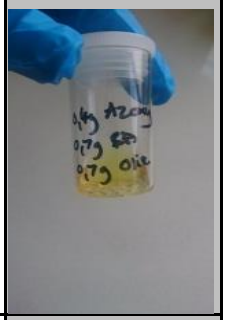
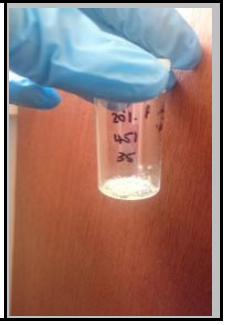
Directly after manufacture	Following dilution with ddH_2O (1:300)
	

Figure 4-1: Azoxystrobin/pro-Pheroid formulation with DMSO as solvent

During the next round of solubility studies, the ratio of the Pheroid components as well as the amount of pro-Pheroid and BnOH was varied and the amount of active ingredient was slightly reduced (see Table 4-2). BnOH was used because of its promising results in the azoxystrobin solubility studies (during initial studies; results not shown) as well as its success in similar formulations. The rationale behind the variations in the formulations was to avoid crystal formation. Unfortunately, this could not be achieved at a concentration of 20.00% - 22.00%.

Table 4-2: Incorporation of different ratios of pro-Pheroid components with BnOH as solvent

Solvent	Azoxystrobin concentration % ($^{w}/_{w}$)	Method	Outcome	
50% BnOH 30% oil phase [Vitamin F Ethyl Esther and Kolliphor RH40 (ratio 40:60)]	20.00%($^{w}/_{w}$)	1. Weigh off compound and add to solvent. 2. Vortex for a few seconds. 3. Heat until boiling point or dissolved.	Unsuccessful; crystallization after 24-48h	
40% BnOH 40% oil phase [Vitamin F Ethyl Esther and Kolliphor RH40 (ratio 30:70)]	20.00%($^{w}/_{w}$)		Unsuccessful; crystallization after 24-48h	
45% BnOH 35% oil phase [Vitamin F Ethyl Esther and Kolliphor RH40 (ratio 40:60)]	22.00%($^{w}/_{w}$)		Unsuccessful; crystallization after 24-48h	
45% BnOH 35% oil phase [Vitamin F Ethyl Esther and Kolliphor RH40 (ratio 70:30)]	20.00%($^{w}/_{w}$)		Unsuccessful; crystallization after 24-48h	
45% BnOH 35% oil phase [Vitamin F Ethyl Esther and Kolliphor RH40 (ratio 60:40)]	20.00%($^{w}/_{w}$)		Unsuccessful; crystallization after 24-48h	

Promising results were obtained during solubility studies with BnOH, ACN, DCM, DMSO and combinations thereof (see Table 4-3). After 5 days, no crystals were present in the formulations and they were subsequently diluted with water (at a ratio of 1:300) to form solutions. All of the solutions precipitated within minutes. Some remixed completely whereas others remixed only partially; however, this was insignificant because of the rapid precipitation rates. Therefore, these formulations produced unstable solutions and were not fit for agricultural application. It was hypothesized that the lack of crystal formation in the concentrates was largely due to the significantly lessened amount of active ingredient in the formulation (in comparison to the formulations in Table 4-1 where similar solvents were used). Thus, succeeding formulations were intentionally less concentrated at first (i.e. less than 25.00% $^{w}/_{w}$) and then incremented until crystallization occurred.

Table 4-3: Azoxystrobin solubility studies with BnOH, ACN, DCM, DMSO, and combinations thereof as solvents

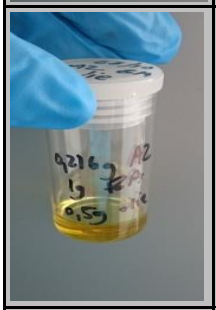
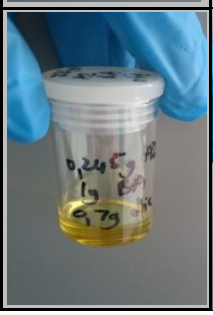
Formula	Concentration % $(^{w}/_{w})$	Method	Outcome		
			After 5 days	After 1:150 dilution with distilled H_2O	
Azoxystrobin	12.70%	1. Weigh off compound and add to solvent. 2. Stir for a few seconds. 3. Heat until compound is dissolved. 4. Add oil phase. 5. Stir for a few seconds. 6. Heat for a few seconds.			Suspension formed; precipitated after a few minutes; remixed well
BnOH	43.70%				
Oil Phase	43.70%				
Azoxystrobin	12.60%				Suspension formed; precipitated after a few minutes; remixed reasonably well
BnOH	51.30%				
Oil Phase	36%				
Azoxystrobin	12.60%				Suspension formed; precipitated after a few minutes; remixed reasonably well
BnOH	58.30%				
Oil Phase	29.10%				

Table 4-3: Azoxystrobin solubility studies with BnOH, ACN, DCM, DMSO, and combinations thereof as solvents (continued)

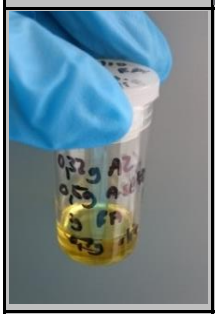
Formula	Concentration % $(^{w}/_{w})$	Method	Outcome		
			After 5 days	After 1:150 dilution with distilled H_2O	
Azoxystrobin	12.60%	1. Weigh off compound and add to BnOH. 2. Stir for a few seconds. 3. Heat until compound is dissolved. 4. Add ACN. 5. Heat for a few seconds. 6. Add oil phase. 7. Stir for a few seconds. 8. Heat for a few seconds.			Suspension formed; precipitated after a few minutes; remixed reasonably well
BnOH	35.00%				
ACN	17.50%				
Oil Phase	35.00%				
Azoxystrobin	11.40%				Suspension formed; precipitated after a few minutes; remixed reasonably well
BnOH	35.50%				
ACN	17.70%				
Oil Phase	35.50%				
Azoxystrobin	12.70%				Suspension formed; precipitated after a few minutes; remixed better than previous two formulations
BnOH	43.70%				
ACN	21.80%				
Oil Phase	21.80%				

Table 4-3: Azoxystrobin solubility studies with BnOH, ACN, DCM, DMSO, and combinations thereof as solvents (continued)

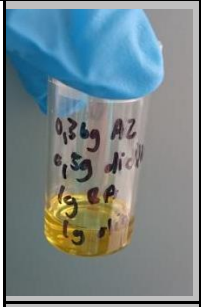
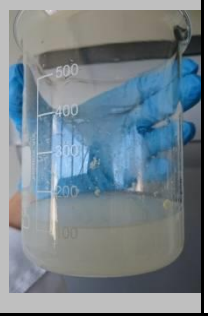
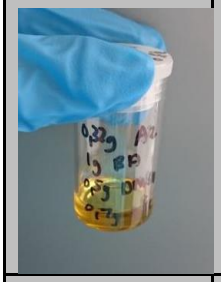
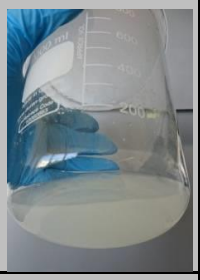
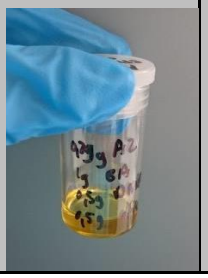
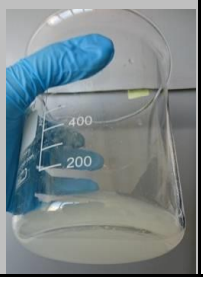
Formula	Concentration % ($^{w}/_{w}$)	Method	Outcome		
			After 5 days	After 1:150 dilution with distilled H_2O	
Azoxystrobin	12.60%	1. Weigh off compound and add to BnOH. 2. Stir for a few seconds. 3. Heat until compound is dissolved. 4. Add DCM. 5. Heat for a few seconds. 6. Add oil phase. 7. Stir for a few seconds. 8. Heat for a few seconds.			Suspension formed; precipitated after a few minutes; remixed well
BnOH	17.50%				
DCM	35.00%				
Oil Phase	35.00%				
Azoxystrobin	12.70%				Suspension formed; precipitated after a few minutes; remixed reasonably well
BnOH	39.70%				
DCM	19.80%				
Oil Phase	27.80%				
Azoxystrobin	12.70%				Suspension formed; precipitated after a few minutes; remixed reasonably well
BnOH	43.70%				
DCM	21.80%				
Oil Phase	21.80%				

Table 4-3: Azoxystrobin solubility studies with BnOH, ACN, DCM, DMSO, and combinations thereof as solvents (continued)

Formula	Concentration % $^{(w}/_{w)}$	Method	Outcome		
			After 5 days	After 1:150 dilution with distilled H_2O	
Azoxystrobin	12.60%	1. Weigh off compound and add to BnOH. 2. Stir for a few seconds. 3. Heat until compound is dissolved. 4. Add DMSO. 5. Heat for a few seconds. 6. Add oil phase. 7. Stir for a few seconds. 8. Heat for a few seconds.			Suspension formed; precipitated within a few minutes (quickly); remixed well
BnOH	17.50%				
DMSO	35.00%				
Oil Phase	35.00%				
Azoxystrobin	12.70%				Suspension formed; precipitated within a few minutes (quickly); remixed well
BnOH	39.70%				
DMSO	19.80%				
Oil Phase	27.80%				
Azoxystrobin	12.70%				Suspension formed; precipitated within a few minutes (quickly); remixed reasonably well
BnOH	43.70%				
DMSO	21.80%				
Oil Phase	21.80%				

Since all the previous solubility studies were carried out to no avail, tetrahydrofurfuryl alcohol (THFA) was obtained. The latter, although well known in the agricultural industry (Tike & Mahajani, 2007), is uncommon to pharmaceutics and was never used in combination with Pheroid technology before. Because the reaction between the solvent and Pheroid was unknown, THFA was combined with BnOH, ACN, DCM or DMSO at first (familiar solvents with a history in Pheroid formulations). Only one formulation, comprising of BnOH and a high quantity THFA, formed crystals (12.60% formulation - refer to Table 4-4). The rest remained stable up until dilution when the same precipitation problems were experienced as in the preceding formulations (Table 4-3). Thereafter, THFA-alone was deemed fit to use in conjunction with the Pheroid system and formulations were prepared with varying amounts of active and THFA (see Table 4-5). All of the formulations were unsuccessful as crystals were present within 4 days.

Table 4-4: Azoxystrobin solubility studies with combinations of organic solvents and THFA

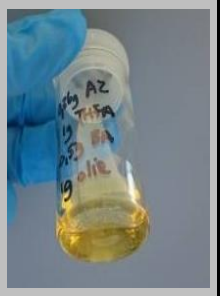
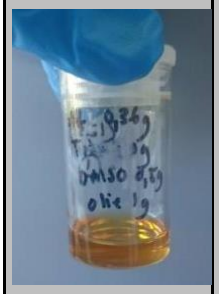
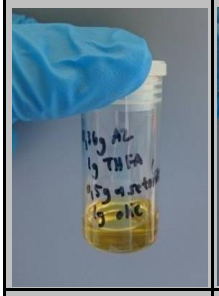
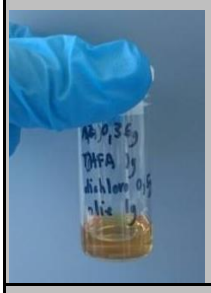
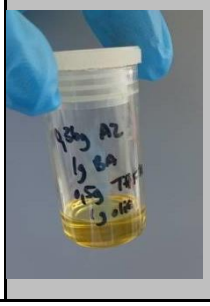
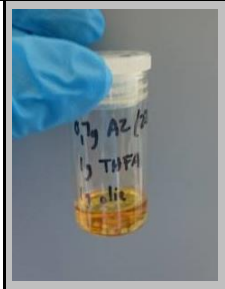
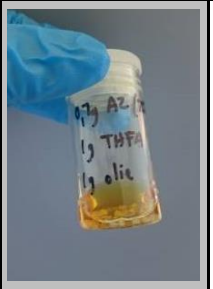
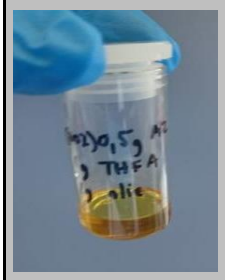
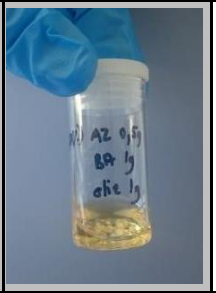
Formula	Concentration % $(^{w}/_{w})$	Method	Outcome		
			Immediately	After 4 days	
Azoxystrobin	12.60%	1. Weigh off compound and add to first solvent. 2. Stir for a few seconds. 3. Heat until compound is dissolved. 4. Add second solvent. 5. Stir for a few seconds. 6. Heat for a few seconds. 7. Add oil phase. 8. Stir for a few seconds. 9. Heat for a few seconds.			Unsuccessful; crystallization
THFA	35.00%				
BnOH	17.40%				
Oil Phase	35.00%				
Azoxystrobin	12.60%				Suspension formed; precipitated after a few minutes; remixed well
THFA	35.00%				
DMSO	17.40%				
Oil Phase	35.00%				
Azoxystrobin	12.60%				Suspension formed; precipitated after a few minutes; remixed reasonably well
THFA	35.00%				
ACN	17.40%				
Oil Phase	35.00%				
Azoxystrobin	12.60%				Suspension formed; precipitated after a few minutes; remixed well
THFA	35.00%				
DCM	17.40%				
Oil Phase	35.00%				
Azoxystrobin	12.60%				Suspension formed; precipitated after a few minutes; remixed reasonably well
BnOH	35.00%				
THFA	17.40%				
Oil Phase	35.00%				

Table 4-5: Azoxystrobin solubility studies with THFA as solvent

Formula	Concentration % ($^{w}/_{w}$)	Method	Outcome		
			Immediately	After 4 days	
Azoxystrobin	26.00%	1. Weigh off compound and add to solvent. 2. Stir for a few seconds. 3. Heat until compound is dissolved. 4. Add oil phase. 5. Stir for a few seconds. 6. Heat for a few seconds.			Unsuccessful; crystallization
THFA	37.00%				
Oil Phase	37.00%				
Azoxystrobin	20.00%				Unsuccessful; crystallization
THFA	40.00%				
Oil Phase	40.00%				
Azoxystrobin	15.60%				Unsuccessful; crystallization
THFA	42.20%				
Oil Phase	42.20%				
Azoxystrobin	13.00%				Unsuccessful; crystallization
THFA	43.50%				
Oil Phase	43.50%				

Futile efforts to deliver an effective azoxystrobin/pro-Pheroid formulation led to the obtainment of three additional variations of the raw material (i.e. three different azoxystrobin compounds). Solubility testing carried out on the initial raw material (from hereto referred to as azoxystrobin) were revisited on the newly acquired actives (further referred to as azoxystrobin 1, 2 and 3 respectively). Selected results are presented in Tables 4-6 to 4-12.

Table 4-6: Solubility studies carried out on azoxystrobin 1

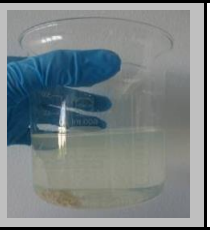
Formula	Concentration % ($^{w}/_{w}$)	Method	Outcome		
			Immediately	After 1:240 dilution with distilled H2O	
Azoxystrobin	20.00%	1. Weigh off compound and add to solvent. 2. Stir for a few seconds. 3. Heat until compound is dissolved. 4. Add oil phase. 5. Stir for a few seconds. 6. Heat for a few seconds.			Unsuccessful; precipitated; did not remix
DMSO	40.00%				
Oil Phase	40.00%				
Azoxystrobin	20.00%			Unsuccessful; crystallization	
BnOH	40.00%				
Oil Phase	40.00%				

Table 4-7: Solubility studies carried out on azoxystrobin 1

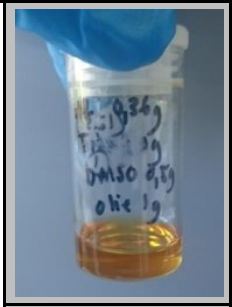
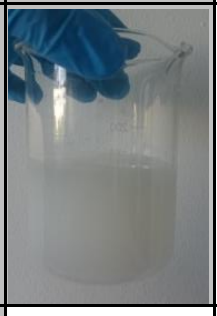
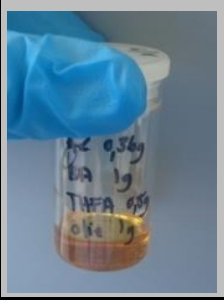
Formula	Concentration % ($^{w}/_{w}$)	Method	Outcome		
			Immediately	After 1:150 dilution with distilled H2O	
Azoxystrobin	12.60%	1. Weigh off compound and add to first solvent. 2. Stir for a few seconds. 3. Heat until compound is dissolved. 4. Add second solvent. 5. Stir for a few seconds. 6. Heat for a few seconds. 7. Add oil phase. 8. Stir for a few seconds. 9. Heat for a few seconds.			Suspension formed; precipitated after a few minutes; remixed reasonably well
THFA	35.00%				
DMSO	17.40%				
Oil Phase	35.00%				
Azoxystrobin	12.60%				Suspension formed; precipitated after a few minutes; remixed reasonably well
THFA	35.00%				
DCM	17.40%				
Oil Phase	35.00%				
Azoxystrobin	12.60%				Suspension formed; precipitated after a few minutes; remixed reasonably well
BnOH	35.00%				
THFA	17.40%				
Oil Phase	35.00%				

Table 4-8: Solubility studies carried out on azoxystrobin 2

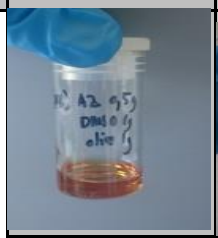
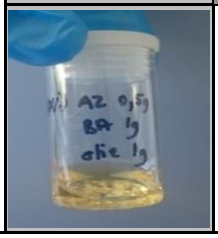
Formula	Concentration % ($^{w}/_{w}$)	Method	Outcome		
			Immediately	After 1:240 dilution with distilled H2O	
Azoxystrobin	20.00%	1. Weigh off compound and add to solvent. 2. Stir for a few seconds. 3. Heat until compound is dissolved. 4. Add oil phase. 5. Stir for a few seconds. 6. Heat for a few seconds.			Unsuccessful; precipitated; did not remix
DMSO	40.00%				
Oil Phase	40.00%				
Azoxystrobin	20.00%			Unsuccessful; crystallization	
BnOH	40.00%				
Oil Phase	40.00%				

Table 4-9: Solubility studies carried out on azoxystrobin 2

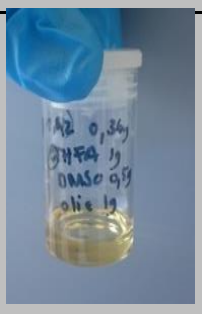
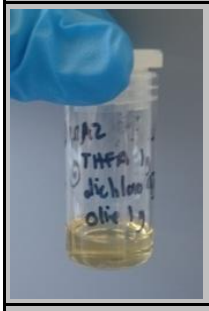
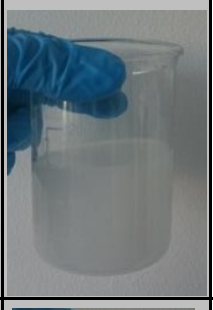
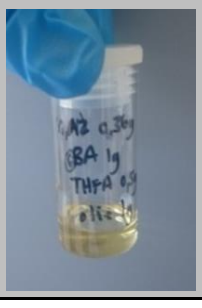
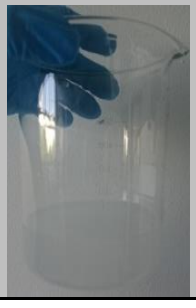
Formula	Concentration % ($^{w}/_{w}$)	Method	Outcome		
			Immediately	After 1:150 dilution with distilled H2O	
Azoxystrobin	12.60%	1. Weigh off compound and add to first solvent. 2. Stir for a few seconds. 3. Heat until compound is dissolved. 4. Add second solvent. 5. Stir for a few seconds. 6. Heat for a few seconds. 7. Add oil phase. 8. Stir for a few seconds. 9. Heat for a few seconds.			Suspension formed; precipitated after a few minutes; remixed reasonably well
THFA	35.00%				
DMSO	17.40%				
Oil Phase	35.00%				
Azoxystrobin	12.60%				Suspension formed; precipitated after a few minutes; remixed reasonably well
THFA	35.00%				
DCM	17.40%				
Oil Phase	35.00%				
Azoxystrobin	12.60%				Suspension formed; precipitated after a few minutes; remixed reasonably well
BnOH	35.00%				
THFA	17.40%				
Oil Phase	35.00%				

Table 4-10: Solubility studies carried out on azoxystrobin 3

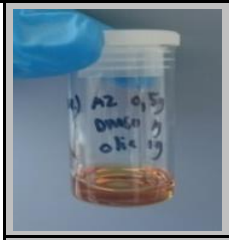
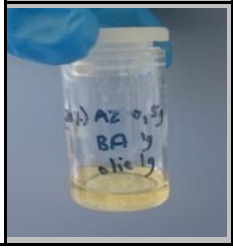
Formula	Concentration % ($^{w}/_{w}$)	Method	Outcome		
			Immediately	After 1:240 dilution with distilled H_2O	
Azoxystrobin	20.00%	1. Weigh off compound and add to solvent. 2. Stir for a few seconds. 3. Heat until compound is dissolved. 4. Add oil phase. 5. Stir for a few seconds. 6. Heat for a few seconds.			Unsuccessful; precipitated; did not remix
DMSO	40.00%				
Oil Phase	40.00%				
Azoxystrobin	20.00%			Unsuccessful; crystallization	
BnOH	40.00%				
Oil Phase	40.00%				

Table 4-11: Solubility studies carried out on azoxystrobin 3

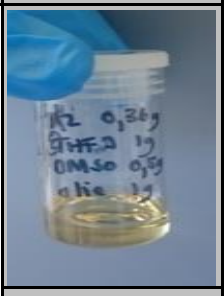
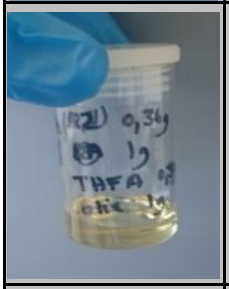
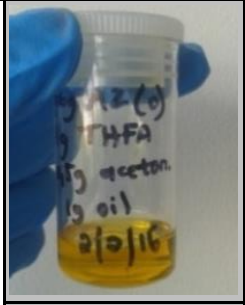
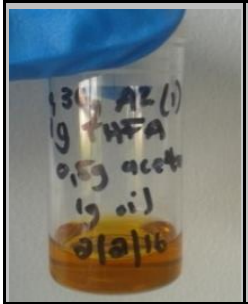
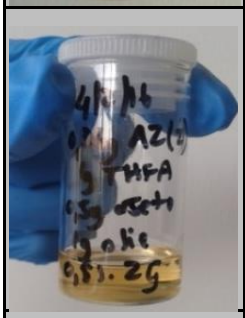
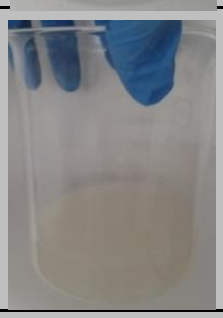
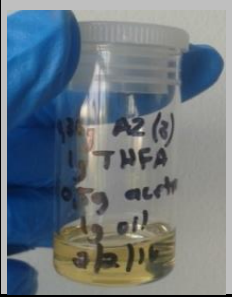
Formula	Concentration % ($^{w}/_{w}$)	Method	Outcome		
			Immediately	After 1:150 dilution with distilled H_2O	
Azoxystrobin	12.60%	1. Weigh off compound and add to first solvent. 2. Stir for a few seconds. 3. Heat until compound is dissolved. 4. Add second solvent. 5. Stir for a few seconds. 6. Heat for a few seconds. 7. Add oil phase. 8. Stir for a few seconds. 9. Heat for a few seconds.			Suspension formed; precipitated after a few minutes; remixed reasonably well
THFA	35.00%				
DMSO	17.40%				
Oil Phase	35.00%				
Azoxystrobin	12.60%				Suspension formed; precipitated after a few minutes; remixed reasonably well
THFA	35.00%				
DCM	17.40%				
Oil Phase	35.00%				
Azoxystrobin	12.60%				Suspension formed; precipitated after a few minutes; remixed reasonably well
BnOH	35.00%				
THFA	17.40%				
Oil Phase	35.00%				

Table 4-12: Solubility of different azoxystrobin compounds in THFA and ACN

Formula	Concentration % ($^{w}/_{w}$)	Method	Outcome	
			After 72 hours	After dilution
Azoxystrobin	12.59	1. Weigh off compound; add to THFA, mix, and heat. 2. Add ACN, mix, and heat until active is dissolved. 3. Add oil phase and mix well.		
THFA	33.30			
ACN	17.15			
Oil Phase	34.96			
Azoxystrobin 1	12.59			
THFA	33.30			
ACN	17.15			
Oil Phase	34.96			
Azoxystrobin 2	12.59			
THFA	33.30			
ACN	17.15			
Oil Phase	34.96			
Azoxystrobin 3	12.59			
THFA	33.30			
ACN	17.15			
Oil Phase	34.96			

Unfortunately, due to instability, no satisfactory formulations were obtained once again and the obligatory decision was made to include other excipients.

