Formulation, characterisation and *in vivo* efficacy of dapsone and proguanil in trimethylated chitosan microparticles

J. van Heerden

12814423

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Promoter: Prof. L.H. du Plessis

Co-promoters: Prof. A.F Kotzé and Prof. J.H. Steenekamp

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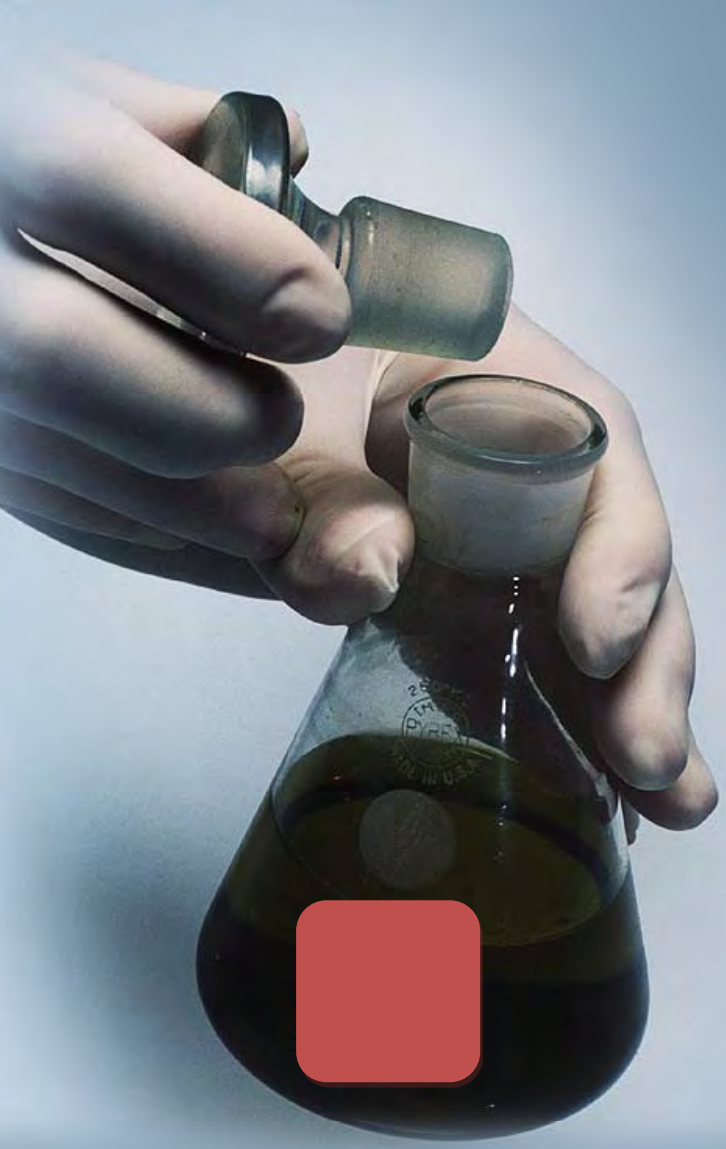


Preface

This thesis is submitted in fulfilment of the requirements of the degree of Doctor of Philosophy in Pharmaceutics. This work was financially supported by the Medical Research Council of South Africa. This thesis is submitted in article format in accordance with the General Academic Rules (A.13.7.3) of the North-West University. Each chapter is written in accordance with specific guidelines as stipulated by the journals intended for publication. A short description about the specific guidelines are given before each chapter. Each chapter has its own list of abbreviations, table of contents, list of figures and list of tables were applicable. The outline of this thesis is as follows:

Chapter 1	Introduction and aim of the study.
Chapter 2	Literature study.
Chapter 3	Article for submission: Formulation, characterisation and *in vivo* efficacy of dapsone and proguanil in trimethylated chitosan microparticles.
Chapter 4	Article for submission: Preparation and characterisation of quaternised *N*-trimethyl chitosan chloride by microwave irradiation compared to the conventional method.
Chapter 5	Conclusion and future prospects.

Annexure A	Determining the UV standard curve for the concentration measurement of dapsone and proguanil
Annexure B	Nuclear Magnetic Resonance and FTIR Spectra of synthesised TMC
Annexure C	Validation of the HPLC method
Annexure D	Additional information regarding the *in vivo* pharmacodynamic evaluation
Annexure E	Complete data set of the *in vivo* pharmacodynamic evaluation
Annexure F	Malvern Mastersizer and Zetasizer data
Annexure G	Bioanalytical report: Bioavailability evaluation of a reference dapsone and dapsone-TMC formulation in mice
Annexure H	Bioanalytical report: Bioavailability evaluation of a reference proguanil and proguanil-TMC formulation in mice

The contribution of each author is as follows:

J. van Heerden	Responsible for the following under supervision of Prof. L.H. du Plessis, Prof. A.F. Kotzé and Prof. J.H. Steenekamp: • Planning and design of study. • Experimental work. • Interpretation of results. • Writing of thesis and articles.
Prof. L.H. du Plessis	As promoter of the candidate, I was responsible for: • Planning and design of study in collaboration with the candidate and co-promoter. • Assisted in interpretation of results. • Supervised writing of thesis and article. • Acts as corresponding author of articles.
Prof. A.F. Kotzé	Responsibilities of co-promoter were as follows: • Assisted in the planning and design of the study in collaboration with the candidate and promoter. • Gave a critical review of the articles and thesis.
Prof. J.H.Steenekamp	Responsibilities of co-promoter were as follows: • Assisted in the planning and design of the study in collaboration with the candidate and promoter.
Dr. H.J.R. Lemmer	Synthesised TMC and microparticles for the bioavailability study.