The Pheroid system is a safe, environmentally friendly, and cost-effective additive to agricultural formulations. It has the potential to modify the toxicity of the active compounds, improve their target specificity on pests, and reduce the development or exacerbation of resistance. Unlike many additives currently used in the industry, it is completely harmless by itself (Grobler, 2009). Consequently, the option of adding another, potentially less safe excipient was avoided until it

became inevitable as none of the prior formulations were plausible as a commercial product. Fortunately, the excipients that were utilized in the final formulations were also not a safety concern.

In order to try and prevent the precipitation that occurred after dilution of the prior formulations, three excipients were selected and incorporated into the manufacturing process of several formulations. The identities of these excipients are regarded as propriety information and will subsequently be referred to as excipient A, B and C. The selected excipients were chosen because orthodox excipients such as PEG 400 and Tween 80 were already used in prior studies to no avail. The exact same method of preparation was applied to all four different azoxystrobin compounds. The use of less active ingredient was favourable since the higher amounts resulted in crystallization of the concentrates (not shown).

In Tables 4-13 to 4-15 the results obtained with different concentrations of excipient A (a thickener, stabilizing agent and suspending agent) are shown. The combination of THFA and ACN as solvents was used throughout these formulations based on the results displayed in Table 4-13. Excipient A (0.01%) was not sufficient to avoid precipitation, but a decline in the rate of precipitation was noted. The subsequent increase in concentration (to 0.50%) had a radical effect on the diluted formulations. The less transparent solution indicated that the particles were more homogeneously distributed. The rate of precipitation reduced significantly but was not yet satisfactory. Consequently, the concentration excipient A was doubled (1.00%) and resulted in concentrates which did not crystallize and, after dilution, a few stable solutions were obtained. After deliberation, it was concluded that the best formulation was obtained with azoxystrobin 1. It was interesting to observe the effects of the different raw materials on the formulations. The active ingredients were the only variable in every formulation and were supposed to be equals since they are all labelled as ‘azoxystrobin 98% TC’. Yet, they not only differed visually (e.g. textures) but behaved differently as well. While all the formulations prepared with 1.00% excipient A delivered good results, some were clearly better than other with the ultimate formulation being obtained with azoxystrobin 1. As is to be expected, the various raw materials were complemented with different excipients (see Table 4.16 and 4.17). Therefore, these results highlight the effect that the physiochemical properties of an ingredient can have on a formulation.

Table 4-13: Effect of 0.01% ($^{w}/_{w}$) excipient A on selected azoxystrobin formulations

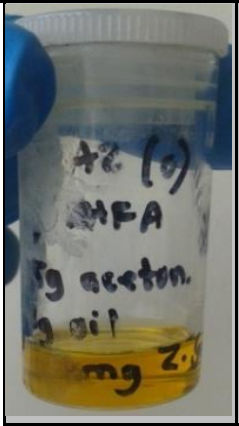
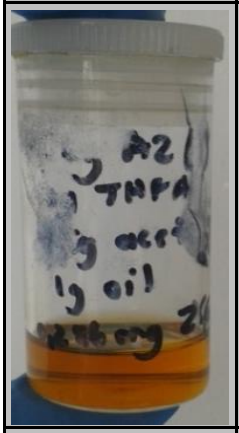
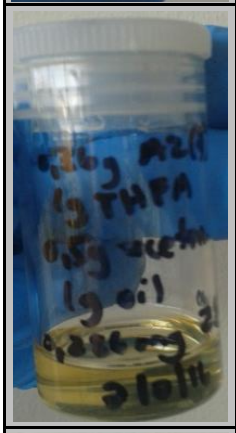
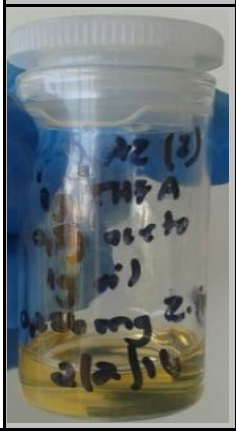
Formula	Concentration % ($^{w}/_{w}$)	Method	Outcome	
			After 72 hours	After dilution
Azoxystrobin	12.59	1. Dissolve excipient A in kolliphor RH40. 2. Combine with rest of oil phase. 3. Separately weigh off compound, add to THFA, mix, and heat. 4. Add ACN, mix, and heat until active is dissolved. 3. Combine the preparations and mix well.		
THFA	34.95			
ACN	17.48			
Excipient A	0.01			
Kolliphor RH40	24.27			
Vitamin F ethyl ester	10.49			
DL-α-tocopherol	0.21			
Azoxystrobin 1	12.59			
THFA	34.95			
ACN	17.48			
Excipient A	0.01			
Kolliphor RH40	24.27			
Vitamin F ethyl ester	10.49			
DL-α-tocopherol	0.21			
Azoxystrobin 2	12.59			
THFA	34.95			
ACN	17.48			
Excipient A	0.01			
Kolliphor RH40	24.27			
Vitamin F ethyl ester	10.49			
DL-α-tocopherol	0.21			
Azoxystrobin 3	12.59			
THFA	34.95			
ACN	17.48			
Excipient A	0.01			
Kolliphor RH40	24.27			
Vitamin F ethyl ester	10.49			
DL-α-tocopherol	0.21			

Table 4-14: Effect of 0.5% ($^{w}/_{w}$) excipient A on selected azoxystrobin formulations

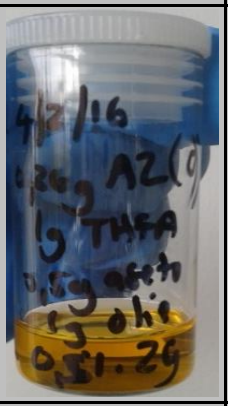
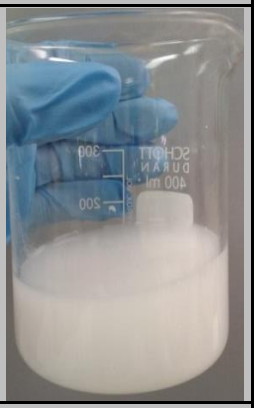
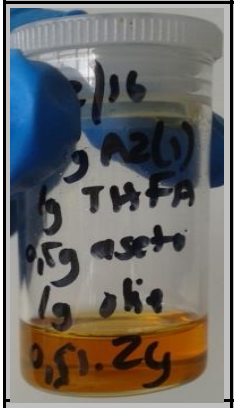
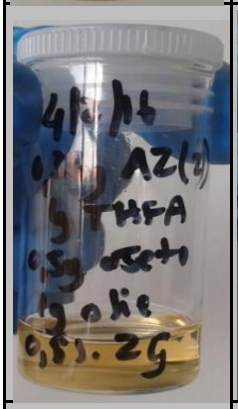
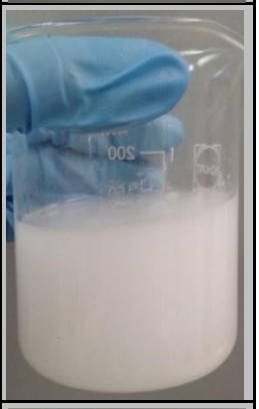
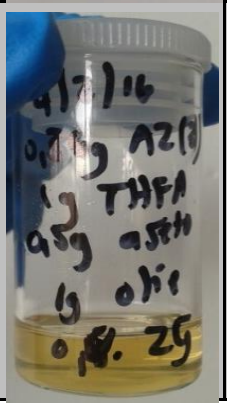
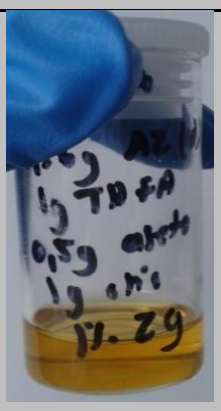
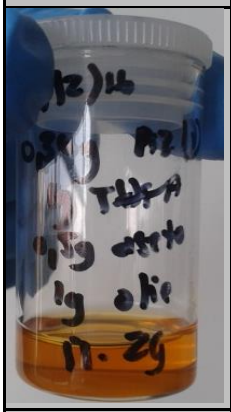
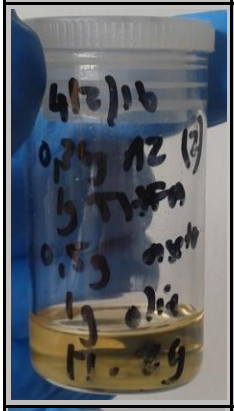
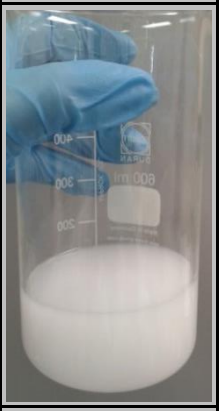
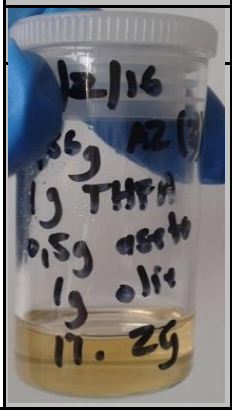
Formula	Concentration % ($^{w}/_{w}$)	Method	Outcome	
			After 72 hours	After dilution
Azoxystrobin	12.52	1. Dissolve excipient A in kolliphor RH40. 2. Combine with rest of oil phase. 3. Separately weigh off compound, add to THFA, mix, and heat. 4. Add ACN, mix, and heat until active is dissolved. 3. Combine the preparations and mix well.		
THFA	34.79			
ACN	17.40			
Excipient A	0.50			
Kolliphor RH40	24.14			
Vitamin F ethyl ester	10.44			
DL-α-tocopherol	0.21			
Azoxystrobin 1	12.52			
THFA	34.79			
ACN	17.40			
Excipient A	0.50			
Kolliphor RH40	24.14			
Vitamin F ethyl ester	10.44			
DL-α-tocopherol	0.21			
Azoxystrobin 2	12.52			
THFA	34.79			
ACN	17.40			
Excipient A	0.50			
Kolliphor RH40	24.14			
Vitamin F ethyl ester	10.44			
DL-α-tocopherol	0.21			
Azoxystrobin 3	12.52			
THFA	34.79			
ACN	17.40			
Excipient A	0.50			
Kolliphor RH40	24.14			
Vitamin F ethyl ester	10.44			
DL-α-tocopherol	0.21			

Table 4-15: Effect of 1% ($^{w}/_{w}$) excipient A on selected azoxystrobin formulations

Formula	Concentration % ($^{w}/_{w}$)	Method	Outcome	
			After 72 hours	After dilution
Azoxystrobin	12.46	1. Dissolve excipient A in kolliphor RH40. 2. Combine with rest of oil phase. 3. Separately weigh off compound, add to THFA, mix, and heat. 4. Add ACN, mix, and heat until active is dissolved. 3. Combine the preparations and mix well.		
THFA	34.60			
ACN	17.31			
Excipient A	1.00			
Kolliphor RH40	24.03			
Vitamin F ethyl ester	10.39			
DL-α-tocopherol	0.21			
Azoxystrobin 1	12.46			
THFA	34.60			
ACN	17.31			
Excipient A	1.00			
Kolliphor RH40	24.03			
Vitamin F ethyl ester	10.39			
DL-α-tocopherol	0.21			
Azoxystrobin 2	12.46			
THFA	34.60			
ACN	17.31			
Excipient A	1.00			
Kolliphor RH40	24.03			
Vitamin F ethyl ester	10.39			
DL-α-tocopherol	0.21			
Azoxystrobin 3	12.46			
THFA	34.60			
ACN	17.31			
Excipient A	1.00			
Kolliphor RH40	24.03			
Vitamin F ethyl ester	10.39			
DL-α-tocopherol	0.21			

Formulations that contained azoxystrobin, excipient A and selected organic solvents (the same solvents that were routinely used during the solubility studies) succeeded the 1.00% excipient A formulations. These solubility studies were carried out in case a superior formulation could be found. Due to the occurrence of extreme precipitation (Table 4-16) and since a successful formulation containing azoxystrobin 1 and excipient A was already found, the focus was turned to the other chosen excipients, namely excipient B (non-ionic emulsifier) and excipient C (dispersing agent) for the following studies.

Table 4-16: Solubility studies with azoxystrobin 1, selected organic solvents, and excipient A

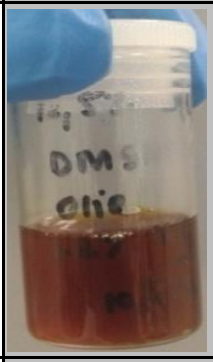
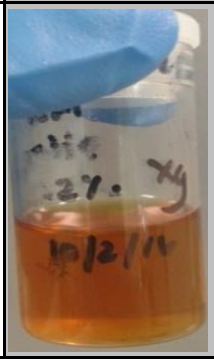
Formula	Concentration % ($^{w}/_{w}$)	Method	Outcome	
			After 60 hours	After dilution
Azoxystrobin 1	12.50	1. Dissolve excipient A in kolliphor RH40. 2. Combine with rest of oil phase. 3. Separately weigh off compound, add to DMSO, mix, and heat until active is dissolved. 3. Combine the preparations and mix well.		
DMSO	53.00			
Excipient A	1.20			
Kolliphor RH40	23.11			
Vitamin F ethyl ester	9.99			
DL-α-tocopherol	0.20			
Azoxystrobin 1	12.50	1. Dissolve excipient A in kolliphor RH40. 2. Combine with rest of oil phase. 3. Separately weigh off compound, add to BnOH, mix, and heat. until active is dissolved. 3. Combine the preparations and mix well.		
BnOH	53.00			
Excipient A	1.20			
Kolliphor RH40	23.11			
Vitamin F ethyl ester	9.99			
DL-α-tocopherol	0.20			
Azoxystrobin 1	12.50	1. Dissolve excipient A in kolliphor RH40. 2. Combine with rest of oil phase. 3. Separately weigh off compound, add to DMSO, mix, and heat. 4. Add BnOH, mix, and heat until active is dissolved. 3. Combine the preparations and mix well.		
DMSO	26.50			
BnOH	26.50			
Excipient A	1.20			
Kolliphor RH40	23.11			
Vitamin F ethyl ester	9.99			
DL-α-tocopherol	0.20			

Table 4-17: Effect of excipient B on selected azoxystrobin formulations

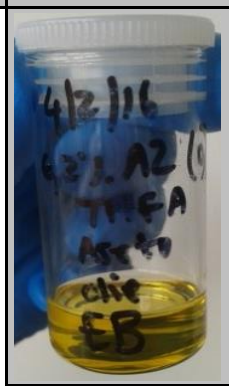
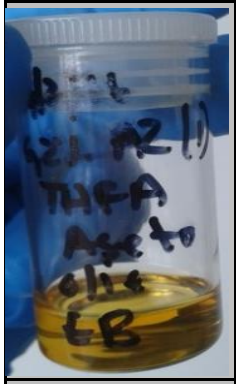
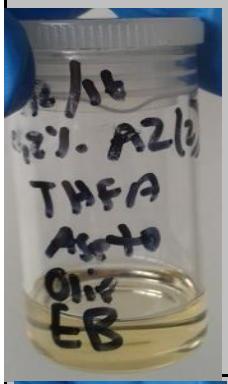
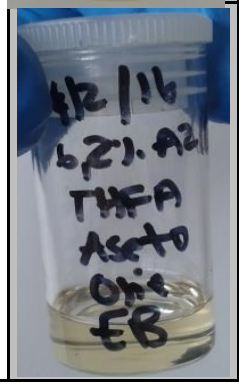
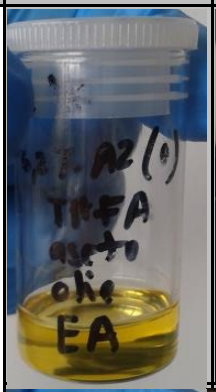
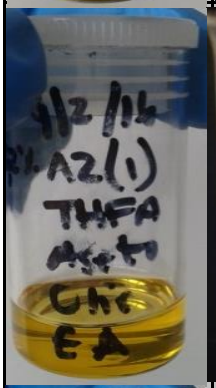
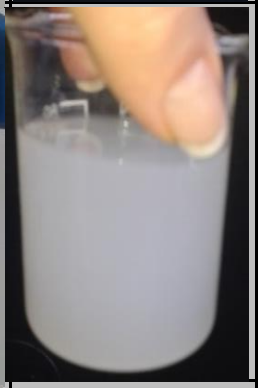
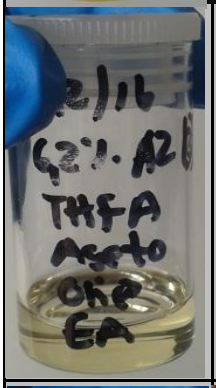
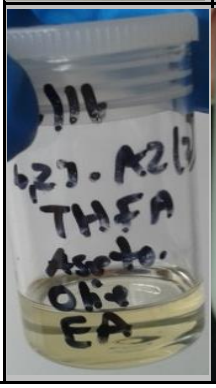
Formula	Concentration % $(^{w}/_{w})$	Method	Outcome	
			After 72 hours	After dilution
Azoxystrobin	6.25	1. Weigh off compound; add to THFA, mix, and heat. 2. Add ACN, mix, and heat until active is dissolved. 3. Add oil phase and mix. 4. Add excipient B and mix well.		
THFA	33.51			
ACN	16.78			
Oil Phase	33.56			
Excipient B	9.90			
Azoxystrobin 1	6.25			
THFA	33.51			
ACN	16.78			
Oil Phase	33.56			
Excipient B	9.90			
Azoxystrobin 2	6.25			
THFA	33.51			
ACN	16.78			
Oil Phase	33.56			
Excipient B	9.90			
Azoxystrobin 3	6.25			
THFA	33.51			
ACN	16.78			
Oil Phase	33.56			
Excipient B	9.90			

Table 4-18: Effect of excipient C on selected azoxystrobin formulations

Formula	Concentration % ($^{w}/_{w}$)	Method	Outcome	
			After 72 hours	After dilution
Azoxystrobin	6.25	1. Weigh off compound; add to THFA, mix, and heat. 2. Add ACN, mix, and heat until active is dissolved. 3. Add oil phase and mix. 4. Add excipient C and mix well.		
THFA	33.51			
ACN	16.78			
Oil Phase	33.56			
Excipient C	9.90			
Azoxystrobin 1	6.25			
THFA	33.51			
ACN	16.78			
Oil Phase	33.56			
Excipient C	9.90			
Azoxystrobin 2	6.25			
THFA	33.51			
ACN	16.78			
Oil Phase	33.56			
Excipient C	9.90			
Azoxystrobin 3	6.25			
THFA	33.51			
ACN	16.78			
Oil Phase	33.56			
Excipient C	9.90			

None of the formulation concentrates that contained excipient C or excipient B (presented in Table 4-18 and 4-18 respectively) crystallized within three days after they were manufactured. However, it was observed that an increased amount of active ingredient would be more likely to form crystals (not shown). Subsequently, the concentrates displayed in the tables above contained only 6.25% active ingredient. After a 1:75 dilution with ddH_2O, all the formulations were in solution. In general, the formulations that included excipient C were less stable than the excipient B formulations. After a few hours, slight precipitation was visible with the excipient C formulations that contained azoxystrobin 1, 2 and 3 – remixing the solutions resulted in the same observation after a while. Azoxystrobin resulted in a better solution than the latter actives, but the formulations that contained excipient B were still superior. The least desirable formulation that contained the latter excipient was obtained with azoxystrobin 1. Fine precipitation was present and, like the former solutions, re-precipitated again after mixing. Azoxystrobin resulted in a promising solution but was disregarded when slight precipitation formed. Azoxystrobin 2 and 3 was both considered for the final formulation, but in the end, the formulation that contained azoxystrobin 3 was more stable. Moreover, the fact that it had prominent colouring, signified that the particles were homogeneously dispersed.

To implement the results that were obtained from the solubility studies, the two chosen formulations were made on a larger scale. Both formulations included THFA, ACN, and pro-Pheroid. The 6.25% ($^{w}/_{w}$) azoxystrobin/pro-Pheroid formulation included azoxystrobin 3 and excipient B in addition while the 12.50% ($^{w}/_{w}$) azoxystrobin/pro-Pheroid formulation incorporated azoxystrobin 1 and excipient A.

4.3.2 The characterization of Pheroid

Every manufactured batch containing the Pheroid delivery system is subject to the standard characterization tests, which include Mastersize, CLSM and colloidal stability testing.

4.3.2.1 Particle size and particle size distribution

4.3.2.1.1 Apparatus and experimental conditions

The particle size and particle size distribution was measured through laser diffraction with the use of a Malvern Mastersize 2000 by means of its wet cell Hydro MU dispersion unit (Malvern Instruments Ltd, Malvern, Worcestershire, UK) and indicated on a graph which displays the percentage volume against particle size. The instrument was switched on 30 minutes prior to the measurement to stabilize the laser. The samples were slightly mixed to ensure uniform distribution before the analysis was initiated.

The instrument is driven by pre-established measurement controls (in this case, specialized for Pheroid formulations) and parameters with step-by-step software wizards. This increases consistency and ease of use while reducing the need for user intervention.

4.3.2.1.2 Method

Once the laser was aligned with deionised water in the dispersion unit, the sample was slowly added and stirred at 1500rpm until an obscuration rate of 10.00 – 20.00% was obtained. The measurement parameters were as follows: the refractive indices for azoxystrobin and the dispersant were 1.458 and 1.330, respectively, and the absorption value of the active ingredient was 0.1. The data were expressed as $d_{0.1}$, $d_{0.5}$, $d_{0.9}$, which are equivalent spherical volume diameters at 10, 50 (i.e. median), and 90% cumulative volume, respectively. Each sample was measured in triplicate.

4.3.2.1.3 Results

The complete Mastersize analysis reports for the two azoxystrobin/pro-Pheroid formulations are available in Annexure A1 and A2. Figure 4-1 and 4-2 illustrates the particle size distribution of the respective formulations as determined through Mastersize analysis. The two formulations presented with similar profiles. Pheroid generally has a bimodal distribution. The largest peak forms in the submicron size range whereas the smaller peak appears within the micron sized range. The desired size range for Pheroid particles is between 200 nm and 2 µm. The azoxystrobin formulations did not present typical Pheroid properties but, because solvents and excipients were combined with the Pheroid, this was to be expected. Nonetheless, due to the solubility struggles experienced with the active, the successful formulations were sent for trial plot studies regardless of the results obtained from the characterization analyses.

4.3.2.1.3.1 The 6.25% azoxystrobin/pro-Pheroid formulation

Particles (90.00%) in the formulation were smaller than 307.751 µm. The submicron size range contained 17.74% of the particles whereas the micron sized range contained 1.08%. Unlike standard Pheroid formulations, the largest peak was observed above 100 µm instead of in the submicron size range (see Figure 4-1). A laser obscuration of 13.42% was obtained during the analysis and could therefore not be the cause of the large particle sizes in the report. However, due to its composition, the addition of a wax type of excipient could have increased the oil phase in the Pheroid formulation and resulted in larger particles.

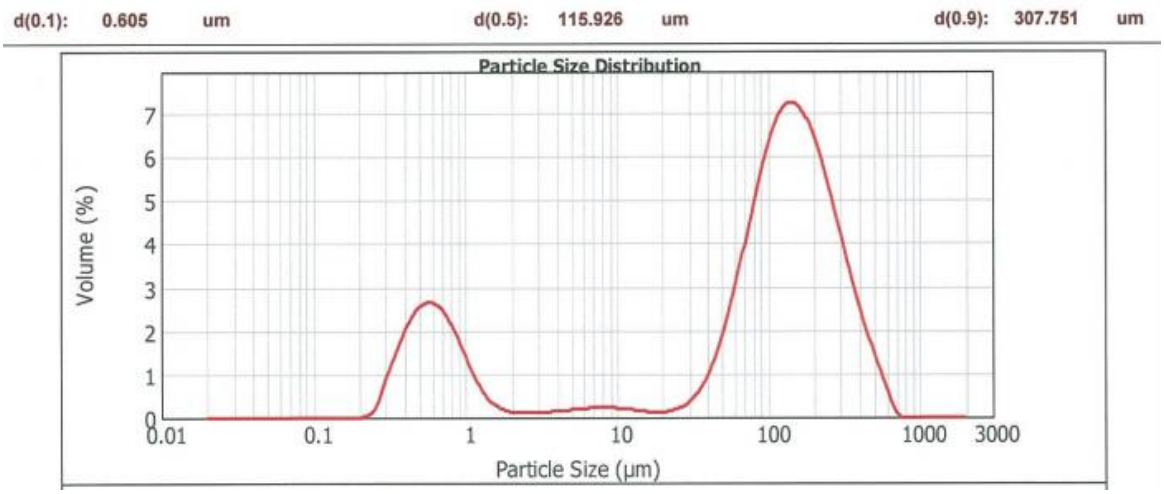


Figure 4-2: Particle size and particle size distribution of the 6.25% azoxystrobin/pro-Pheroid formulation

4.3.2.1.3.2 The 12.50% azoxystrobin/pro-Pheroid formulation

Of all the particles in the formulation, 10.00% were larger than 193.336 µm. The submicron range comprised of 12.81% of the particles while only 0.11% of particles was between 1.5 and 5 µm in size. The particle sizes peaked more or less at 100 µm while a much smaller peak was observed in and above the submicron sized range (see Figure 4-2). The laser obscuration fell into the stipulated range during the analysis (13.00%) and could not have influenced the measurement.

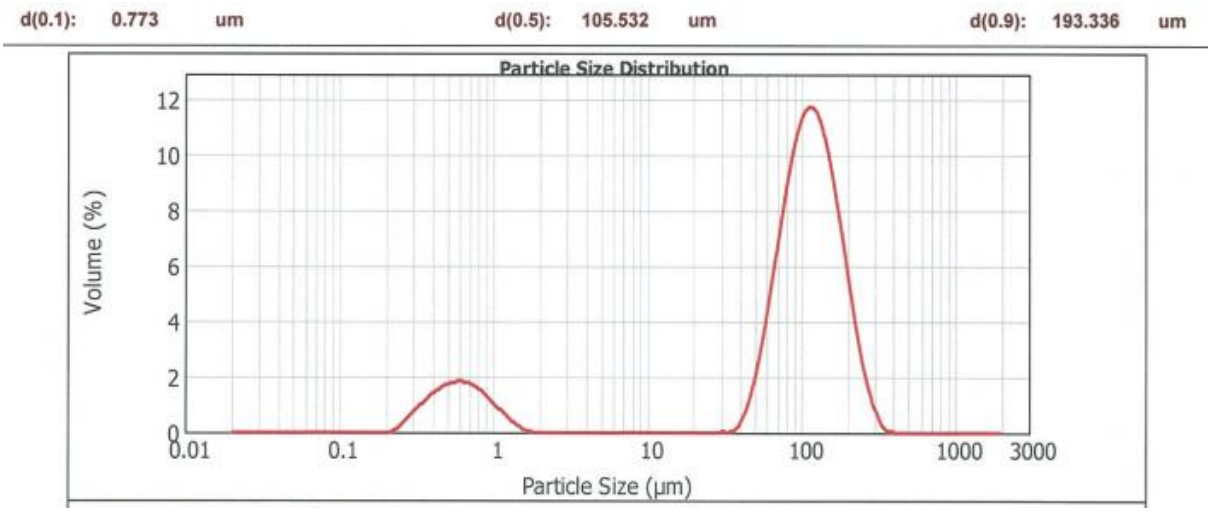


Figure 4-3: Particle size and particle size distribution of the 12.50% azoxystrobin/pro-Pheroid formulation

4.3.2.2 Confocal laser scanning microscopy (CLSM)

4.3.2.2.1 Apparatus and experimental conditions

The qualitative analysis was performed with the use of a confocal laser scanning microscope. The images were taken with a Nikon D – eclipse confocal laser scanning microscope. A 60x plan apochromatic oil immersion objective was used to record tungsten sourced light and fluorescent images of the sample.

4.3.2.2.2 Method

A quantity of 50µl appropriately diluted sample was labelled with 1 µl of a 1mg/ml solution of the fluorophore Nile Red (Molecular Probes, Thermo Fisher Scientific Inc. Waltham, MA, USA). The mixture was vortexed, left to incubate for 15 minutes in the dark and vortexed again. Thereafter, 20 µl was placed on a microscope slide, covered with a glass coverslip, and analysed for its characteristics. The sample was analysed with the use of three lasers and operated as follows: excitation at 405, 488 and 543 nm wavelength lasers led to fluorescence light emissions in three wavelength bands, at emission wavelengths 567 to 643 nm, 500 to 530nm and 432 to 468 nm. The emitted photons were captured and automatically reinterpreted as an image. Point illumination by means of a small spatial pinhole was used to exclude out of focus emissions.

4.3.2.2.3 Results

The results obtained from the confocal laser scanning microscope (Figure 4.3 and 4.4) compiled out of four images each. Image 1 was obtained with tungsten sourced light. Image 2 displays an overlay of the fluorescence detected in wavelengths 500 to 530 nm and 568 to 642 nm. Image 3 represents the fluorescence detected in the 568 and 642 nm waveband only and Image 4 is that seen in the 500 to 530 nm waveband.

4.3.2.2.3.1 The 6.25% azoxystrobin/pro-Pheroid formulation

Several small Pheroid of less than 1 µm were present although a small fraction was up to 10 µm in size (see Figure 4-3). Nile Red fluorescence was detected in the 568 and 642 nm waveband showing Pheroidal structures with few oil droplets. The concentration of Pheroid within the 200 to 5000 nm diameter range was 527 x 10^8 per ml with an average size of 863 nm. These measurements represent the average of four aliquots of sample. The results obtained from the confocal analysis indicated that the 6.25% azoxystrobin/pro-Pheroid formulation was satisfactory.

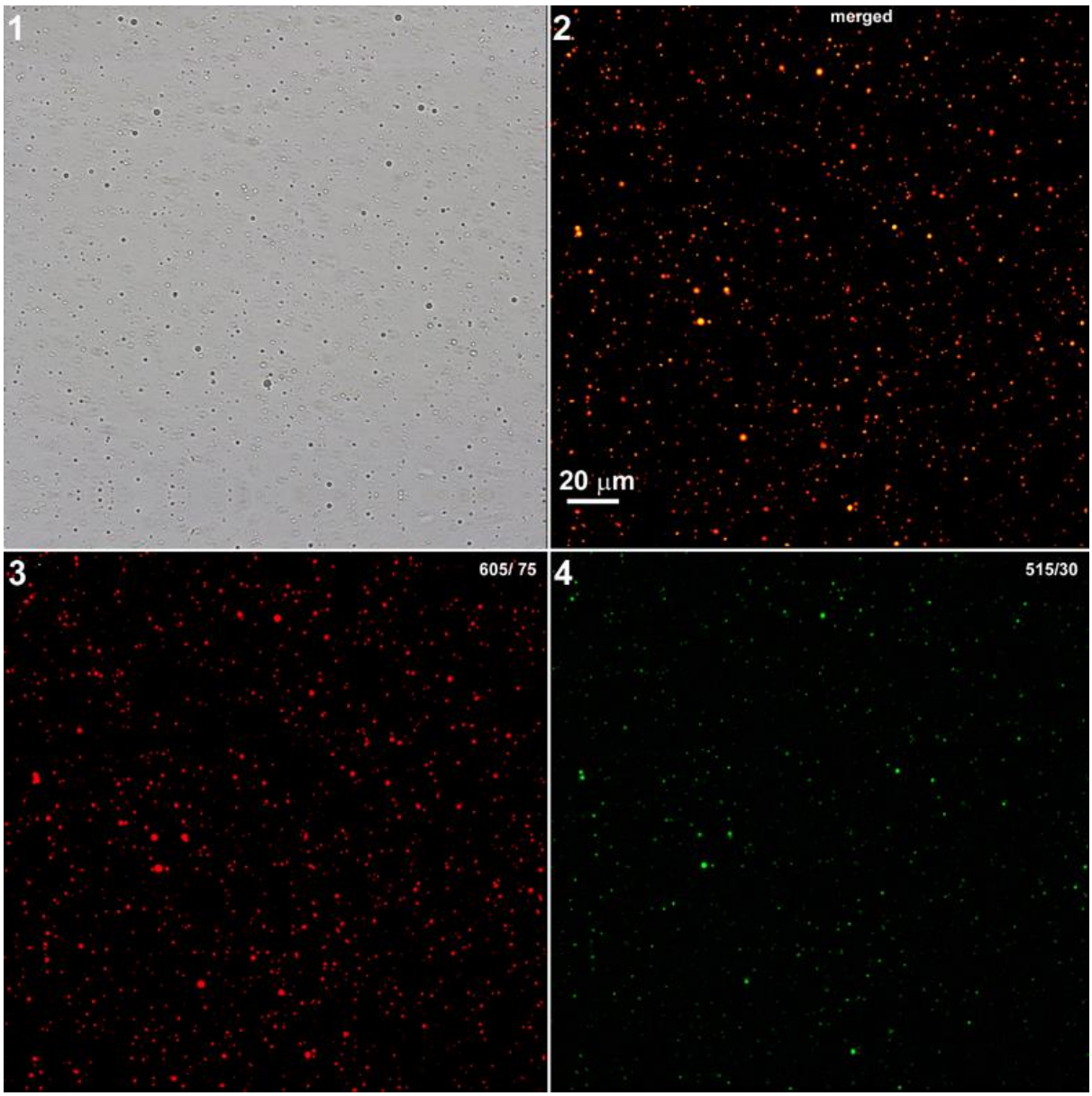


Figure 4-4: CLSM-imaging of 6.25% azoxystrobin/pro-Pheroid formulation

4.3.2.2.3.2 The 12.50% azoxystrobin/pro-Pheroid formulation

Small Pheroid of less than 1 µm were common but a considerable fraction was up to 8 µm in size and irregular in shape with uneven edges suggesting that components were not mixing adequately (refer to Figure 4.4). Nile Red fluorescence was detected in the 568 and 642 nm waveband and revealed Pheroidal structures with a few oil droplets. The average Pheroid was 930 nm in size while the concentration of Pheroid within the 200 to 5000 nm diameter range was 229.7 x 10^8 per ml. These measurements represent the average of four aliquots of sample.

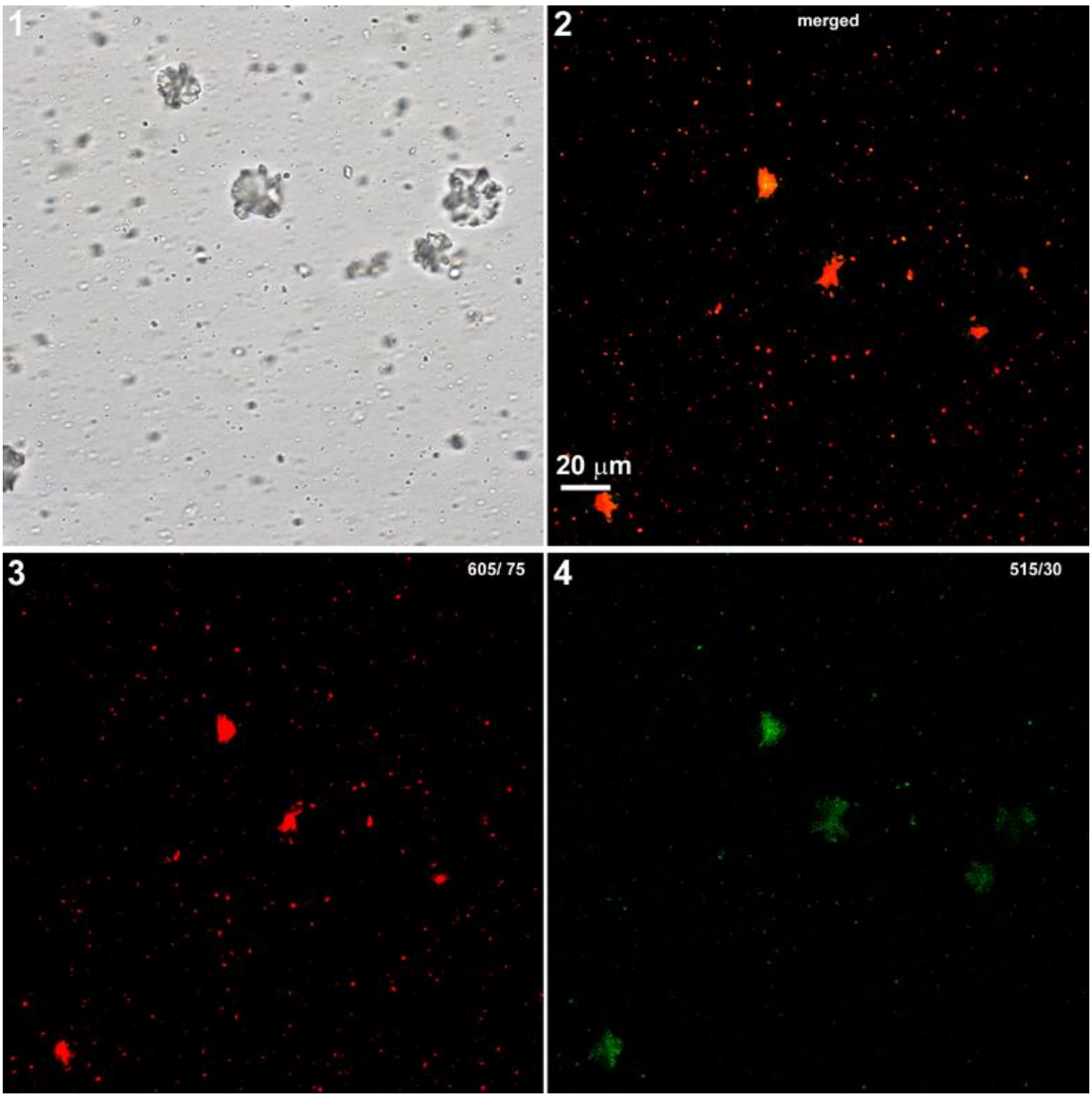


Figure 4-5: CLSM-imaging of 12.50% azoxystrobin/pro-Pheroid formulation

It was established during the confocal analysis that the 12.50% azoxystrobin/pro-Pheroid sample contained large irregularly shaped non-pheroidal type structures with a relatively low concentration of Pheroid. The formulation was subsequently regarded as unsatisfactory. Regardless, both samples were still sent for trial plot studies to evaluate their efficacy and phytotoxicity. This was used as an opportunity to compare the formulation's value as established via characterization methods with that obtained during trials.

4.3.2.3 Zeta potential

4.3.2.3.1 Apparatus and experimental conditions

The zeta potential was measured with the use of a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd, Worcestershire, United Kingdom). Air bubbles or particles such as dust in the cell interfere with measurements and were avoided. Furthermore, care was taken to prevent contact to the two brass electrodes and the front and back of the cell (through which the light beam passes) as it may also disrupt the procedure. To remove air bubbles in the cell, it is gently tapped on the side until the bubbles disappear before insertion into the measurement chamber.

4.3.2.3.2 Method

The instrument was switched on at least 30 minutes prior to analysis to warm up as required. A 5000x dilution of the sample was prepared using distilled water (1µl sample diluted with ~5ml ddH_2O) and carefully mixed with a Pasteur pipette. Using a syringe, 1mL sample was used to rinse out the zetasizer cell. The cell was emptied and carefully injected with the diluted sample until halfway full and rotated upright to fill the rest of the cell. The cell was closed with the use of two stoppers to avoid leakage and placed in the measurement chamber. The measurement was started, results recorded and the process was repeated as required. Each measurement was carried out in triplicate.

4.3.2.3.3 Results

The 6.25% azoxystrobin/pro-Pheroid formulation had a zeta potential measurement of -13.91 mV whereas -0.5 mV were obtained with 12.50% azoxystrobin/pro-Pheroid. Although the measurement for the former formulation indicated a higher stability than the latter, both formulations were unstable and had a tendency to aggregate. This is in line with the large particle sizes that were observed during the Mastersize and confocal analysis although the 6.25% formulation (with a higher stability) contained the largest particles (see Figure 4-1 and 4-2).

4.4 Efficacy evaluation of the azoxystrobin/pro-Pheroid formulations (Experimental studies)

After manufacture and characterization, the formulations were sent to Agricultural Science Consultants (ASC) for antifungal trial plot studies to test the efficacy and possible phytotoxicity of both the 12.50% and 6.25% azoxystrobin/pro-Pheroid formulations (AZ/PPA and AZ/PPB in turn) on late blight (tomatoes) and white blister (cabbage). The trials were conducted between May and July 2016 in Pongola, Kwa-Zulu-Natal and Phillipi, Western Cape respectively. Thirty-

six plots, each sized 1 m x 10 m (10 m^2), were distributed in a randomized complete block (total plot size: 360 m^2). The nine treatment formulations were randomly replicated in four blocks. A mist blower was used to apply the treatments in such a manner that the plants were fully covered but no run off occurred. The disease severity ratings of the tomato plants and cabbage leaves were subjected to statistical analysis (briefly described in section 4.4.3). Any signs of possible adverse effects on growth (e.g. chlorosis (yellowing), stunting, necrosis or node curl) were monitored for possible phytotoxic reactions to the applications. The information below was extracted from reports compiled by ASC.

4.4.1 Azoxystrobin/pro-Pheroid formulations for the control of *Phytophtora infestans* (late blight) in tomatoes

4.4.1.1 Study details

The efficacy of AZ/PPA and AZ/PPB was compared to the commercial product, Amistar 250 SC (suspension concentrate) and an untreated control in order to determine the ability of the Pheroid-based formulations to control late blight on tomato in terms of disease severity (see Table 4-19 for a description of the treatments).

Plants were treated at a volume of 1000 L/ha and watered with the use of pivot irrigation. The first application occurred during the 1st fruit cluster (BBCH 71 – described in Annexure B.1). Assessments on late blight were done prior to each application as well as 7 days after the last application (for a total of nine assessments – see Table 4-20). Assessments were done on five plants from each plot. A percentage disease severity was allocated to each tomato plant.

Table 4-19: Abbreviation for the respective treatments utilized during the efficacy studies on late blight

Treatment product	Abbreviation
Untreated Check	Control
Amistar 250 SC 60 mL/100 L	0.5X AZ
Amistar 250 SC 120 mL/100 L	1X AZ
12.50% azoxystrobin/pro-Pheroid formulation 120 mL/100 L	0.5X AZ/PPB
12.50% azoxystrobin/pro-Pheroid formulation 240 mL/100 L	1X AZ/PPB
12.50% azoxystrobin/pro-Pheroid formulation 480 mL/100 L	2X AZ/PPB
6.25% azoxystrobin/pro-Pheroid formulation 240 mL/100 L	0.5X AZ/PPA
6.25% azoxystrobin/pro-Pheroid formulation 480 mL/100 L	1X AZ/PPA
6.25% azoxystrobin/pro-Pheroid formulation 960 mL/100 L	2X AZ/PPA

In practice, Amistar 250 SC is concomitantly used with Bravo 720 SC (alternating every seven days) for the treatment of late blight in tomatoes. The study was launched to compare the efficacy of the respective azoxystrobin/pro-Pheroid formulations with Amistar 250 SC and therefore Bravo 720 SC (fixed ingredient and therefore not included in Table 4-19) was also used in combination with the test formulations (Table 4-20 presents the application timeline). To simplify reading, all the azoxystrobin formulations in Table 4-20 are referred to as AZ while Bravo 720 SC (380 mL/ 100L) is referred to as Bravo. A dash (–) is used to signify that no treatment was applied.

Table 4-20: Study design of the treatment applications against late blight on tomatoes

Assessment	1	2	3	4	5	6	7	8	9
Application	Bravo	Bravo	AZ	Bravo	AZ	Bravo	AZ	Bravo	–

The weather conditions during applications and assessments are displayed in Table 4-21 and 4-22 respectively.

Table 4-21: Weather conditions at time of applications

Application Information	Application 1	Application 2	Application 3	Application 4
BBCH*	71	71	75	75
Date	26-May-2016	02-Jun-2016	09-Jun-2016	16-Jun-2016
Time of application	12H05	14H00	10H30	11H00
Temperature (°C)	17	16	21	14
Relative humidity (%)	59	18	59	48
Wind speed (m/s)	1.9	2.5	1.4	2.2
Rain (mm)	0	0	0	0

Table 4-21: Weather conditions at time of applications (continued)

Application Information	Application 5	Application 6	Application 7	Application 8
BBCH*	75	80	81	83
Date	23-Jun-2016	30-Jun-2016	05-Jul-2016	12-Jul-2016
Time of application	15H00	14H00	15H00	14H00
Temperature (°C)	18	16	18	16
Relative humidity (%)	55	39	55	39
Wind speed (m/s)	2.5	2.1	3.1	2.9
Rain (mm)	0	0	0	0

*The BBCH-scale is a scale used to identify the phenological development stages of a plant (see Appendix B1 and B2 for a description of the BBCH-values displayed in Table 4-21 and 4-25)

Table 4-22: Weather conditions at time of assessments

Assessment Information	Assessment 1	Assessment 2	Assessment 3	Assessment 4	Assessment 5
Date	26-May-2016	02-Jun-2016	09-Jun-2016	16-Jun-2016	23-Jun-2016
Time of assessment	12H05	14H00	10H30	11H00	15H00
Temperature (°C)	17	16	21	14	18
Relative humidity (%)	59	18	59	48	55
Wind speed (m/s)	1.9	2.5	1.4	2.2	2.5
Rain (mm)	0	0	0	0	**0**

Table 4-22: Weather conditions at time of assessments (continued)

Assessment Information	Assessment 6	Assessment 7	Assessment 8	Assessment 9
Date	30-Jun-2016	05-Jul-2016	12-Jul-2016	19-Jul-2016
Time of assessment	14H00	15H00	14H00	14H15
Temperature (°C)	16	18	16	19
Relative humidity (%)	39	55	39	44
Wind speed (m/s)	2.1	3.1	2.9	1.7
Rain (mm)	0	0	0	0

4.4.2 Azoxystrobin/pro-Pheroid formulations for the control of *Albugo candida* (white blister) in cabbage

4.4.2.1 Study details

The efficacy of AZ/PPA and AZ/PPB was compared to the commercial product, Amistar 250 SC and an untreated control in order to determine the ability of the Pheroid-based formulations to control white blister on cabbage in terms of disease severity on cabbage leaves (see Table 4-23 for a description of the treatments). Plants were treated at a volume of 450 to 700 L/ha and watered by means of sprinkler irrigation. The treatment was initiated when nine or more true leaves had unfolded (BBCH 19 – refer to Annexure B.2 for a description). Assessments on white blister were done prior to each application, during treatment, and 4 and 11 days after the last application (for a total of six assessments – see Table 4-24). Assessments were done on 25 leaves from each plot. A percentage disease severity was allocated to each cabbage leaf.

Table 4-23: Abbreviation for the respective treatments utilized during the efficacy studies on white blister

Treatment product	Abbreviation
Untreated Check	Control
Amistar 250 SC 375 mL/100 L	0.5X AZ
Amistar 250 SC 750 mL/100 L	1X AZ
12.50% azoxystrobin/pro-Pheroid formulation 750 mL/100 L	0.5X AZ/PPB
12.50% azoxystrobin/pro-Pheroid formulation 1500 mL/100 L	1X AZ/PPB
12.50% azoxystrobin/pro-Pheroid formulation 3000 mL/100 L	2X AZ/PPB
6.25% azoxystrobin/pro-Pheroid formulation 1500 mL/100 L	0.5X AZ/PPA
6.25% azoxystrobin/pro-Pheroid formulation 3000 mL/100 L	1X AZ/PPA
6.25% azoxystrobin/pro-Pheroid formulation 6000 mL/100 L	2X AZ/PPA

In practice, Amistar 250 SC is concomitantly used with Bravo 720 SC and Ridomil Gold-Flo (alternating Amistar with two consecutive sprays of Bravo and Ridomil Gold-Flo every 7 days) for the treatment of white blister in cabbage. The study was launched to compare the efficacy of

the azoxystrobin/pro-Pheroid formulations with Amistar 250 SC and therefore Bravo 720 SC and Ridomil Gold-Flo (as fixed ingredients) were also used in combination with the test formulations and were therefore not described in Table 4-23. See Table 4-24 for a timeline of the treatment applications. To simplify reading, Bravo 720 SC (2 L/ ha) and Ridomil Gold-Flo (2 L /ha) is respectively referred to as Bravo and RGF in Table 4-24, while all the azoxystrobin formulations are referred to as AZ. A dash (–) is used to represent the fact that no treatment was applied.

Table 4-24: Study design of the treatment applications against white blister in cabbage

Assessment	1	2	3	4	5	6
Application	Bravo	AZ RGF	AZ RGF	Bravo	–	–

The weather conditions during applications and assessments are displayed in Table 4-25 and 4-26 respectively.

Table 4-25: Weather conditions at time of applications

Application Information	Application 1	Application 2	Application 3	Application 4	Application 5	Application 6
BBCH	19	19	39	40	–	–
Date	06-Jun-2016	11-Jun-2016	20-Jun-2016	27-Jun-2016	–	–
Time of application	12H11	10H45	14H02	16H14	–	–
Temperature (°C)	15.1	16.1	13.3	15.4	–	–
Relative humidity (%)	68.6	40.94	77.16	78.6	–	–
Wind speed (m/s)	3.3	1.35	2.21	1.2	–	–
Rain (mm)	0	0	0	0	–	–

Table 4-26: Weather conditions at time of assessments

Assessment Information	Assessment 1	Assessment 2	Assessment 3	Assessment 4	Assessment 5	Assessment 6
Date	06-Jun-2016	11-Jun-2016	20-Jun-2016	27-Jun-2016	01-Jul-2016	08-Jul-2016
Time of assessment	12H11	10H45	14H02	16H14	16H48	17H41
Temperature (°C)	15.1	16.1	13.3	15.4	12	16.50
Relative humidity (%)	68.6	40.94	77.16	78.6	57.0	62.49
Wind speed (m/s)	3.3	1.35	2.21	1.2	1.4	1.83
Rain (mm)	0	0	0	0	0	0

4.4.3 Data obtained from the experimental studies

ASC was not only responsible for the plot design and execution of the antifungal studies but performed the particular study's statistical data analysis as well. The analyses were executed

with the use of Descriptive Statistics, Analysis of variance (ANOVA), Bartlett's test (homogeneity of variances (De Muth, 2014:225)) and Student-Newman-Keuls (identifies sample means that are significantly different from each other (De Muth, 2014:251)). These are the standard tests in agriculture with regards to statistical analysis of pesticide effectively and phytotoxicity.

4.4.4 Efficacy and possible phytotoxicity of the azoxystrobin/pro-Pheroid formulations

The treatment rates of the azoxystrobin/pro-Pheroid formulations were adjusted in order to be equivalent to the comparator (25.00% azoxystrobin – see Figure 4-20 and 4-24). Treatments that are identified by the same X-values in Table 4-27 and 4-28 (e.g. 0.5X or 1X) are parallel in strength. The X-value also serves as an indication of the factor with which the formulations differ (e.g. the concentration of 2X- formulations is fourfold that of 0.5X-formulations).

4.4.4.1 Results: Azoxystrobin/pro-Pheroid formulations in the control of late blight on tomatoes

Results of late blight severity are presented in Table 4-27. Efficacy was determined in terms of percentage disease severity. No late blight was found at assessments 1 – 4, therefore only assessments 5 – 9 are shown in Table 4-27 and are subsequently discussed. Means followed by the same letter do not significantly differ (p=0.05, established with Student-Newman-Keuls). None of the formulations caused any visible symptoms of phytotoxicity (e.g. yellowing, stunting, necrosis, or node curl) on the plants at any given time of the trial studies (see Annexure C1). With the 5th assessment, the azoxystrobin treatments had been applied on one occasion, two weeks earlier (Bravo 720 SC was utilized for the first two treatments). After the assessment, the azoxystrobin treatments were applied for the second time and, after two weeks, the final azoxystrobin treatments were applied (presented in Table 4-19). Figure 4-5 is a visual representation of the data in Table 4-27.

Table 4-27: The effect of treatments on late blight severity of tomato plants

Assessment		5		6		7		8		9	
Treatment number	**Treatment name**										
1	**Control**	1.00 ± 0.71	a	3.25 ± 0.83	a	9.50 ± 1.50	a	15.25 ± 2.68	a	16.75 ± 1.30	a
2	**0.5X AZ**	0.25 ± 0.43	a	0.50 ± 0.50	bc	1.75 ± 0.83	c	5.25 ± 2.49	b	5.25 ± 1.30	c
3	**1X AZ**	0.25 ± 0.43	a	0.50 ± 0.50	bc	2.25 ± 1.09	c	3.75 ± 2.68	b	4.75 ± 0.83	c
4	**0.5X AZ/PPB**	0.75 ± 0.43	a	2.00 ± 1.00	b	4.25 ± 1.48	c	5.75 ± 1.64	b	7.25 ± 2.17	bc
5	**1X AZ/PPB**	0.50 ± 0.87	a	0.75 ± 0.83	bc	2.75 ± 0.83	c	5.25 ± 2.59	b	5.00 ± 1.87	c
6	**2X AZ/PPB**	0.25 ± 0.43	a	0.50 ± 0.50	bc	2.50 ± 1.12	c	3.50 ± 1.12	b	4.75 ± 0.83	c
7	**0.5X AZ/PPA**	0.75 ± 0.83	a	1.75 ± 0.43	bc	7.00 ± 2.24	b	8.00 ± 2.55	b	8.75 ± 1.48	b
8	**1X AZ/PPA**	0.25 ± 0.43	a	0.25 ± 0.43	c	3.75 ± 1.48	c	4.50 ± 1.12	b	5.00 ± 0.71	c
9	**2X AZ/PPA**	0.25 ± 0.43	a	0.50 ± 0.50	bc	2.75 ± 1.30	c	3.25 ± 1.30	b	4.25 ± 1.79	c
LSD (p =.05)		1.030		1.065		2.423		3.712		2.509	
Standard Deviation		0.705		0.730		1.660		2.544		1.719	
CV		149.39		65.67		40.94		42.0		25.06	
Bartlett's X2		4.589		4.767		4.42		5.588		6.196	
p (Bartlett's X2)		0.8		0.782		0.817		0.693		0.625	
Replicate F		0.205		1.391		0.282		0.372		0.486	
Replicate Prob (F)		0.8922		0.2696		0.8378		0.7739		0.6955	
Treatment F		0.684		7.643		9.584		8.644		21.442	
Treatment Prob (F)		0.7015		0.0001		0.0001		0.0001		0.0001	

Means followed by the same letter do not significantly differ (p=.05, Student-Newman-Keuls)
Mean comparisons performed only when Analysis of Variance (AOV) Treatment Prob (F) is significant at mean comparison observed significance level (OSL).
* = significant at p = 0.05 (Bartlett's test)

None of the formulations completely eliminated the threat. As is to be expected, the late blight severity in the control plots increased with every assessment. For the duration of the study, the mean percentage disease severity in the control plots superseded those observed for the other treatments (see Figure 4-5). The plants that were treated with 0.5X AZ had the least percentage mean late blight severity up until assessment 7. During assessment 8, the mean disease severity observed in the plants that were treated with 0.5X AZ increased by 3.50% and remained unchanged during assessment 9. Plots treated with1X AZ had the same mean disease severity as the 0.5X AZ plots during assessment 5 and 6, but had 0.50% less mean disease severity than 0.5X AZ during the last assessment. During the course of the trial, plants

treated with 0.5X AZ/PPB and 0.5X AZ/PPA had a higher percentage mean disease severity compared to the other treatments (with the exclusion of the control). The plots that were treated with 1X AZ/PPB and 1X AZ/PPA had a mean disease severity of 5.00% at the conclusion of the study. With the final assessment, plants treated with 2X AZ/PPA had the lowest mean percentage late blight severity. During assessment 9, no statistical difference was observed between the 1X formulations but there were between the 0.5X formulations.

The AZ/PPB formulations had a good dose response between the three formulations until assessment 9 when saturation was achieved. The AZ/PPA formulations also presented a good dose response without achieving saturation at assessment 9, meaning that a concentration increase (from 2X AZ/PPA) would most likely result in a more efficacious formulation. To the contrary, the mean percentage pest severity did not present an enhanced response to the more concentrated AZ formulation with assessment 6 and 7 although a higher efficacy was observed with the 8th and 9th assessment.

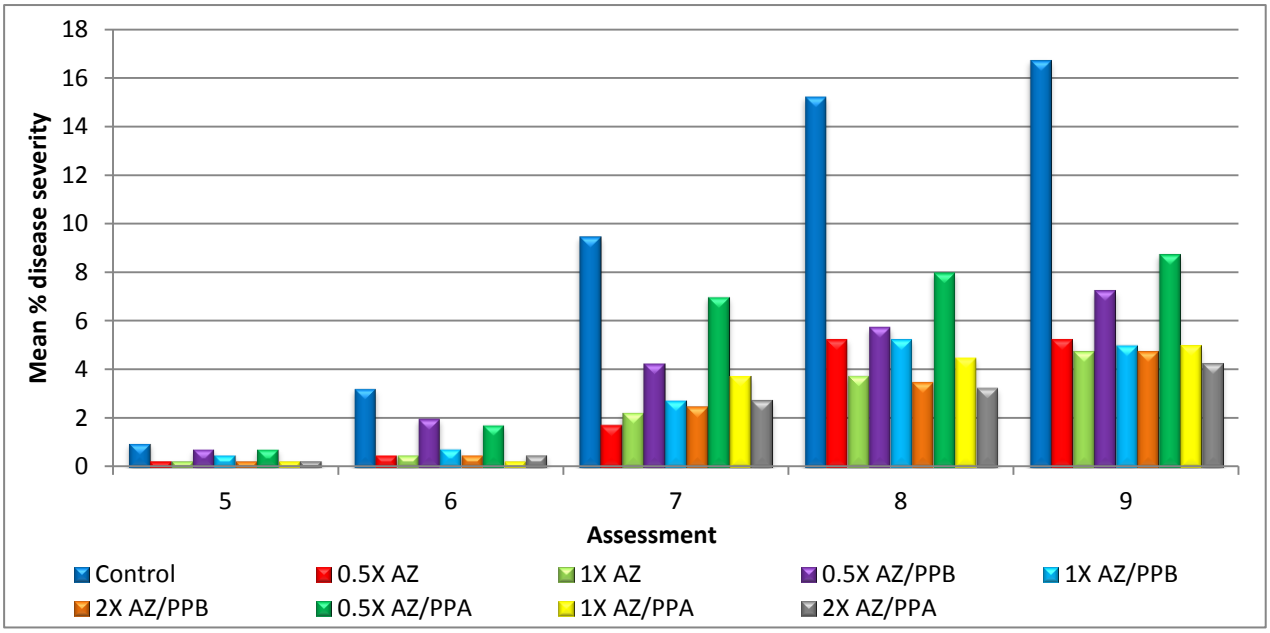


Figure 4-6: The effect of treatments on late blight severity of tomato plants

Figure 4-6 compares the mean percentage disease severity that was observed with the 0.5X formulations as well as the control. A high percentage disease severity during the first assessment is preferable to a lower percentage as it is more reliable. The smaller the initial percentage is, the smaller change in disease severity is required to obtain a high efficacy. With a higher disease severity a larger change in severity is required to obtain the same efficacy as the latter. Since 0.5X AZ had significantly less late blight severity during assessment 5 it was highly likely that the treatment would result in the lowest mean percentage disease severity with

the following assessment (as it did). The 0.5X AZ/PPB formulation experienced a constant increase in disease severity and therefore had a constant effect of control on the disease (compared to the control treatment). During the last three assessments (from assessment 7 to 9), 0.5X AZ/PPA increased significantly less in disease severity than the other 0.5X treatments (only 1.75%). This signifies that, in the long run, this treatment might result in far better late blight control than the other treatments.

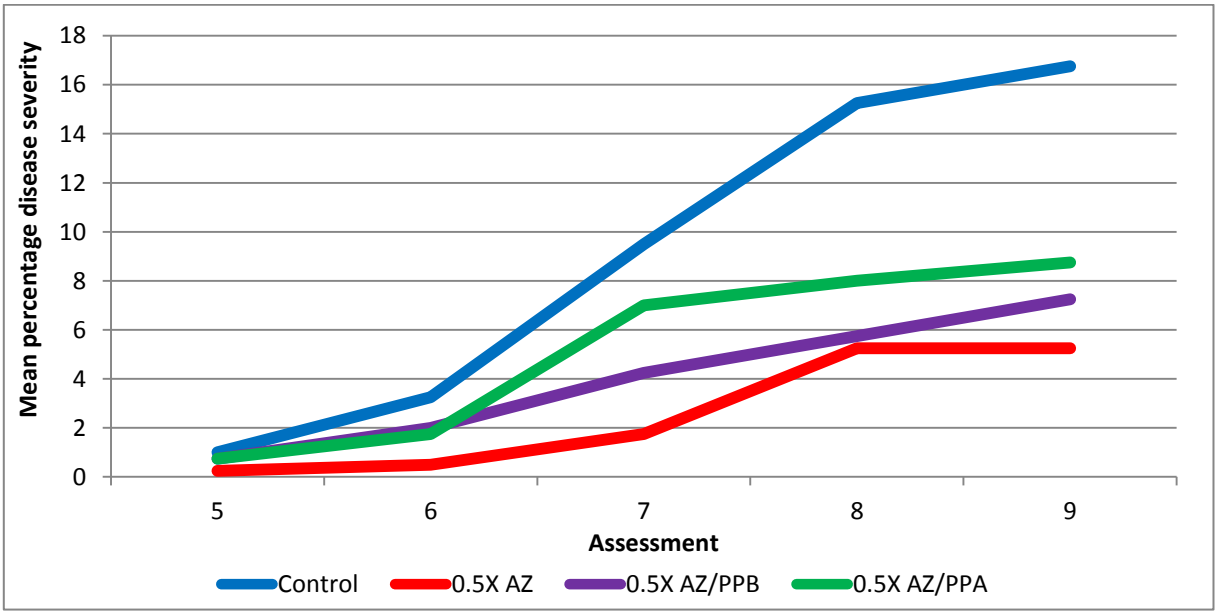


Figure 4-7: Comparison of mean percentage late blight severity between the control and the various 0.5X formulations throughout the study

Figure 4-7 compares the mean late blight severity observed with the control and the 1X formulations throughout the study. The AZ formulations had similar disease severity distributions throughout the study. The 1X AZ/PPB formulation did not present the same constant disease control as the 0.5X AZ/PPB formulation, but the 1X AZ did. From assessment 8 to 9, a reduction of 0.25% was observed (from a low percentage of 5.25 to 5.00%) in disease severity with the 1X AZ/PPB treatment. Although this is a small difference, it should be noted that this was the only treatment to result in a decrease in disease severity at any stage of the study. The three 1X treatments had similar mean percentage disease severities at the final assessment (4.75 to 5.00%).

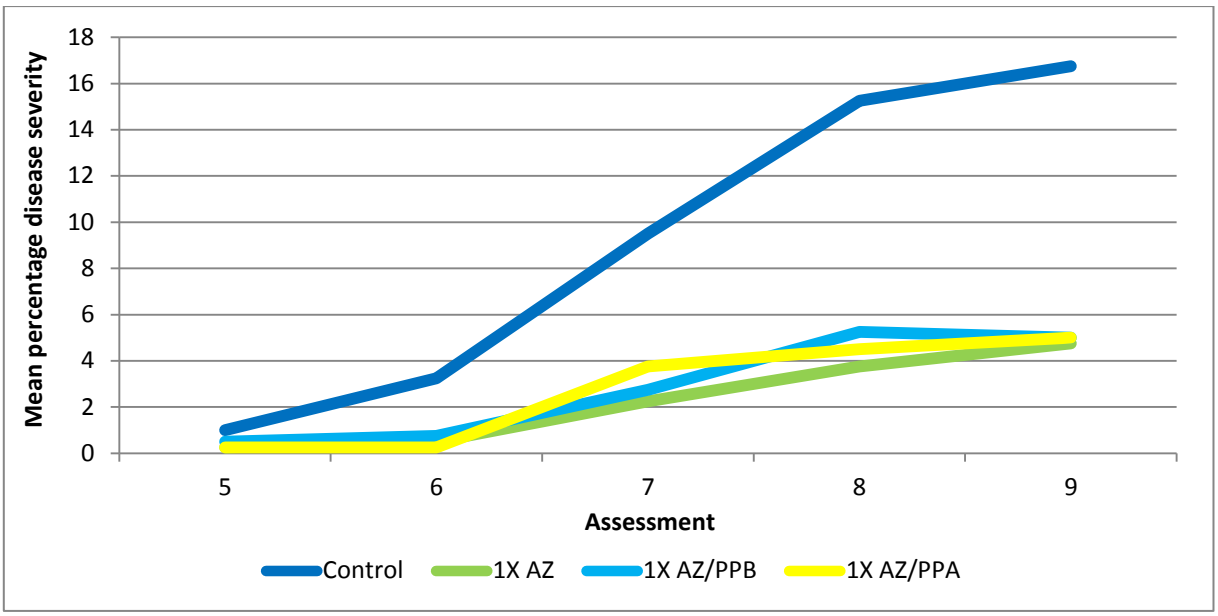


Figure 4-8: Comparison of mean percentage late blight severity between the control and the various 1X formulations throughout the study

However, Table 4-27 and Figures 4-5 to 4-7 is only a reflection of the mean disease severity that was observed during the different assessment days in relation to the treatments that were applied and does not consider that the plots had different mean disease severity values to begin with. The corrected level of effectiveness results on tomato leaves are presented in Table 4-28. The corrected level of effectiveness (percentage inhibition relative to the control) was calculated by expressing the mean percentage disease severity in treated plots as a percentage of mean percentage disease severity in the control plots. Since no late blight was found at assessments 1 – 4, only assessments 5 – 9 are shown in Table 4-28.

Table 4-28: The corrected level of effectiveness for the treatments against late blight

Treatment	Assessment				
	5	6	7	8	9
Control	0.00	0.00	0.00	0.00	0.00
0.5X AZ	75.00	84.62	81.58	65.57	68.66
1X AZ	75.00	84.62	76.32	75.41	71.64
0.5X AZ/PPB	25.00	38.46	55.26	62.30	56.72
1X AZ/PPB	50.00	76.92	71.05	65.57	70.15
2X AZ/PPB	75.00	84.62	73.68	77.05	71.64
0.5X AZ/PPA	25.00	46.15	26.32	47.54	47.76
1X AZ/PPA	75.00	92.31	60.53	70.49	70.15
2X AZ/PPA	75.00	84.62	71.05	78.69	74.63

Table 4-28 indicates that treatment 2X AZ/PPA was the most efficacious treatment compared to the control. 2X AZ/PPB also presented with a high corrected efficacy; however, both formulations were double the strength of 1X AZ, which had the same corrected level of effectiveness as 2X AZ/PPB. The azoxystrobin/pro-Pheroid formulations that were parallel to 0.5X AZ, namely 0.5X AZ/PPA and 0.5X AZ/PPB had significantly smaller corrected level of effectiveness (68.66% compared to 47.78% and 56.72% in turn). Formulation 1X AZ, 1X AZ/PPA and 1X AZ/PPB was equal in strength and had similar corrected levels of effectiveness (71.64%, 70.15% and 70.15% respectively). 1X AZ was proved to be the most effective treatment for the control of late blight in tomatoes but did not differ significantly from treatment AZ/PPB and AZ/PPA.

The treatments that started with the lowest mean disease severity resulted in the highest corrected level off efficacy throughout. This is to be expected since a reduction from a smaller initial value results in a higher percentage reduction compared to an equal reduction in a value with a higher starting value. The three treatments had very different corrected level of effectiveness distributions (see Figure 4-8). Treatment 0.5X AZ had fluctuating efficacies with the various assessments with a ~3% increase as the trial concluded. Treatment 0.5 AZ/PPA had a very unorthodox distribution that alternated with low and higher percentages. Treatment 0.5X AZ/PPB increased in corrected efficacy with every assessment until the last assessment when a reduction of 6.00% was observed.

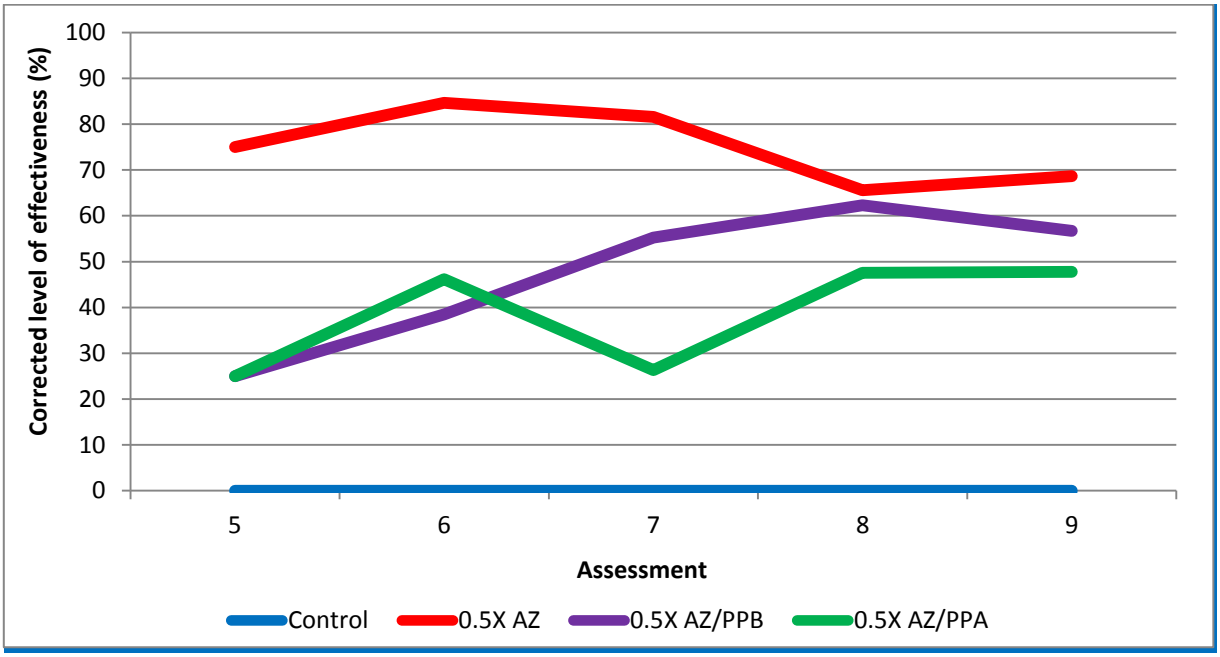


Figure 4-9: Comparison of the efficacies obtained with the various 0.5X formulations relative to the control against late blight

The corrected level of effectiveness obtained for the 1X formulations compared to the control are illustrated in Figure 4-9. All three treatments had an increase in efficacy from assessment 5 to 6, followed by a reduction at assessment 7. For assessment 6, treatment 1X AZ/PPA obtained the highest corrected level of effectiveness of all treatments at any stage of the study (92.31%). The 1X treatments fluctuated significantly less than the 0.5X treatments. When the study concluded, the three formulations had efficacies between 70.15% and 71.64%.

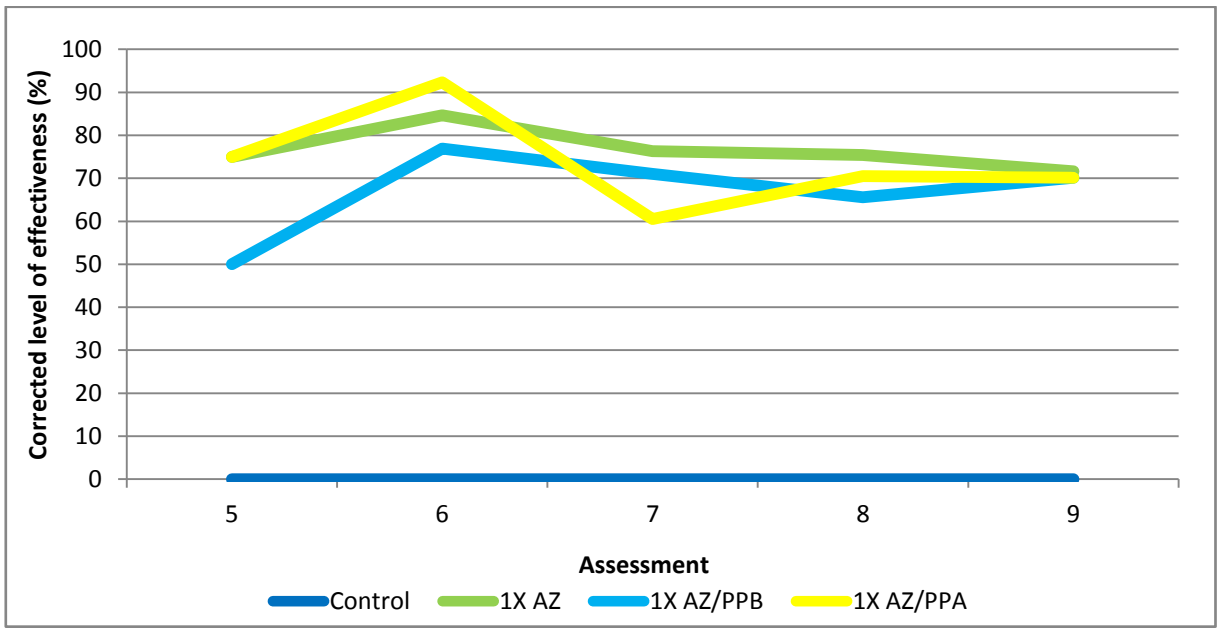


Figure 4-10: Comparison of the efficacies obtained with the various 1X formulations relative to the control against late blight

4.4.4.2 Results: Azoxystrobin/pro-Pheroid formulations in the control of white blister on cabbage

Results of white blister severity are presented in Table 4-29. Efficacy was determined in terms of percentage disease severity. No white blister was found at assessments 1 and 2, therefore only assessments 3 – 6 are shown in Table 4-29. Means followed by the same letter do not significantly differ (p=0.05, established with Student-Newman-Keuls) while a p-value of 0.05 (obtained by Bartlett's test) was regarded as statistically significant. No phytotoxic effects were experienced with any of the formulations at any given time of the trial studies (presented in Annexure C2). The mean values in Table 4-29 are reflected in Figure 4-10 for ease of comparison.

Table 4-29: The effect of treatments on white blister severity of cabbage leaves

Assessment		3		4		5		6	
Treatment number	**Treatment name**								
1	**Control**	2.95 ± 0.83	a	3.05 ± 1.50	a	9.65 ± 2.68	a	13.90 ± 1.30	a
2	**0.5X AZ**	0.55 ± 0.50	c	0.70 ± 0.83	c	1.40 ± 2.49	d	2.55 ± 1.30	bc
3	**1X AZ**	0.65 ± 0.50	c	0.80 ± 1.09	c	1.10 ± 2.68	d	1.75 ± 0.83	c
4	**0.5X AZ/PPB**	2.45 ± 1.00	ab	2.30 ± 1.48	b	3.70 ± 1.64	b	4.65 ± 2.17	b
5	**1X AZ/PPB**	0.7 ± 0.83	c	0.65 ± 0.83	c	2.05 ± 2.59	cd	3.30 ± 1.87	bc
6	**2X AZ/PPB**	0.45 ± 0.50	c	0.70 ± 1.12	c	1.60 ± 1.12	d	1.80 ± 0.83	c
7	**0.5X AZ/PPA**	2.15 ± 0.43	b	2.05 ± 2.24	b	3.20 ± 1.55	bc	3.45 ± 1.48	bc
8	**1X AZ/PPA**	0.8 ± 0.50	c	0.65 ± 1.48	c	1.35 ± 1.12	d	2.85 ± 0.71	bc
9	**2X AZ/PPA**	0.65 ± 0.50	c	0.90 ± 1.30	c	2.00 ± 1.30	cd	2.30 ± 1.79	bc
LSD (p =.05)		0.588		0.529		1.089		1.733	
Standard Deviation		0.403		0.362		0.747		1.187	
CV		31.93		27.64		25.79		29.24	
Bartlett's X2		11.231		7.848		13.422		19.263	
p (Bartlett's X2)		0.189		0.448		0.098		0.014*	
Replicate F		0.902		0.429		0.619		1.501	
Replicate Prob (F)		0.4545		0.7342		0.6097		0.2396	
Treatment F		23.111		25.13		51.503		40.925	
Treatment Prob(F)		0.0001		0.0001		0.0001		0.0001	

Means followed by the same letter do not significantly differ (p =.05, Student-Newman-Keuls)
Mean comparisons performed only when Analysis of Variance (AOV) Treatment Prob (F) is significant at mean comparison observed significance level (OSL).
* = significant at p = 0.05 (Bartlett's test)

By the time that assessment 3 took place, all of the fungicides had been applied once (refer to Table 4-23 for a description of the study design). The disease severity in the control plots increased with every assessment and presented with the highest mean disease severity throughout the study. Although the plots that were treated with 0.5X AZ/PPA had the third highest white blister severity at the last assessment, it only increased by 1.50% since assessment 3. The highest disease severity was obtained with treatment 0.5X AZ/PPB at the conclusion of the study whereas treatment 1X AZ and 2X AZ/PPB had the least (see Figure 4-10).

The dose response for treatment AZ/PPA and AZ/PPB were more promising against late blight than white blister although AZ/PPB was not saturated at assessment 9 (occurred against late blight).

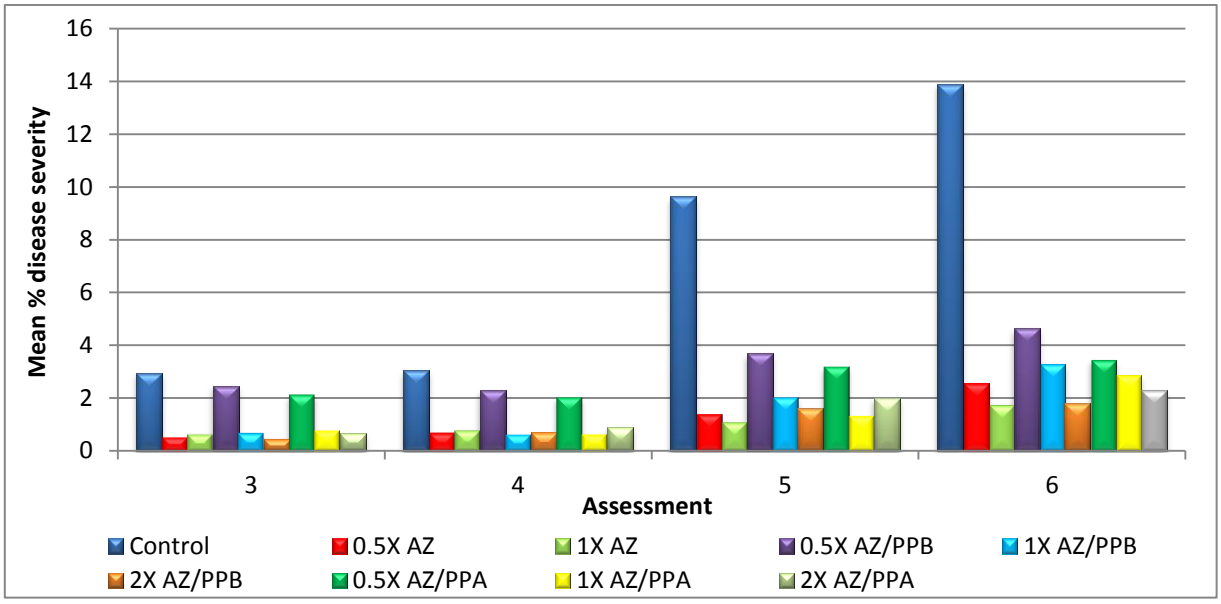


Figure 4-11: The effect of treatments on white blister severity of cabbage leaves

Figure 4-11 displays the mean percentage white blister severity for the control and 0.5X treatments. Treatment 0.5X AZ/PPB and 0.5X AZ/PPA had similar mean disease severities throughout the study. During assessment 3 and 4, the control had a mean disease severity that were closely distributed to the other treatments but differed significantly after the 4th assessment.

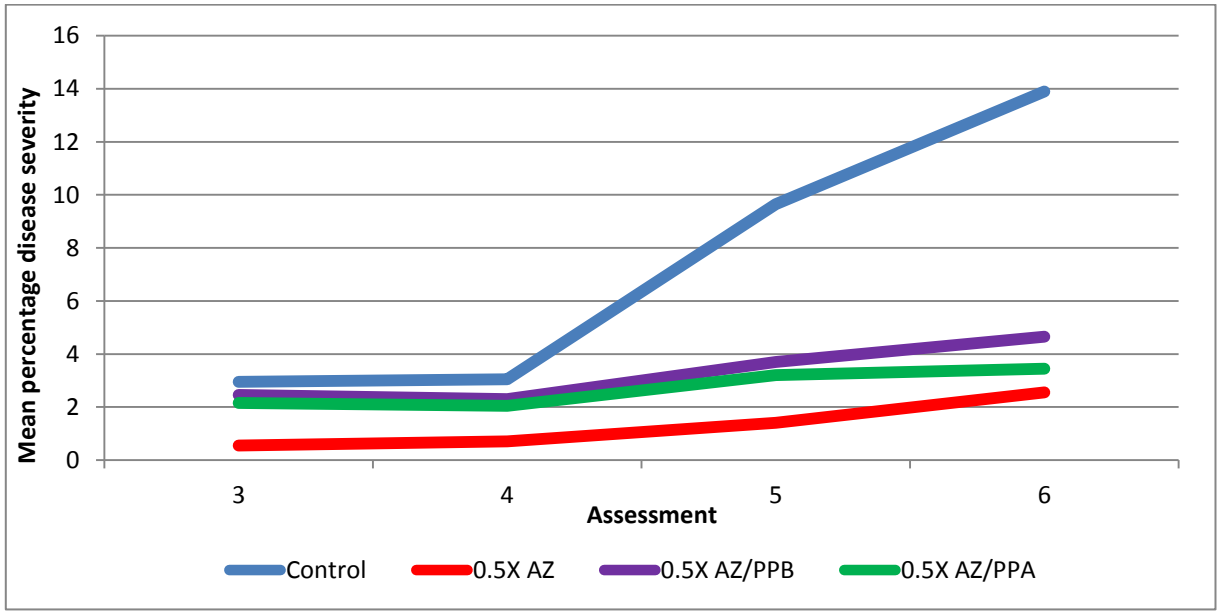


Figure 4-12: Comparison of mean percentage white blister severity between the control and the various 0.5X formulations throughout the study

Formulations that received treatment 1X AZ, 1X AZ/PPB and 1X AZ/PPA had similar mean percentage disease severities throughout the study that was significantly lower than that obtained with the control. The 1X formulations resulted in lower mean disease severities than the 0.5X formulations which is to be expected.

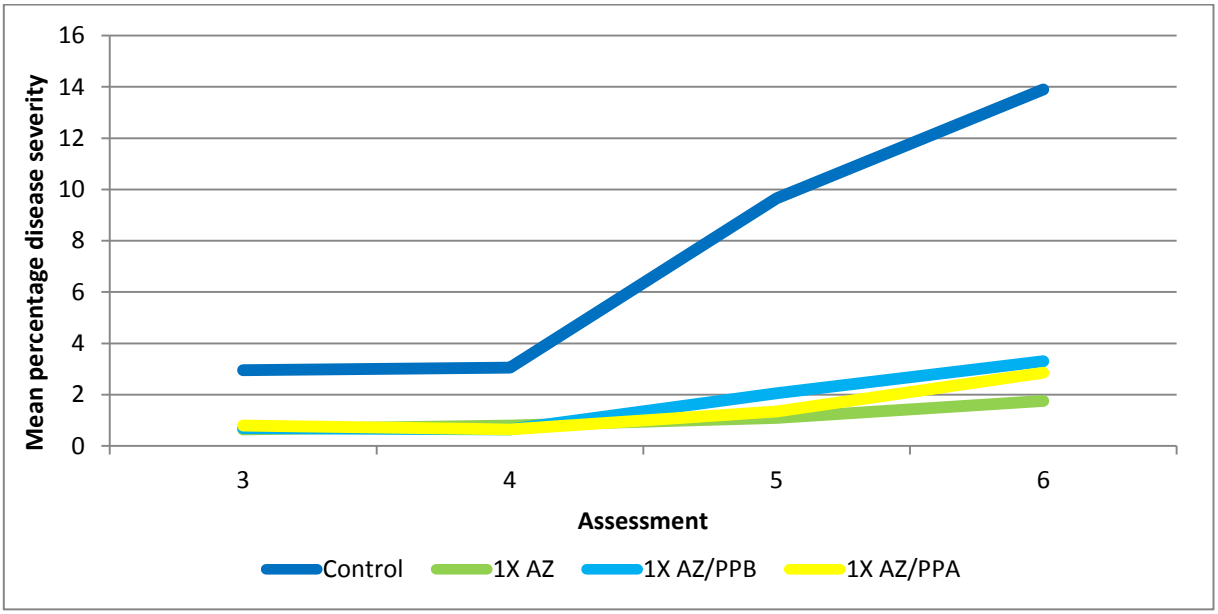


Figure 4-13: Comparison of mean percentage white blister severity between the control and the various 1X formulations throughout the study

However, the initial disease severity (assessment 3) was not taken into account in Table 4-29 and Figures 4-10 to 4-12 and therefore is not an accurate representation of the efficacies that were obtained with the various treatments, but merely an illustration of the mean disease severity observed for each treatment during the various assessments. Table 4-30, on the other hand, reflects the corrected level of effectiveness results on the cabbage leaves. This is an indication of the percentage white blister inhibition relative to the control and was calculated by expressing the mean percentage disease severity in the treated plots as a percentage of mean percentage disease severity in the control plots. No white blister was found at assessment 1 and 2, therefore only assessments 3-6 are shown in Table 4-30.

Table 4-30: The corrected level of effectiveness for the treatments against white blister

Treatment	Assessment			
	3	4	5	6
Control	0.00	0.00	0.00	0.00
0.5X AZ	81.36	77.05	85.49	81.65
1X AZ	77.97	73.77	88.60	87.41
0.5X AZ/PPB	16.95	24.59	61.66	66.55
1X AZ/PPB	76.27	78.69	78.76	76.26
2X AZ/PPB	84.75	77.05	83.42	87.05
0.5X AZ/PPA	27.12	32.79	66.84	75.18
1X AZ/PPA	72.88	78.69	86.01	79.50
2X AZ/PPA	77.97	70.49	79.27	83.45

0.5X AZ/PPB, with a corrected level of effectiveness of 66.55% was the least effective treatment against white blister on cabbage. The comparator was significantly more effective (81.65%). Treatment 2X AZ/PPA and 2X AZ/PPB was less effective than the half-strength comparator, 1X AZ (with corrected levels of effectiveness of 83.45%, 87.05% and 87.41% respectively. At equal strengths, 1X AZ was significantly more effective than 1X AZ/PPA (79.50%) and 1X AZ/PPB (76.26%).

A comparison of the corrected level of effectiveness between the various 0.5X treatments is presented in Figure 4-13. With assessment 3, treatment 0.5X AZ had a much higher efficacy that the other two formulations. However, this is because the plants treated with 0.5X AZ had a much smaller incidence of the disease than the other 0.5X treatments. From assessment 4 to 5, treatment 0.5X AZ/PPB and 0.5X AZ/PPA had a significant increase in efficacy compared to treatment 0.5X AZ. Treatment 0.5X AZ controlled the white blister with a relatively constant corrected efficacy of between 77.05% and 85.49% during the course of the study and had only 0.29% more mean white blister severity at the conclusion of the study than with assessment 3.

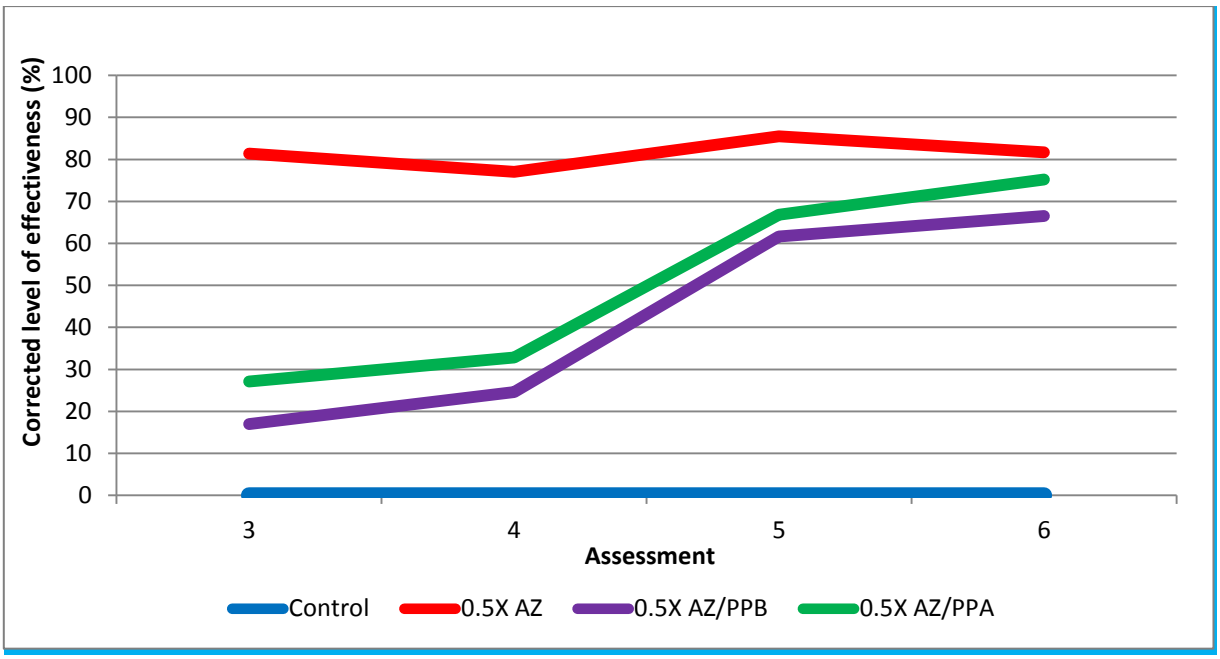


Figure 4-14: Comparison of the efficacies obtained with the various 0.5X formulations relative to the control against white blister

The 1X treatments presented with similar corrected level of effectiveness distributions that varied between 72.88% and 88.60% (see Figure 4-14). Treatment 1X AZ/PPB presented with a constant level of effectiveness throughout the study that ranged between 76.26% and 78.76%.

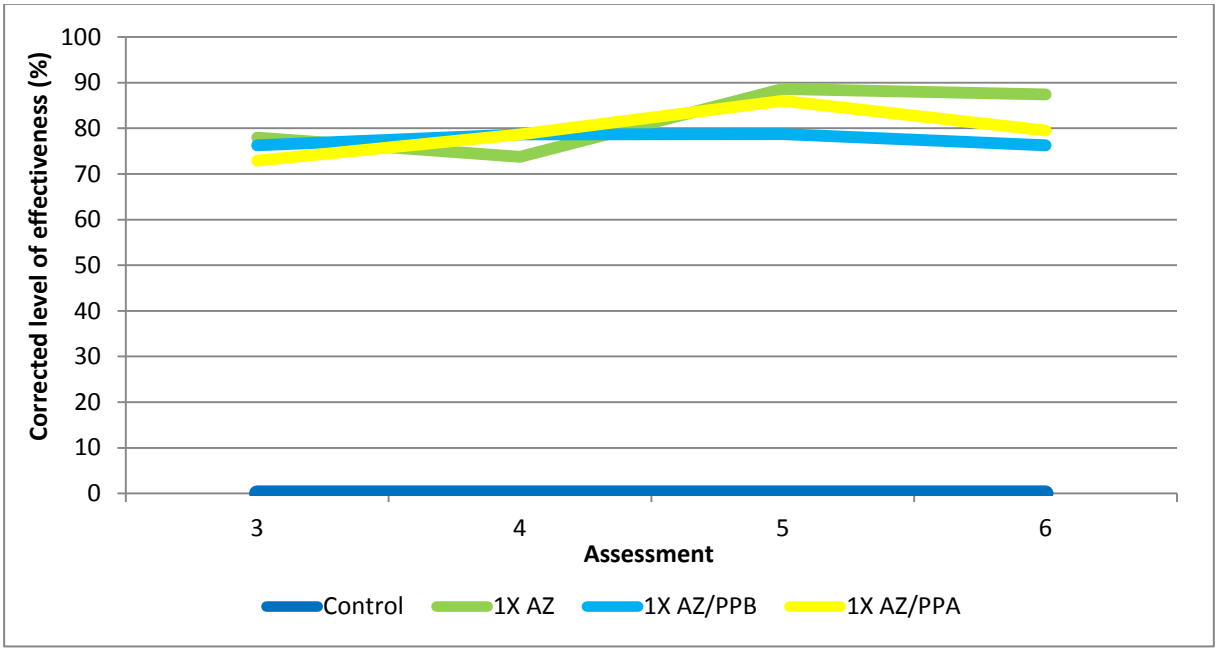


Figure 4-15: Comparison of the efficacies obtained with the various 1X formulations relative to the control against white blister

4.5 Conclusion

Due to insufficient data, the Pheroid specifications could not be described and formalized as aimed and could therefore not be applied to or verified by the azoxystrobin formulations. It was decided to proceed with the azoxystrobin formulations taking the obvious impact of solubility observed during the analysis into account despite the fact that specifications could not be established for use as guidelines. After numerous solubility studies were carried out, two formulations were deemed fit for manufacture. These formulations contained 12.50% and 6.25% azoxystrobin each whereas the desired strength (and that of the comparator) was 25% because it was observed that the formulations with higher amounts of active were more prone to crystallization. Appropriate adaptation of the dilution factor for the 12.50% and 6.25% formulations resulted in formulations that were equivalent in strength to the 25.00% comparator.

The characterization analyses that were carried out on the formulation showed unsatisfactory results but despite the struggles that were experienced with crystallization of the concentrates or precipitation of the diluted formulations, the formulations were still sent for efficacy and possible phytotoxicity studies against late blight (*Phytophthora infestans*) on tomatoes and white blister (*Albugo candida*) on cabbage. Unfortunately, and as could be expected from the characterization studies, the azoxystrobin/pro-Pheroid formulations proved to be less effective than the same strength comparator with both trials.

In the control of late blight in tomatoes, Amistar 250 SC (250 g/L) was the most effective formulation with a corrected level of effectiveness of 71.64%. Similar efficacies were obtained with the 6.25% and 12.50% azoxystrobin/pro-Pheroid formulations (both 70.15%) at equal strength. Amistar 250 SC (250 g/L) was also the best treatment against white blister on cabbage (87.61% corrected level of effectiveness). The azoxystrobin/pro-Pheroid formulations were significantly less effective than the comparator – even at double the concentrations.

The azoxystrobin/pro-Pheroid had good dose responses during both trial studies which signifies that increased efficacy could be obtained when a smaller dilution ratio is applied.

The Pheroid based formulations, AZ/PPA and AZ/PPB had the same corrected level of effectiveness against late blight on tomatoes, but against white blister on cabbage, AZ/PPA was more effective than AZ/PPB. This could be due to the fact that AZ/PPA contained double the amount of pro-Pheroid as the other formulation. Since AZ/PPA is a 12.50% formulation, double the amount of formulation used for the 12.50% formulation was required to obtain equally concentrated dilutions (the undiluted formulations contained ~34.00% pro-Pheroid). On the other hand, the results could also have been influenced by the excipients that were used. It

would be valuable to focus future research on the determining the influential factor and the degree to which the factors influenced the results.

Neither of the azoxystrobin/pro-Pheroid formulations had increased efficacy compared to the comparator although the results were promising. Therefore, the solubility studies should be revisited as signified by the results obtained from the characterization analyses.

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CHAPTER 5 EFFICACY EVALUATION OF CHLORPYRIFOS WITH PHEROID® TECHNOLOGY FOR AGRICULTURAL APPLICATION

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5.1 Introduction to the chapter

The article was written for the Agriculture, Ecosystems and Environment journal (see Annexure D for the author guidelines). Like the title implies, the journal is focussed on research that integrates agriculture and the environment. The chlorpyrifos manuscript intended for submission to this journal focussed on the incorporation of chlorpyrifos in Pheroid to reduce its environmental impact. Chlorpyrifos is one of the most commonly used insecticides worldwide, although parties concerned about the environment are opposed to its use. Regardless of their efforts to raise awareness on the subject, the product remains in high demand. Consequently, it was decided that, since it seems that the use of chlorpyrifos will be decreased radically in the near future, its impact on the environment will have to be lessened. In pursuit of that aim, chlorpyrifos was formulated in Pheroid technology, after which trial plot studies were conducted to determine the formulation's efficacy and possible phytotoxicity against African Bollworm on tomatoes and cabbage aphids.

Abstract

Despite the increasing population, the agricultural productivity is drastically decreasing in especially developing countries for reasons of poor rainfall and pests, consequently, threatening these countries' long-term food security. Pesticides are commonly employed to address this problem but are not always safe, environmentally suitable and cost effective. In a novel approach to overcome those barriers, one of the most frequently used organophosphates, chlorpyrifos, was formulated with the Pheroid® delivery system. The efficacy and phytotoxicity of the formulation were determined via trial plot studies on *Helicoverpa armigera* (tomatoes) and *Brevicoryne brassicae* (cabbage). Increased efficacy was obtained with the Pheroid®-based chlorpyrifos formulation without any signs of phytotoxicity. At half the concentration of the commercial standard, the Pheroid® entrapped chlorpyrifos proved to be 15.30% more effective against African bollworm and at the same strength as the comparator, 14.90% more effective in the control of cabbage aphids. This translates into a reduced environmental effect associated with the insecticide.

Keywords

Keywords: Chlorpyrifos, organophosphate, insecticide, Pheroid®, delivery system, bollworm, aphid, phytotoxicity

5.2 Introduction

Many smallholders depend on the production of crops for nutrition, income or animal feed (Romney *et al.*, 2003; Baiphethi & Jacobs, 2009; Thornton, 2010; Fischer & Hajdu, 2015). In developing countries such as South Africa, crop production reduced by more than 44.00% over the past two years (Crop Estimates Committee, 2015) while a 59.00% decrease is expected from 2016 to 2017 (Crop Estimates Committee, 2016) due to factors such as drought and pests. With an average annual growth of nearly 2.00% (Statistics South Africa, 2015), the population (~55 million in 2015) is expected to reach 82 million by 2035. By that time, it would be essential that the country's current food production is doubled to ensure adequate nourishment (WWF - SA, 2010). This is in line with the worldwide trend of population growth (an average increase of 1.18% per annum) (FAO, 2015).

Worldwide, 14.10% of crop losses are due to plant diseases (including attacks by insects) which amount to about $220 billion in losses (Agrios, 2005:4). One of the methods used to address this problem is the application of pesticides such as chlorpyrifos. Chlorpyrifos (*O,O*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothioate) is used in agriculture, horticulture and forestry against several members of the Dipteran, Coleopteran, Homopterous and Lepidopteran orders (Paranjape *et al.*, 2014:98), in and around the home against various pests (including fire ants, termites, lice, cockroaches, flies and roundworms) (Greene, 2013:217; Pohanish, 2014:136)

and even for insect control on animals (Dikshith, 2016:173). Nonetheless, it is mainly utilized for its insecticidal properties on corn (US EPA, 1999).

Pesticides are formulated to be toxic to living organisms and, unfortunately, humans and animals are no exceptions. After exposure, chlorpyrifos is metabolically activated into a strong inhibitor of acetyl cholinesterase (AChE), chlorpyrifos-oxon. The breakdown of the acetylcholine neurotransmitter is inhibited, accumulation occurs at cholinergic synapses, and overstimulation of the neuronal cells takes place (Costa, 2006). This results in neurotoxicity (typically referred to as "cholinergic syndrome") which may cause dizziness, nausea, headaches and, at high exposure rates, respiratory paralysis and ultimately death (Mileson *et al.*, 1998; Karanth & Pope, 2000; Pohanish, 2014:137). It is estimated that close to half a million kilograms of this compound are utilized each year by the United States alone (US EPA, 2005). In Sprague-Dawley rats, chlorpyrifos has been shown to have an acute oral median lethal dose (LD_{50}) of 229 mg/kg, and a dermal LD_{50} of >2000 mg/kg, and has therefore been classified as moderately hazardous by the World Health Organization (WHO/IPCS, 2010:67). Chlorpyrifos has a higher acute toxicity in rats than most organophosphates (OP's). In contrast; studies indicate that it is the least toxic OP to humans (Eddleston *et al.*, 2005). Chlorpyrifos has been shown to be a high risk to birds, aquatic invertebrate species, fish and small mammals after a single application. After multiple applications, these species are at a prolonged risk, either by directly influencing their existence, breeding or development, or indirectly by decreasing their food sources or altering their habitat (i.e. residues in soil or water) (US EPA, 2009; Loewy *et al.*, 2011; Köhler & Triebskorn, 2013). Therefore, a reduction in the current use of the compound is crucial.

Despite the fact that numerous studies have been carried out on chlorpyrifos in the past few decades demonstrating its environmental and ecological impact, it remains one of the most frequently used insecticides worldwide (Jarvinen *et al.*, 1988; van den Brink *et al.*, 1995; US EPA, 1999; DeLorenzo *et al.*, 2001; US EPA, 2009; John & Shaike, 2015; Tsaboula *et al.*, 2016). Since (a) there is no current good alternative in conventional farming practices, and (b) it seems that, regardless of the available information, individuals demonstrate a lack of willingness to adapt its usage accordingly, this study approached the predicament from another perspective and aimed to formulate a chlorpyrifos formulation with increased efficacy at a lower concentration. This way, the use of the product can be continued but with less impact on the surroundings. The aim was approached by incorporating chlorpyrifos into the pro-Pheroid® system.

The use of Pro-Pheroid® in plants has been patented by the North-West University, Potchefstroom, South Africa (Grobler, 2012). Pheroid® technology underlies an environmentally friendly colloidal delivery system comprising mainly of modified fatty acids. Pro-Pheroid®,

consisting of an oil phase and an interspersed gas phase, is only one of the Pheroid® types, which have the ability to be altered according to indication with regards to morphology, size, structure, and function (Grobler *et al.*, 2008; Grobler, 2009). Pro-Pheroid® was chosen above the other water based Pheroid® types due to the hydrophobic nature of chlorpyrifos (log K_{ow} of 4.7 at 24.5°C) (Tomlin, 2006:186; Paranjape *et al.*, 2014; Pohanish, 2014:137). Pheroid® technology is versatile and safe for animal and human consumption (Elgar, 2008; Grobler, 2009). Pheroid®, referring to unique lipid-based submicron- and micron-sized vesicles evenly distributed in the dispersion medium, has the ability to entrap, transport and deliver active compounds over most physiological barriers (including cellular membranes of plants or organisms) (Grobler *et al.*, 2008; Grobler, 2009).

In agriculture, Pheroid®, unlike many additives currently used in the industry, is completely harmless by itself and exhibit a peculiar ability to combine an array of functions of additives (Grobler, 2009). The carrier system, without containing any active ingredient, was shown to increase crop yield, stimulate plant growth stages, enhance physical appearance and improve disease resistance (Grobler, 2012). It has the potential to modify the toxicity of the active compounds and improve their target specificity on pests (Grobler, 2009). This application of the delivery system is known as 'enabling technology', and is used to formulate non-toxic, user-friendly and effective products.

Therefore, a novel pro-Pheroid® formulation containing chlorpyrifos was developed and evaluated for its efficacy and possible phytotoxicity against African bollworm (tomatoes) and aphids (cabbage) through trial plot studies. The hypothesis of the study was that the incorporation of chlorpyrifos in the Pheroid® system would result in an efficacious insecticide that has reduced toxic effects on the environment.

5.3 Materials and methods

5.3.1 Materials

Chlorpyrifos (98.00% TC) was obtained from Plum Agrochemical Consulting & Service Co., Ltd, Shanghai, China. Pro-Pheroid® is made up from vitamin F ethyl ester (Chemisches Laboratorium Dr. Kurt Richter GmbH, Germany), DL-α-tocopherol (Chempure (Pty) Ltd, South Africa), kolliphor™ RH40 (BASF SA (Pty) Ltd, South Africa) and N_2O (Afrox, South Africa) in unique patented ratios. The general method of preparation for the system is described in Grobler *et al.* (2014). To protect Intellectual Property (IP) any other excipient(s) used in the formulation as well as the specific method of preparation is not specified in this article. For morphology characterisation, the chlorpyrifos/pro-Pheroid® formulation was labelled with the fluorophore, Nile red (Molecular Probes, Thermo Fisher Scientific Inc. Waltham, MA, USA).

5.3.2 Methods

5.3.2.1 Characterization of the formulation

Both qualitative (CLSM) and quantitative (particle size and particle size distribution and zeta potential) studies were carried out on the formulation.

The mean particle size and particle size distribution were measured through light scattering by means of a particle size analyser (Malvern Mastersizer 2000, Malvern Instruments Ltd, Malvern, Worcestershire, UK United Kingdom). Once the laser was aligned with deionised water in the dispersion unit, the sample was slowly added and stirred at 1500rpm until an obscuration rate of 10 – 20% was obtained. The results were presented in Figure 5-1 showing the distribution of different particles sizes and the volume (%) they represent in the sample. The analysis was carried out in triplicate.

The formulation's morphological features were assessed by via confocal laser scanning microscopy (CLSM). The images were captured with a Nikon D-eclipse C1 confocal laser scanning microscope (Nikon, the Netherlands). A 60x plan apochromatic objective was used to record tungsten sourced light and fluorescent micrographs of the sample. The sample was labelled with Nile Red (N-1142, excitation/emission maxima ~552/636 nm). The fluorophore does only emit red fluorescence when in association with a lipid. Because of its lipophilic nature, Nile red associates with the lipid molecules in Pheroid® formulations. Therefore, the lipophilic bilayer of the Pheroid® fluoresces with a prominent red colour while the non-fluorescent aqueous core is dark. Photon emissions were collected within three wavelength bands (above 650 nm, 540 to 640 nm and 485 to 545 nm) and merged to form an image. Point illumination by means of a small spatial pinhole is used to exclude photons emitted from the out of focus parts of the sample.

To measure the surface charge, 1 µl sample was diluted with 5 ml ddH_2O and injected into the Malvern Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, Worcestershire, United Kingdom). The measurement was carried out in triplicate.

5.3.2.2 Trial plot studies

Field trials were conducted as from March 2016 by Agricultural Science Consultants™ (ASC), Western Cape, South Africa to evaluate the efficacy and possible phytotoxicity of the chlorpyrifos/pro-Pheroid 480 EC (emulsifiable concentrate) formulation (CPF/PP) on both African bollworm (tomatoes) and aphids (cabbage) and took place in Malmesbury and Joostenbergvlakte (Western Cape Province, South Africa) respectively. The plants and pests

were chosen based on their accessibility and suitability in the South African climate as well as their general association with chlorpyrifos (Obopile *et al.*, 2008) . Treatments were applied with a mist blower at a volume of 500 L/ha to ensure thorough coverage of the plants without run off. The plants were watered by means of sprinkler irrigation. Plants were monitored for any signs of possible adverse effects on growth (e.g. chlorosis (yellowing), stunting, necrosis or node curl) and possible phytotoxic reactions. The percentage reduction in the number of fruit damaged (tomatoes) or aphid count (cabbage) was calculated with the following formula (Ahmad *et al.*, 2007):

$$Mean\ \%\ reduction = \left(\frac{Mean\ pre\ treatment\ count - Mean\ post\ treatment\ count}{Mean\ pre\ treatment\ count}\right) x\ 100$$

5.3.2.2.1 Study design: African bollworm (tomatoes)

The efficacy of CPF/PP at three different dosage rates (75 mL/100 L [0.5X], 150 mL/100 L [1X] and 300mL/100 L [2X]) was compared to a standard chlorpyrifos 480 EC (CPF) (at dosage rate 150mL/100 L [1X]) and an untreated control in order to determine the ability of CPF/PP to control African bollworm on tomato plants in terms of damage incidence on fruit. Twenty plots, each sized 4 m x 5 m (20 m^2), were distributed in a randomized complete block (total plot size: 400m^2). The five treatment formulations were randomly replicated in four blocks. The first application occurred during the 4th fruit cluster (BBCH 74). Applications were carried out in triplicate. Assessments on tomato plants damaged by African bollworm were done prior to each application as well as 7 days after the last application (see Table 5-1). Assessments were done on fifty fruit from each plot by counting the number of fruit damaged by African bollworm and expressing it as a percentage.

5.3.2.2.2 Study design: Aphids (cabbage)

CPF/PP at three different dosage rates (25 mL/100L [0.5X], 50 mL/100 L [1X], and 100 mL/100L [2X]) was compared to a standard Chlorpyrifos 480 EC (CPF) (at dosage rate 50mL/100L [1X]) and an untreated control in order to determine the ability of CPF/PP to control aphids (*Brevicoryne brassicae*) on cabbage with regards to pest incidence on plants. Twenty plots were distributed in a randomized complete block (total plot size: 200 m^2), each comprising of twenty plants (10 m^2). The five treatment formulations were randomly replicated in four blocks. The pesticide was applied once only - when 40.00% of the expected cabbage head size was reached (BBCH 44). Assessments of cabbage aphid infestation were done prior to application as well as 7, 14 and 21 days after the application (see Table 5-2). Assessments were done on five plants from the centre of each plot by counting the numbers of aphids present.

5.3.3 Statistical analysis

The analyses were executed with the use of Descriptive Statistics, ANOVA, Bartlett's test (used to determine the homogeneity of variance) and Student-Newman-Keuls (identifies sample means that are significantly different from each other). A p-value of 0.05 (obtained by Bartlett's test) was regarded as statistically significant.

5.4 Results and Discussion

5.4.1 Characterization of the formulation

The particle size distribution is displayed in Figure 5-1. The particle diameters were determined at the 10th, 50th, and 90th percentile of the particles and are indicated by $d_{0.1}$, $d_{0.5}$ and $d_{0.9}$ respectively. The $d_{0.1}$ of 0.178 µm indicates that 90% of the particles in the formulation are above that size. The $d_{0.5}$ of 1.987 µm reveals the mean particle size of the sample whereas 90% of the particles present in the formulation are below the $d_{0.9}$ of 11.192 µm in size. The results represent a standard particle size distribution for pro-Pheroid® agricultural products.

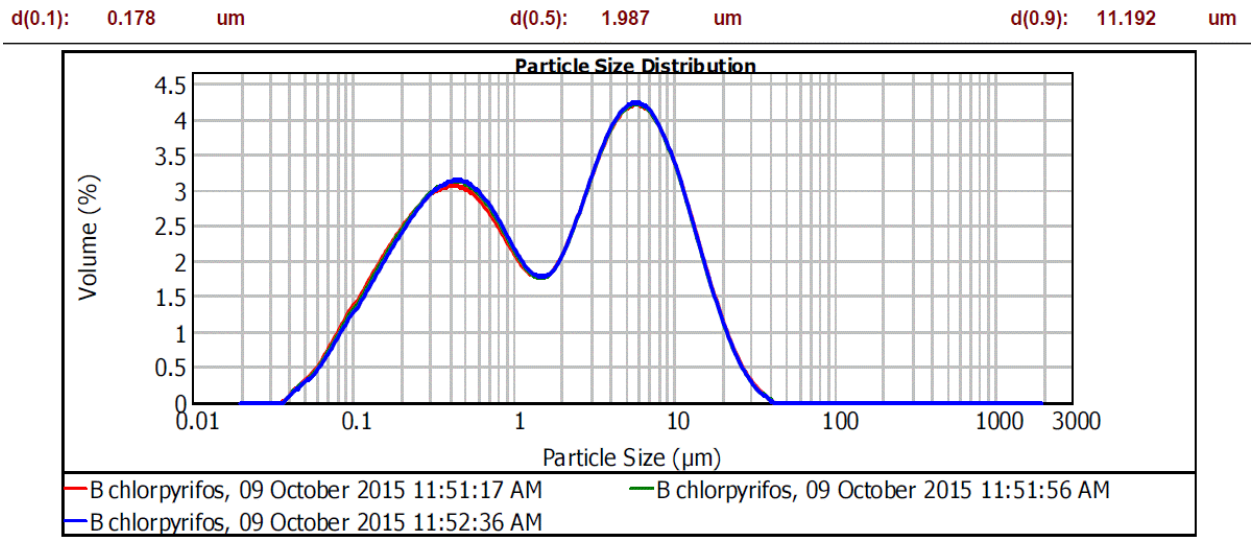

Figure 5-1: CPF/PP particle size distribution curve

The CLSM-images (displayed in Figure 5-2) reveals the successful formation of spherical particles. The sample contained pheriodal structures with internal structure. The latter depends on the types of Pheroid® structures that formed. Present in the formulation were both Pheroid® vesicles and micro-sponges which have internal cavities. The active ingredient(s) were contained in those cavities upon the addition of aqueous media (such as dilution prior to pesticide administration on crops). Some artefacts and/or oil droplets were also observed (image 1 and 2). The moderate fluorescence in image 4 is indicative of the entrapped

compound inside the Pheroid® that emits photons in the 540 to 640 nm wavelength band. Most Pheroid® vesicles and microsponges were smaller than 1 µm in diameter, although a few had a diameter of up to 12 µm according to this technique. The concentration of the pheroidal structures was 1183.1 x 10^8/ml and the average size 1223 nm. These measurements denote the average of four aliquots of sample.

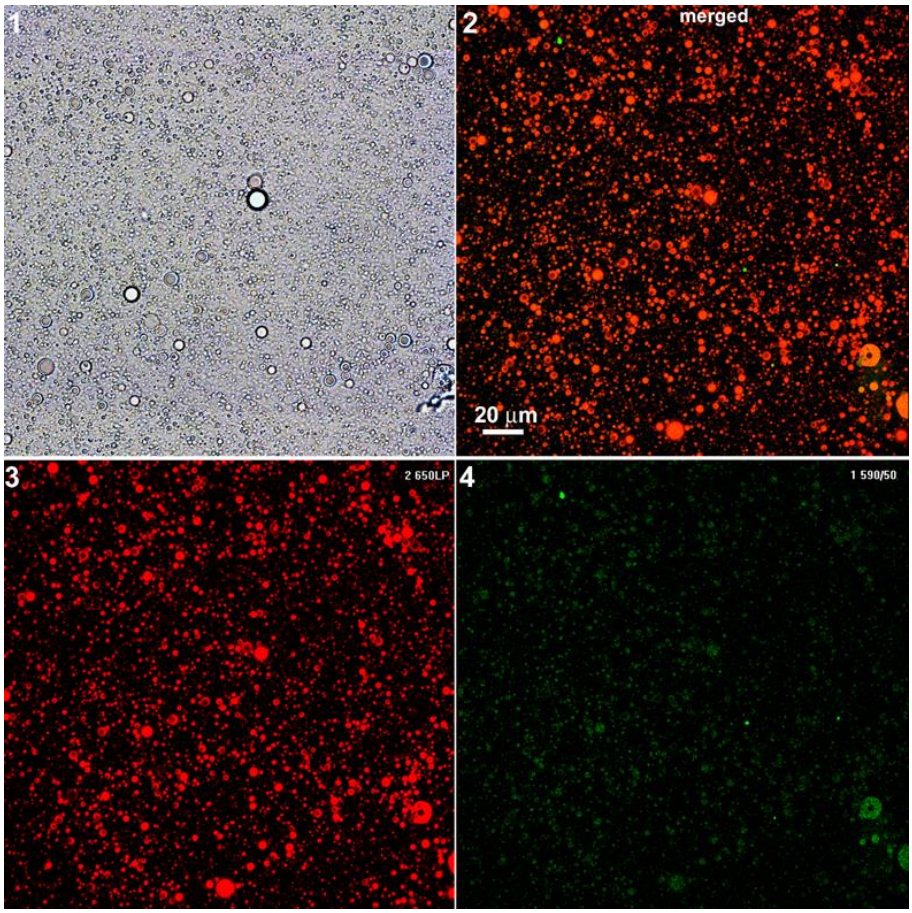

Figure 5-2: (1) Micrograph obtained with tungsten sourced light, (2) Micrograph representing a combination of 590/ 50 and 650 nm long pass (LP) photon emissions, (3) Fluorescence signal detected in wavelengths longer than 650nm and (4) Fluorescence signal detected in the 540 to 640nm wavelength band. Magnification was 60x using Nile Red as label.

The zeta potential measures the electro kinetic potential in colloidal systems in millivolts (mV) and is used as an indicator of the formulation's stability (Hunter *et al.*, 2013:159-160). A high zeta potential (from ≥ ±25) will confer stability as the repulsive forces in the formulation exceed the attractive forces and particles are dispersed in the emulsion (Roland *et al.*, 2003). When the potential is low (0 to <±25), attraction exceeds repulsion and the dispersion will aggregate in time. A zeta potential of -9.79 mV was obtained for CPF/PP which signifies an unstable formulation. However, prior to analysis, the sample was diluted according to procedure. The addition of an aqueous phase converted the oil-based pro-Pheroid® to the water based Pheroid®. Therefore, the acquired zeta potential is not a measure of the concentrates stability,

but rather that of the diluted formulation. In practise, the concentrate is diluted with water (as prescribed) shortly prior to application on crops. This is done to decrease volume to be transported and increase stability and shelf life of the concentrate.

5.4.2 Efficacy studies

The results from the efficacy study on African bollworm are presented in Table 5-2 and Figure 5-4, while that of the aphid study are displayed in Table 2 and Figure 4. Although CPF/PP surpassed the standard CPF in both trials, the most promising results were obtained with CPF/PP in the treatment of African bollworm (tomatoes). During both trials, no phytotoxic effects were observed at any stage or with any of the formulations.

5.4.2.1 African bollworm (tomatoes)

One week after the first application on tomatoes, all of the treatments led to a decrease in the mean percentage of fruit damaged by the African bollworm (see Table 5-1). A week later, the treatments had reduced the mean percentage fruit damage even further and resulted in means that were significantly different from that obtained for the previous assessment but not from each other or from the following week (indicated by "*") (see Figure 5-3). With the final assessment (1 week after the last application), 0.5X CPF/PP and 2X CPF/PP experienced a slight increase in the mean percentage of damaged fruit (not statistically significant) although both were still more efficacious than the standard formulation. For the duration of the study, 1X CPF, 0.5X CPF/PP, and 2X CPF/PP resulted in a 63.60%, 73.70% and 75.00% reduction in mean fruit damaged respectively when compared to the untreated plots. The plants that received 1X CPF/PP had the least amount of damaged fruit present when the study concluded (reduction of 78.90%).

Table 5-1: Mean percentage fruit damaged by African bollworm

Evaluation day	0	7	14	21
Treatment product				
Untreated Check (Control)	12.00 ± 3.74	13.50 ± 3.84	17.00 ± 4.12	13.50 ± 3.57
CPF 150 ml in 100 L (1X CPF)	11.00 ± 5.39	8.00 ± 2.83	5.00 ± 2.24*	4.00 ± 1.41*
CPF/PP 75 ml in 100 L (0.5X CPF/PP)	9.50 ± 3.84	6.50 ± 1.66	2.00 ± 1.41*	2.50 ± 2.18*
CPF/PP 150 ml in 100 L (1X CPF/PP)	9.50 ± 1.66	7.00 ± 3.32	2.00 ± 1.41*	2.00 ± 1.41*
CPF/PP 300 ml in 100 L (2X CPF/PP)	12.00 ± 5.48	7.50 ± 2.60	1.50 ± 0.87*	3.00 ± 1.00*
Bartlett's X2	3.712	1.892	7.71	5.504
p (Bartlett's X2)	0.446	0.756	0.103	0.239
Treatment F	0.259	2.81	28.516	16.228
Treatment Prob (F)	0.8984	0.074	0.0001	0.0001

Means followed by the same symbol (e.g. "*") do not significantly differ from each other (p =0.05, Student-Newman-Keuls)

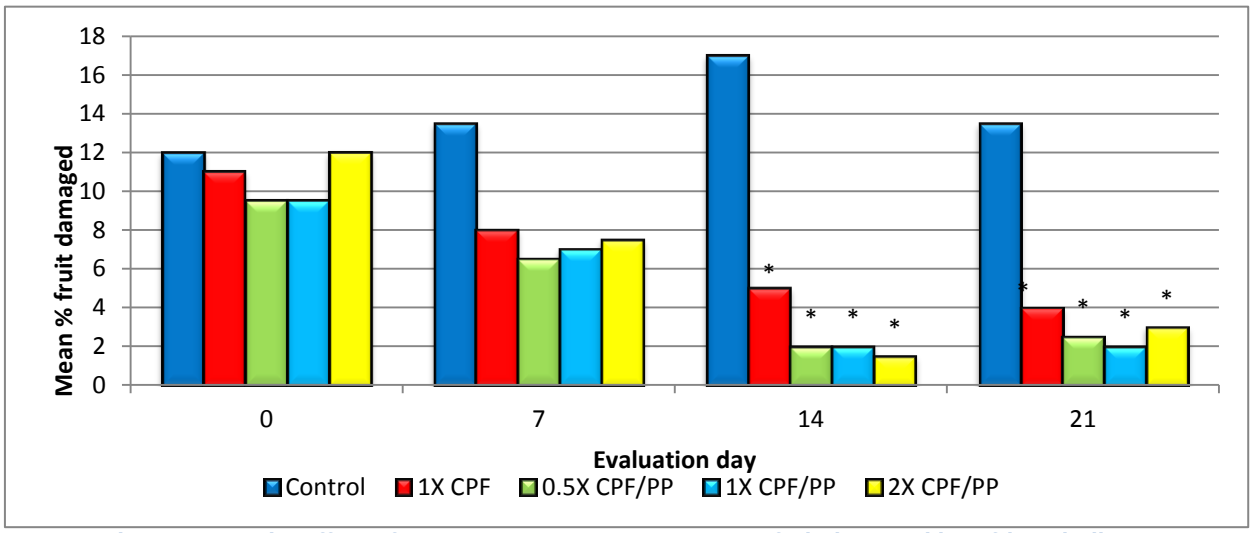

Figure 5-3: The effect of treatments on mean percentage fruit damaged by African bollworm

Duffield and Jordan (2000) found that chlorpyrifos, applied at a rate of 2 L/ha, resulted in 74.20% less *Helicoverpa armigera* larvae on soybean leaves over the 7-day post-application period. In another study, the effect of chlorpyrifos 40 EC (2.5 L/ha) in the control of Gram Pot Borer (*Helicoverpa armigera* Hubner) on chickpea was evaluated (Rashid *et al.*, 2003). The average larval population reduced with 68.10% in the 7 days after the insecticide was administered. The CPF/PP trial plot study on tomatoes focused on the mean amount of fruit damaged over time and not on the mean number of pests as in the above-mentioned studies. Although these efficacies are not directly comparable, a reduction in the number of insects would effectively result in less damaged fruit. The most successful CPF/PP formulation, 1X

CPF/PP, reduced the number of damaged fruit by 26.30% within the 7 days after the first application. This formulation contained only 0.75 L/ha chlorpyrifos whereas the treatments used in the previously mentioned studies contained more than twice that amount. The Pheroid® treatment containing an equal amount of active compound to that in the described studies had reduced the amount of damaged fruit by 78.90% during the study period with the use of less active ingredient and would, therefore, be less harmful to the environment.

5.4.2.2 Aphids (cabbage)

Seven days after the treatments were applied to the cabbage (assessment 2), mean aphid counts that were significantly different from the previous week were obtained by for all treatments (see Table 5-2 - indicated by "*"). With the third evaluation (day 14), 1X CPF, 1X CPF/PP, and 2X CPF/PP displayed results which were significantly different from the previous week (indicated by "**"). Treatment with formulations 1X CPF and 1X CPF/PP showed a slight increase in mean aphid counts (not statistically significant) whereas treatment with 0.5X CPF/PP resulted in a mean count that exceeded the number of aphids at the commencement of the study (see Figure 5-4). On the 21st day of the study, all treatments had results that were similar to the 7th day (assessment 2). All the formulations were considerably more effective in controlling the insects than the untreated control. Treatments 1X CPF, 0.5X CPF/PP, and 2X CPF/PP resulted in a 63.40%, 27.50% and 69.00% reduction in mean aphid count in comparison to the untreated plots. Treatment 1X CPF/PP was the most effective formulation with a 78.50% reduction in mean aphid count since the initiation of the study.

Table 5-2: The effect of treatments on mean aphid counts on plants

Evaluation day	0	7	14	21
Treatment product				
Untreated Check (Control)	155.25 ± 57.91	174.00 ± 57.15	206.25 ± 9.50	203.75 ± 36.02
CPF 50 ml in 100 L (1X CPF)	112.75 ± 20.34	52.50 ± 17.53*	63.75 ± 7.50**	41.00 ± 12.43*
CPF/PP 25 ml in 100 L (0.5X CPF/PP)	104.50 ± 23.04	85.75 ± 13.27*	111.00 ± 22.51*	75.75 ± 30.20*
CPF/PP 50 ml in 100 L (1X CPF/PP)	121.00 ± 55.52	31.25 ± 12.95*	46.75 ± 17.85**	26.00 ± 5.34*
CPF/PP 100 ml in 100 L (2X CPF/PP)	104.75 ± 24.37	36.25 ± 16.89*	35.50 ± 13.16**	32.50 ± 9.60*
Bartlett's X2	5.518	10.583	4.041	11.235
p (Bartlett's X2)	0.238	0.032†	0.4	0.024†
Treatment F	0.798	14.313	64.419	32.699
Treatment Prob (F)	0.549	0.0002	0.0001	0.0001

Means followed by the symbol (e.g. "*") do not significantly differ from each other (p =.05, Student-Newman-Keuls)

† = significant at p = 0.05

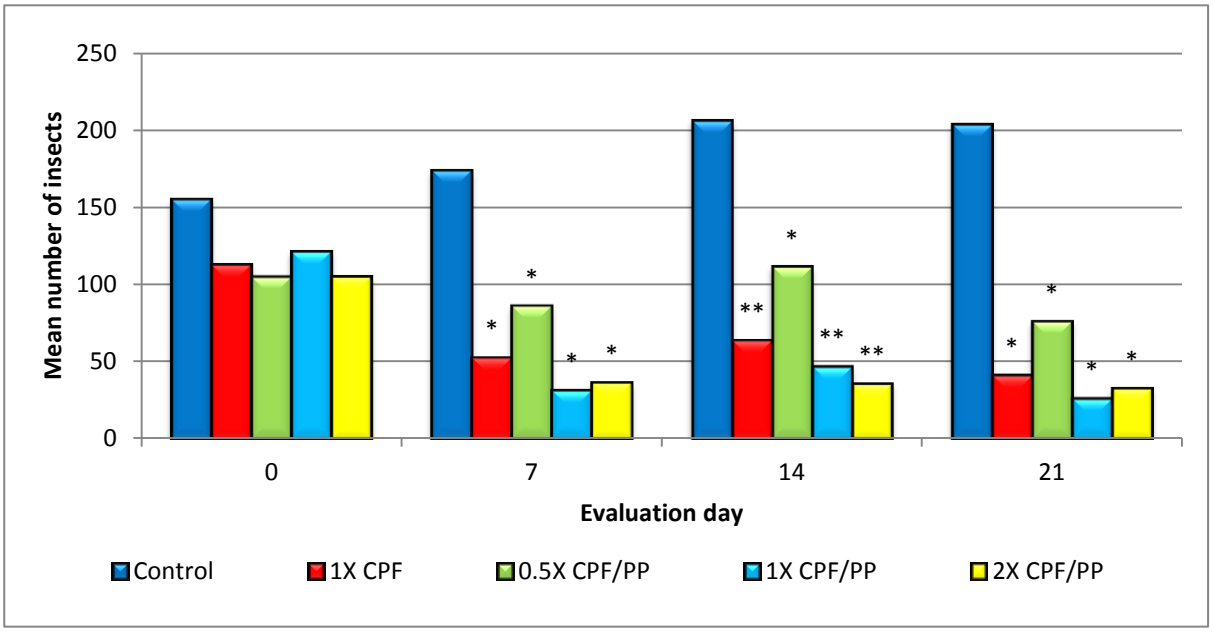

Figure 5-4: The effect of treatments on mean aphid counts on plants

Gupta *et al.* (2014) evaluated the efficacy of the combination formulation Action 505 (chlorpyriphos + cypermethrin) on cabbage aphids. The most promising dose (1.6 L/ha) resulted in a 70.15% reduction in the aphid population 10 days after a single application of the treatment. The treatment, which contained 800g chlorpyrifos/ ha, was significantly more concentrated than any of the CPF/PP formulations used for the trial plot studies in Table 5-2 (the most concentrated formulation, 2X CPF/PP, was diluted (with H_2O) to contain 480g/500 mL

chlorpyrifos per hectare). 1X CPF/PP (120g/ 250 mL/ha) reduced the number of aphids by 74.20% 7 days after the formulation was applied. Therefore, 1X CPF/PP was more effective and safe than Action 505.

5.5 Conclusion

The addition of Pheroid® technology to chlorpyrifos has delivered a formulation with increased efficacy in comparison to the commercially available product. In the control of African bollworm in tomatoes, half the standard strength was 15.30% more effective than the comparator, while equal strength was 14.90% more effective in controlling cabbage aphids than the same strength comparator. During both trial studies, CPF/PP has proved to be more effective than the commercial product with varying levels of supremacy. Therefore, in future studies, the efficacy of CFP/PP will be investigated against pests in other crops where chlorpyrifos is routinely utilized to identify the ideal dosage rate for each indication. Additional results similar to that of the African bollworm study would substantiate the fact that CPF/PP can have enhanced efficacy against pest at lower dosages and would subsequently have less impact on the environment.

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CHAPTER 6 CONCLUSION AND FUTURE PROSPECTS

Standard specifications provide the framework of a product and comprise of a list of assessments, references to analytical procedures and acceptance criteria embodied by numerical limits or ranges to describe the size, shape, form and any other important information about the product. Specifications serves as a primary means of control; adherence is therefore mandatory for a product to be regarded as satisfactory for its intended application and to ensure consistency among manufactured batches. In the formulation industry, specifications have functions that include: behavioural prediction of a compound or formulation, the confirmation of formulation stability and the compilation of dossiers.

The aim of this study was to set standardized specifications for anti-infective Pheroid products that would ultimately promote quality control and the reproducibility of formulations. The intention was to describe and formalize existing ranges for different types of Pheroid formulations with anti-infective properties and validate the results through the formulation of two classes of anti-infective compounds, azoxystrobin (fungicide) and chlorpyrifos (insecticide) in the Pheroid delivery system according to the determined ranges. The use of a plant model to verify the formalized ranges is effective and much less time consuming than an animal or human model. Anti-infective agents are amongst the most frequently used drugs in the world and resistance to those drugs is developing into a peril to public health. Since previous studies on anti-infective Pheroid formulations showed promising results, including escalated drug plasma levels at lower dosages, increased bioavailability, and reduced side effects, they were chosen for further investigation.

To achieve the stated aim, data was collected from anti-infective Pheroid formulations as well as reference Pheroid formulations (i.e. containing only Pheroid ingredients) that were manufactured between 2008 and 2015 (Pheroid technology is a relatively new concept). The data was grouped according to the type of Pheroid contained in the respective formulations (i.e. Pheroid vesicles, pro-Pheroid and Pheroid micro-sponges) and subdivided according to the variables (e.g. active ingredient or additives used). The data was then statistically analysed in terms of the characterization results that were obtained for the various formulations after which the collection of anti-infective data was compared with that collected for reference. The characterization analyses comprised exclusively of Mastersize analysis (particle size distribution) CLSM (average particle size and concentration), and zeta potential measurements (stability).

During the statistical data analysis, some formulations had concomitant confocal data that was most likely the result of an error at some point during the analysis. Particle size and

concentration are inversely proportional, therefore, between equivalent formulations; one formulation should in theory not contain an abundance of large particles. CLSM is more suited to analyse the internal structure of particles than the number of particles. In addition, the CLSM leaves room for human error. Although the implication of measuring the particle sizes with the incorrect laser obscuration was also noticed during the study. Therefore, the Malvern, following the SOP in order to reduce/eliminate error, should be used instead of CLSM to determine particle sizes whereas CLSM should be focussed on analysing the internal structure of particles.

The oil and water phases did not seem to influence the characterization results for the formulations. However, since the standard oil and water phases (with specific ingredients at predetermined ratios) were applied to the majority of formulations, there was not enough data available on formulations that contained phases other than the standard so that concrete conclusions could be drawn.

Active ingredients, preservatives, antioxidants and other excipients were shown to influence the characterization of the particles. For instance, none of the anti-infective vesicle formulations that included a preservative and/or antioxidant was over ~1500 nm in size (see Figures 3-27 and 3-28). Additionally, the reference pro-Pheroid formulations were on average between 500 and 1000 nm in size while several anti-infective pro-Pheroid formulations had average particle sizes that ranged up to 3000 nm in size. Active ingredient 10 and 11 were present in pro-Pheroid formulations that contained similar ingredients (which included the same antioxidant). One formulation of each active contained a certain excipient (and had 100.00% submicron sized particles) whereas the other formulation contained another excipient (and both formulations had a total of ~10% particles in the submicron and micron size range – presented in Table 3-62).

As a result of insufficient data and unpredictable behaviour observed with several formulations, the Pheroid specification ranges could not be precisely formalized and the primary aim could not be met. Although an azoxystrobin/Pheroid formulation could not be formulated in accordance with the formalized ranges as intended, it was manufactured via an alternative means nonetheless. According to the literature, azoxystrobin had the highest solubility in organic solvents and solubility studies were initiated accordingly. It soon became clear that the target strength of the formulation, 25.00% ($^{w}/_{w}$), was going to be difficult to achieve when using solvents that are fairly safe. Most of the concentrates (i.e. prior to dilution for agricultural application) crystallized within a few days. It was later hypothesized that the crystallization was a result of the high concentration of active ingredient. For that reason, the succeeding formulations were initially manufactured at a low concentration of active ingredient and, if promising results were obtained, pursued by more concentrated formulations. Through trial and error, success was achieved and two formulations were manufactured on a large scale,

characterized, and sent for trial plot studies by a reputable research and development company that is certified for Good Laboratory Practises (GLP's).

The two formulations, 6.25% azoxystrobin/pro-Pheroid (AZ/PPA) and 12.50% azoxystrobin/pro-Pheroid (AZ/PPB), were tested for their efficacy and possible phytotoxicity against late blight (*Phytophthora infestans*) on tomatoes and white blister (*Albugo candida*) on cabbage. Relative to the control, the comparator (0.15 µm /mL) was slightly more effective than the same-strength azoxystrobin/pro-Pheroid® formulations (71.64% compared to equal effectiveness of 70.15%) against late blight on tomatoes. In the control of white blister on cabbage, the comparator (0.94 µm /mL) was more effective that the double strength azoxystrobin/pro-Pheroid® formulations and significantly more effective than the same-strength azoxystrobin/pro-Pheroid® formulations relative to the control.

The Pheroid based formulations, AZ/PPA and AZ/PPB, had the same corrected level of effectiveness against late blight on tomatoes, while treatment AZ/PPA was more effective than AZ/PPB against white blister on cabbage. After dilution, the AZ/PPA treatment (6.25% formulation) contained double the amount of pro-Pheroid than the other formulation (12.50% formulation) did due to the varying dilution factors. Consequently, the higher amount of pro-Pheroid could have led to the formulation's increased efficacy against white blister. The results could also have been influenced by the different excipients that were used in the formulations or by solubility (as indicated by the characterization data).

In a similar manner to the azoxystrobin/pro-Pheroid formulations, a chlorpyrifos/pro-Pheroid formulation was manufactured and evaluated for efficacy and possible phytotoxicity through trial plot studies on African Bollworm (*Helicoverpa armigera)* on tomatoes and cabbage aphids (*Brevicoryne brassicae*). During both trials, the chlorpyrifos/pro-Pheroid formulation proved to be more effective than the commercial product with varying levels of superiority. In the control of African bollworm in tomatoes, half the standard strength chlorpyrifos/pro-Pheroid (0.36 µg /mL) was 15.30% more effective than the full strength comparator (0.72 µg /mL). At the same strength than the comparator (0.24 µg /mL), it was 14.90% more effective in controlling cabbage aphids than the comparator.

The incorporation of azoxystrobin in Pheroid technology was not superior to the comparator and was therefore unsuccessful. The chlorpyrifos/pro-Pheroid formulation, on the other hand, was superior to the comparator in the control of late blight and African Bollworm on tomatoes as well as cabbage aphids. The chlorpyrifos/pro-Pheroid formulation was more efficacious at the same concentration (and a lower concentration in some instances) as the comparator against the selected pests and diseases. Increased efficacy at the same or lower doses implies that less

product would be required to achieve the desired outcome. Consequently, the environmental and ecological impact would be reduced. In addition, improved crop yield and quality would, in turn, lead to increased food security and agricultural revenue.

Recommendations for future studies are:

- The repeated manufacture of a wide range of formulations, one variable at a time, and with various characteristics so that the Pheroid specifications can be described and formalized.
- Determination of the influential factor behind the unpredictable behaviour of Pheroid formulations.
- In-depth investigation of solubility influences more specific to Pheroid and Pheroid formulations.
- Revisiting of the solubility studies for azoxystrobin/pro-Pheroid formulation.
- Investigation of the azoxystrobin/pro-Pheroid formulations' environmental impact.
- Establishment of the chlorpyrifos/pro-Pheroid formulation's efficacy against pests in other crops where chlorpyrifos is routinely utilized.
- Investigation of the environmental impact of the chlorpyrifos/pro-Pheroid formulations.

ANNEXURES

Annexure A: Mastersize analyses reports

A.1 6.25% azoxystrobin/pro-Pheroid formulation

MASTERSIZER 2000

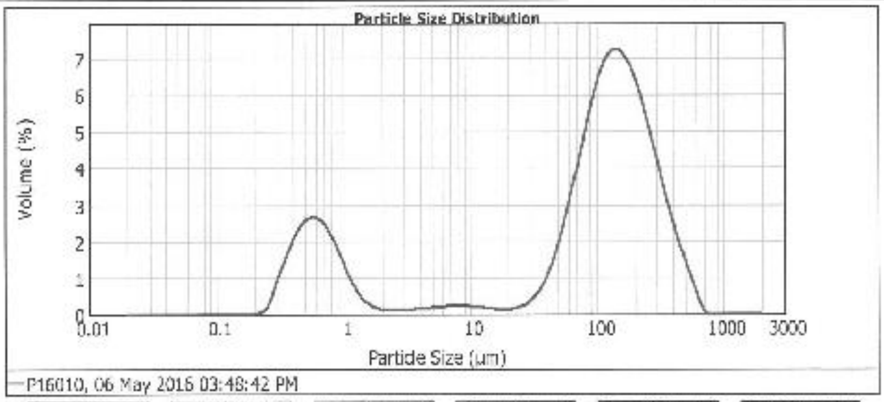
Result Analysis Report

Sample Name: P16010	**SOP Name:** Nick Esparch (Hydro 2000 SM)	**Measured:** 06 May 2016 03:48:42 PM
Sample Source & type: Anon Paters	**Measured by:** lesley	**Analysed:** 06 May 2016 03:48:43 PM
Sample bulk lot ref: MA 439	**Result Source:** Measurement	

Particle Name: Oleic Acid	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 1.458	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 13.42 %
Dispersant Name: Water	**Dispersant RI:** 1.330	**Weighted Residual:** 1.974 %	**Result Emulation:** Off

Concentration: 0.0134 %Vol	**Span :** 2.650	**Uniformity:** 0.807	**Result units:** Volume
Specific Surface Area: 2.16 m²/g	**Surface Weighted Mean D[3,2]:** 2.782 um	**Vol. Weighted Mean D[4,3]:** 139.907 um	

d(0.1): 0.605 um **d(0.5):** 115.925 um **d(0.9):** 307.751 um



Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %
0.010	0.00	0.105	0.00	1.096	0.77	11.482	0.15	120.226	6.45	1258.925	0.00
0.011	0.00	0.120	0.00	1.259	0.45	13.183	0.12	138.038	6.51	1445.440	0.00
0.013	0.00	0.138	0.00	1.445	0.24	15.136	0.10	158.489	6.39	1659.587	0.00
0.015	0.00	0.158	0.00	1.660	0.14	17.378	0.26	181.970	5.97	1905.461	0.00
0.017	0.00	0.182	0.00	1.905	0.05	19.953	0.11	208.930	5.33	2187.762	0.00
0.020	0.00	0.209	0.03	2.188	0.05	22.909	0.15	239.883	4.52	2511.886	0.00
0.023	0.00	0.240	0.21	2.512	0.08	26.303	0.25	275.423	3.86	2884.032	0.00
0.026	0.00	0.275	0.77	2.884	0.08	30.200	0.42	316.228	3.14	3311.311	0.00
0.030	0.00	0.316	1.24	3.311	0.10	34.674	0.69	363.078	2.43	3801.894	0.00
0.035	0.00	0.363	1.71	3.802	0.12	39.811	1.00	416.869	1.77	4365.158	0.00
0.040	0.00	0.417	2.08	4.365	0.14	45.709	1.55	478.630	1.17	5011.872	0.00
0.046	0.00	0.479	2.31	5.012	0.15	52.481	2.29	549.541	0.64	5754.399	0.00
0.052	0.00	0.550	2.37	5.754	0.17	60.256	3.06	630.957	0.15	6606.934	0.00
0.060	0.00	0.631	2.20	6.607	0.18	69.183	3.84	724.436	0.00	7585.776	0.00
0.069	0.00	0.724	1.89	7.586	0.19	79.433	4.70	831.764	0.00	8709.636	0.00
0.079	0.00	0.832	1.50	8.710	0.19	91.201	5.54	954.993	0.00	10000.000	
0.091	0.00	0.955	1.17	10.000	0.17	104.713	6.12	1096.478	0.00		
0.105		1.096		11.482		120.226		1258.925			

Operator notes: 6.25% AZOXYL / Pro-Pheroid formulation diluted with ddH2O

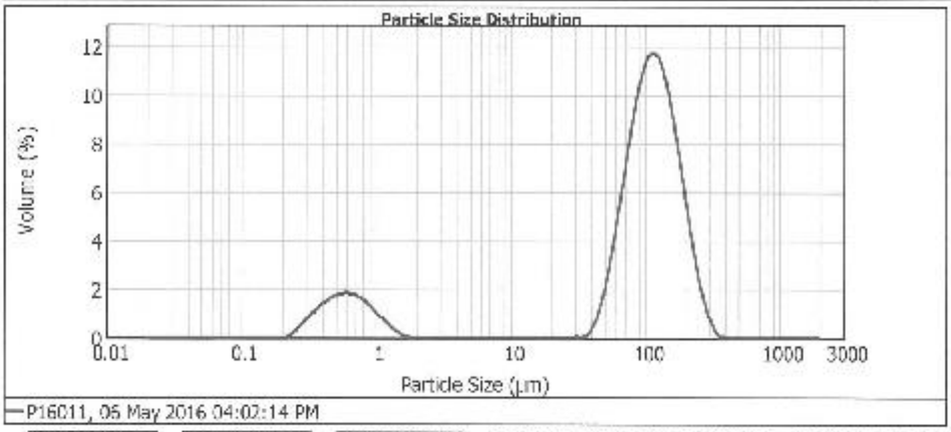
A.2 12.50% azoxystrobin/pro-Pheroid formulation

MASTERSIZER 2000

Result Analysis Report

Sample Name:	SOP Name:	Measured:	
P16011	Nick Espach (Hydro 2000 SM)	05 May 2016 04:02:14 PM	
Sample Source & type:	**Measured by:**	**Analysed:**	
Anri Peters	lesley	05 May 2016 04:02:15 PM	
Sample bulk lot ref:	**Result Source:**		
MA 440	Measurement		
Particle Name:	**Accessory Name:**	**Analysis model:**	**Sensitivity:**
Oleic Acid	Hydro 2000SM (A)	General purpose	Enhanced
Particle RI:	**Absorption:**	**Size range:**	**Obscuration:**
1.456	0.1	0.020 to 2000.000 um	13.00 %
Dispersant Name:	**Dispersant RI:**	**Weighted Residual:**	**Result Emulation:**
Water	1.330	1.109 %	Off
Concentration:	**Span :**	**Uniformity:**	**Result units:**
0.0180 %Vol	1.825	0.48	Volume
Specific Surface Area:	**Surface Weighted Mean D[3,2]:**	**Vol. Weighted Mean D[4,3]:**	
1.58 m²/g	3.787 um	108.493 um	

d(0.1): 0.773 um **d(0.5):** 105.632 um **d(0.9):** 193.336 um



Size (μm)	Volume In %
0.010	0.00
0.011	0.00
0.013	0.00
0.015	0.00
0.017	0.00
0.020	0.00
0.023	0.00
0.026	0.00
0.030	0.00
0.035	0.00
0.040	0.00
0.046	0.00
0.052	0.00
0.060	0.00
0.069	0.00
0.079	0.00
0.091	0.00
0.105	

Size (μm)	Volume In %
0.105	0.01
0.120	0.00
0.138	0.00
0.158	0.00
0.182	0.00
0.209	0.04
0.240	0.33
0.275	0.61
0.316	0.87
0.363	1.19
0.417	1.47
0.479	1.67
0.550	1.83
0.631	1.84
0.724	1.48
0.832	1.19
0.955	0.89
1.096	

Size (μm)	Volume In %
1.096	0.69
1.259	0.31
1.445	0.11
1.660	0.00
1.905	0.00
2.188	0.00
2.512	0.00
2.884	0.00
3.311	0.00
3.802	0.00
4.365	0.00
5.012	0.00
5.754	0.00
6.607	0.00
7.586	0.00
8.710	0.00
10.000	0.00
11.482	

Size (μm)	Volume In %
11.482	0.00
13.183	0.00
15.136	0.00
17.378	0.00
19.953	0.00
22.909	0.00
26.303	0.00
30.200	0.00
34.674	0.07
39.811	0.62
45.709	1.61
52.481	3.08
60.256	4.87
69.183	6.82
79.433	8.62
91.201	9.91
104.713	10.69
120.226	

Size (μm)	Volume In %
120.226	10.92
138.038	9.28
158.489	7.69
181.970	5.79
208.930	3.91
239.883	2.19
275.423	0.94
316.228	0.14
363.078	0.01
416.869	0.00
478.630	0.00
549.541	0.00
630.957	0.00
724.436	0.00
831.764	0.00
954.993	0.00
1096.478	0.00
1258.925	

Size (μm)	Volume In %
1258.925	0.00
1445.440	0.00
1659.587	0.00
1905.461	0.00
2187.762	0.00
2511.886	0.00
2884.032	0.00
3311.311	0.00
3801.894	0.00
4365.158	0.00
5011.872	0.00
5754.399	0.00
6606.934	0.00
7585.776	0.00
8709.636	0.00
10000.000	

Operator notes: *12.5% AZOXYL / Pro-Pheroid formulation diluted with ddH2O*

Annexure B: Description of the BBCH values used during the azoxystrobin/pro-Pheroid trial plot studies (adapted from Feller *et al.* (1995))

B.1: BBCH-scale of solaneous fruit (e.g. tomatoes)

Phenological growth stages and BBCH-identification keys of solanaceous fruits		
Code (2-digit)	**Code (3-digit)**	**Description**
Principal growth stage 0: Germination		
00	000	Dry seeds
01	001	Beginning of seed imbibition
03	003	Seed imbibition complete
05	005	Radicle emerged from seed
07	007	Hypocotyl with cotyledons breaking through seed coat
09	009	Emergence: coryledons break through soil surface
Principal growth stage 1: Leaf development		
10	100	Cotyledons completely unfolded
11	101	First true leaf on main shoot fully unfolded
12	102	2nd leaf on main shoot unfolded
13	103	3rd leaf on main shoot unfolded
1 .	10 .	Stages continuous till . . .
19	109	9 or more leaves on main shoot unfolded
Principal growth stage 2: Formation of side shoots[1]		
21	201	First primary apical side shoot visible
22	202	2nd primary apical side shoot visible
2 .	20 .	Stages continuous till . . .
29	209	9 or more apical primary side shoots visible
–	221	First secondary apical side shoot visible
–	22 .	Stages continuous till . . .
–	229	9th secondary apical side shoot visible
–	231	First tertiary apical side shoot visible
–	23 .	Stages continuous till . . .
–	2NX	Xth apical side shoot of the Nth order visible
Principal growth stage 5: Inflorescence emergence		
51	501	First inflorescence visible (first bud erect)[2] First flower bud visible3
52	502	2nd inflorescence visible (first bud erect)[2]

		2nd flower bud visible[3]
53	503	3rd inflorescence visible (first bud erect)[2] 3rd flower bud visible[3]
5 .	50 .	Stages continuous till . . .
59	509	9 or more inflorescences visible (2digit), 9th inflorescence visible(first bud erect) (3digit)[2] 9 or more flower buds already visible (2digit), 9th flower bud visible (3digit)[3]
–	510	10th inflorescence visible (first bud erect)[2] 10th flower bud visible[3]
–	51 .	Stages continuous till . . .
–	519	19th inflorescence visible (first bud erect)[2] 19th flower bud visible[3]
Principal growth stage 6: Flowering		
61	601	First inflorescence: first flower open[2] First flower open[3]
62	602	2nd inflorescence: first flower open[2] 2nd flower open[3]
63	603	3rd inflorescence: first flower open[2] 3rd flower open[3]
6 .	60 .	Stages continuous till . . .
69	609	9 or more inflorescences with open flowers (2digit) 9th inflorescence: first flower open (3digit)[2] 9 or more flowers already open (2digit) 9th flower open (3digit)[3]
–	610	10th inflorescence: first flower open[2] 10th flower open[3]
–	61 .	Stages continuous till . . .
–	619	19th inflorescence: first flower open[2] 19th flower open[3]
Principal growth stage 7: Development of fruit		
71	701	First fruit cluster: first fruit has reached typical size[2] First fruit has reached typical size and form[3]
72	702	2nd fruit cluster: first fruit has reached typical size[2] 2nd fruit has reached typical size and form[3]
73	703	3rd fruit cluster: first fruit has reached typical size[2] 3rd fruit has reached typical size and form[3]
7 .	70 .	Stages continuous till . . .
79	709	9 or more fruit clusters with fruits of typical size (2digit) 9th fruit cluster: first fruit has reached typical size (3digit)[2] 9 or more fruits have reached typical size and form (2digit) 9th fruit has reached typical size and form(3digit)[3]
–	710	10th fruit cluster: first fruit has reached typical form and size[2] 10th fruit has reached typical form and size[3]
–	71 .	Stages continuous till . . .

		19th fruit has reached typical form and size[3]
–	719	19th fruit cluster: first fruit has reached typical form and size[2]
Principal growth stage 8: Ripening of fruit and seed		
81	801	10% of fruits show typical fully ripe colour
82	802	20% of fruits show typical fully ripe colour
83	803	30% of fruits show typical fully ripe colour
84	804	40% of fruits show typical fully ripe colour
85	805	50% of fruits show typical fully ripe colour
86	806	60% of fruits show typical fully ripe colour
87	807	70% of fruits show typical fully ripe colour
88	808	80% of fruits show typical fully ripe colour
89	809	Fully ripe: fruits have typical fully ripe colour[3]
Principal growth stage 9: Senescence		
97	907	Plants dead
99	909	Harvested product

B.2: BBCH-scale for leafy vegetables forming heads (e.g. cabbage)

Growth stage	Code	Description
0: Germination	00	Dry seed
	01	Beginning of seed imbibition
	03	Seed imbibition complete
	05	Radicle emerged from seed
	07	Hypocotyl with cotyledons breaking through seed coat
	09	Emergence: cotyledons break through soil surface
1: Leaf development (Main shoot)	10	Cotyledons completely unfolded; growing point or true leaf initial visible
	11	First true leaf unfolded
	12	2nd true leaf unfolded
	13	3rd true leaf unfolded
	1 .	Stages continuous till . . .
	19	9 or more true leaves unfolded
4: Development of harvestable vegetative plant parts	41	Heads begin to form: the two youngest leaves do not unfold
	42	20% of the expected head size reached
	43	30% of the expected head size reached
	44	40% of the expected head size reached
	45	50% of the expected head size reached
	46	60% of the expected head size reached
	47	70% of the expected head size reached
	48	80% of the expected head size reached
	49	Typical size, form and firmness of heads reached

5: Inflorescence emergence	51	Main shoot inside head begins to elongate
	53	30% of the expected height of the main shoot reached
	55	First individual flowers of main inflorescence visible (still closed)
	57	First individual flowers of secondary inflorescences visible (still closed)
	59	First flower petals visible; flowers still closed
6: Flowering	60	First flowers open (sporadically)
	61	Beginning of flowering: 10% of flowers open
	62	20% of flowers open
	63	30% of flowers open
	64	40% of flowers open
	65	Full flowering: 50% of flowers open
	67	Flowering finishing: majority of petals fallen or dry
	69	End of flowering
7: Development of fruit	71	First fruits formed
	72	20% of fruits have reached typical size
	73	30% of fruits have reached typical size
	74	40% of fruits have reached typical size
	75	50% of fruits have reached typical size
	76	60% of fruits have reached typical size
	77	70% of fruits have reached typical size
	78	80% of fruits have reached typical size
	79	Fruits have reached typical size
8: Ripening of fruit and seed	81	Beginning of ripening: 10% of fruits ripe, or 10% of seeds of typical colour, dry and hard
	82	20% of fruits ripe, or 20% of seeds of typical colour, dry and hard

	83	30% of fruits ripe, or 30% of seeds of typical colour, dry and hard
	84	40% of fruits ripe, or 40% of seeds of typical colour, dry and hard
	85	50% of the fruits ripe, or 50% of seeds of typical colour, dry and hard
	86	60% of fruits ripe, or 60% of seeds of typical colour, dry and hard
	87	70% of fruits ripe, or 70% of seeds of typical colour, dry and hard
	88	80% of fruits ripe, or 80% of seeds of typical colour, dry and hard
	89	Fully ripe: seeds on the whole plant of typical colour and hard
9: Senescence	92	Leaves and shoots beginning to discolour
	95	50% of leaves yellow or dead
	97	Plants dead
	99	Harvested product (seeds)

Annexure C: Phytotoxicity results obtained during trial plot studies

C.1 Azoxystrobin/pro-Pheroid formulations in the control of *Phytophtora infestans* (late blight) in tomatoes

Rating Date				26-May-2016		02-Jun-2016		09-Jun-2016		16-Jun-2016		23-Jun-2016	
Part rated				Plant		Plant		Plant		Plant		Plant	
Rating Type				PHYGEN		PHYGEN		PHYGEN		PHYGEN		PHYGEN	
Trt no.	**Treatment name**	**Rate**	**Rate Unit**										
1	Untreated Check			0.00	a	0.00	a	0.00	a	0.00	a	0.00	a
2	Amistar 250 SC Bravo 720 SC	60 380	ml/100 l ml/100 l	0.00	a	0.00	a	0.00	a	0.00	a	0.00	a
3	Amistar 250 SC Bravo 720 SC	120 380	ml/100 l ml/100 l	0.00	a	0.00	a	0.00	a	0.00	a	0.00	a
4	12.5% azoxystrobin/pro-Pheroid Bravo 720 SC	120 380	ml/100 l ml/100 l	0.00	a	0.00	a	0.00	a	0.00	a	0.00	a
5	12.5% azoxystrobin/pro-Pheroid Bravo 720 SC	240 380	ml/100 l ml/100 l	0.00	a	0.00	a	0.00	a	0.00	a	0.00	a
6	12.5% azoxystrobin/pro-Pheroid Bravo 720 SC	480 380	ml/100 l ml/100 l	0.00	a	0.00	a	0.00	a	0.00	a	0.00	a
7	6.25% azoxystrobin/pro-Pheroid Bravo 720 SC	240 380	ml/100 l ml/100 l	0.00	a	0.00	a	0.00	a	0.00	a	0.00	a
8	6.25% azoxystrobin/pro-Pheroid Bravo 720 SC	480 380	ml/100 l ml/100 l	0.00	a	0.00	a	0.00	a	0.00	a	0.00	a
9	6.25% azoxystrobin/pro-Pheroid Bravo 720 SC	960 380	ml/100 l ml/100 l	0.00	a	0.00	a	0.00	a	0.00	a	0.00	a
LSD (P=.05)				.		.		.		.		.	
Standard Deviation				0.000		0.000		0.000		0.000		0.000	
CV				0.0		0.0		0.0		0.0		0.0	
Bartlett's X2				0.0		0.0		0.0		0.0		0.0	
P(Bartlett's X2)				.		.		.		.		.	
Replicate F				0.000		0.000		0.000		0.000		0.000	
Replicate Prob(F)				1.0000		1.0000		1.0000		1.0000		1.0000	
Treatment F				0.000		0.000		0.000		0.000		0.000	
Treatment Prob(F)				1.0000		1.0000		1.0000		1.0000		1.0000	

Means followed by same letter do not significantly differ (P=.05, Student-Newman-Keuls)

Mean comparisons performed only when Analysis of Variance (AOV) Treatment Prob (F) is significant at mean comparison observed significance level (OSL).

* = significant at P = 0.05 PHYGEN = phytotoxicity - general

C.2 Azoxystrobin/pro-Pheroid formulations in the control of Albugo candida (white blister) in cabbage

Rating Date				11-Jun-2016		20-Jun-2016		27-Jun-2016		01-Jul-2016		08-Jul-2016	
Part rated				Plant		Plant		Plant		Plant		Plant	
Rating Type				PHYGEN		PHYGEN		PHYGEN		PHYGEN		PHYGEN	
Trt no.	**Treatment name**	**Rate**	**Rate Unit**										
1	Untreated Check			0.00	a	0.00	a	0.00	a	0.00	a	0.00	a
2	Amistar 250 SC Bravo 720 SC	60 380	ml/100 l ml/100 l	0.00	a	0.00	a	0.00	a	0.00	a	0.00	a
3	Amistar 250 SC Bravo 720 SC	120 380	ml/100 l ml/100 l	0.00	a	0.00	a	0.00	a	0.00	a	0.00	a
4	12.5% azoxystrobin/pro-Pheroid Bravo 720 SC	120 380	ml/100 l ml/100 l	0.00	a	0.00	a	0.00	a	0.00	a	0.00	a
5	12.5% azoxystrobin/pro-Pheroid Bravo 720 SC	240 380	ml/100 l ml/100 l	0.00	a	0.00	a	0.00	a	0.00	a	0.00	a
6	12.5% azoxystrobin/pro-Pheroid Bravo 720 SC	480 380	ml/100 l ml/100 l	0.00	a	0.00	a	0.00	a	0.00	a	0.00	a
7	6.25% azoxystrobin/pro-Pheroid Bravo 720 SC	240 380	ml/100 l ml/100 l	0.00	a	0.00	a	0.00	a	0.00	a	0.00	a
8	6.25% azoxystrobin/pro-Pheroid Bravo 720 SC	480 380	ml/100 l ml/100 l	0.00	a	0.00	a	0.00	a	0.00	a	0.00	a
9	6.25% azoxystrobin/pro-Pheroid Bravo 720 SC	960 380	ml/100 l ml/100 l	0.00	a	0.00	a	0.00	a	0.00	a	0.00	a
LSD (P=.05)				.		.		.		.		.	
Standard Deviation				0.000		0.000		0.000		0.000		0.000	
CV				0.0		0.0		0.0		0.0		0.0	
Bartlett's X2				0.0		0.0		0.0		0.0		0.0	
P(Bartlett's X2)				.		.		.		.		.	
Replicate F				0.000		0.000		0.000		0.000		0.000	
Replicate Prob(F)				1.0000		1.0000		1.0000		1.0000		1.0000	
Treatment F				0.000		0.000		0.000		0.000		0.000	
Treatment Prob(F)				1.0000		1.0000		1.0000		1.0000		1.0000	

Means followed by same letter do not significantly differ (P=.05, Student-Newman-Keuls)

Mean comparisons performed only when Analysis of Variance (AOV)

Treatment Prob (F) is significant at mean comparison observed significance level (OSL).

* = significant at P = 0.05

PHYGEN = phytotoxicity - general

Annexure D: Author guidelines for the Agriculture, Ecosystems, and Environment journal

AGRICULTURE, ECOSYSTEMS & ENVIRONMENT
An International Journal for Scientific Research on the Interaction Between Agroecosystems and the Environment

DESCRIPTION

AGRICULTURE, ECOSYSTEMS AND ENVIRONMENT

An International Journal for Scientific Research on the Interaction Between Agro-ecosystems and the Environment

Agriculture, Ecosystems and Environment publishes scientific articles dealing with the interface between **agro-ecosystems** and the **natural environment**, specifically how **agriculture** influences the environment and how changes in that environment impact agro-ecosystems. Preference is given to papers from experimental and observational research at the field, system or landscape level, from studies that enhance our understanding of processes using data-based biophysical modelling, and papers that bridge scientific disciplines and integrate knowledge. All papers should be placed in an international or wide comparative context.

The focus is on the following areas:

- Biological and physical characteristics and dynamics of agro-ecosystems.
- Ecology, diversity, and sustainability of agricultural systems.
- Relationships between agro-ecosystems and the natural environment, including land, air, and water.
- Agro-ecosystem and global environmental changes including climate change, greenhouse gases and air pollution.
- Ecological consequences of intensification, soil degradation, waste application, irrigation, and mitigation options.
- Environmental implications of agricultural land use and land use change.

All manuscripts are initially screened on their topic suitability and linguistic quality. The following topics are discouraged unless they provide new information regarding processes operating at the agro-ecosystem-environment interface: inventory and survey analysis and impact assessment, including life cycle and energy analysis; greenhouse or laboratory-based studies;

development of models or methodologies and pure model application; studies that are purely agronomic, socio-economic, or political.

AUDIENCE

Scientists in Agriculture, Forestry, Ecology and the Environment, Administrators and Policy-Makers in these fields.

IMPACT FACTOR

2015: 3.564 © Thomson Reuters Journal Citation Reports 2016

INTRODUCTION

Agriculture, Ecosystems and Environment deals with the interface between agriculture and the environment. Preference is given to papers that develop and apply interdisciplinary, bridge scientific disciplines, integrate scientific analyses derived from different perspectives of agro-ecosystem sustainability, and are put in as wide an international or comparative context as possible. It is addressed to scientists in agriculture, food production, agroforestry, ecology, environment, earth and resource management, and administrators and policy-makers in these fields.

The journal regularly covers topics such as: ecology of agricultural production methods; influence of agricultural production methods on the environment, including soil, water and air quality, and use of energy and non-renewable resources; agro-ecosystem management, functioning, health, and complexity, including agro-biodiversity and response of multi-species ecosystems to environmental stress; the effect of pollutants on agriculture; agro-landscape values and changes, landscape indicators and sustainable land use; farming system changes and dynamics; integrated pest management and crop protection; and problems of agro-ecosystems from a biological, physical, economic, and socio-cultural standpoint.

Types of papers

Original papers (Regular Papers) should report the results of original research. The material should not have been published previously elsewhere, except in a preliminary form.

Reviews should cover a part of the subject of active current interest. They may be submitted or invited.

A Short Communication is a concise, but complete, description of a limited investigation, which will not be included in a later paper. Short Communications should be as completely documented, both by reference to the literature and description of the experimental procedures employed, as a

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The section 'Opinion Paper' offers a forum for discussion of emerging or controversial ideas, or new approaches and concepts, in all areas covered by the journal. Contributions to this section should not occupy more than 2 printed pages (about 4 manuscript pages).

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Graphical Abstracts / Highlights files (where applicable) *Supplemental files* (where applicable)

Further considerations

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Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

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State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

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The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

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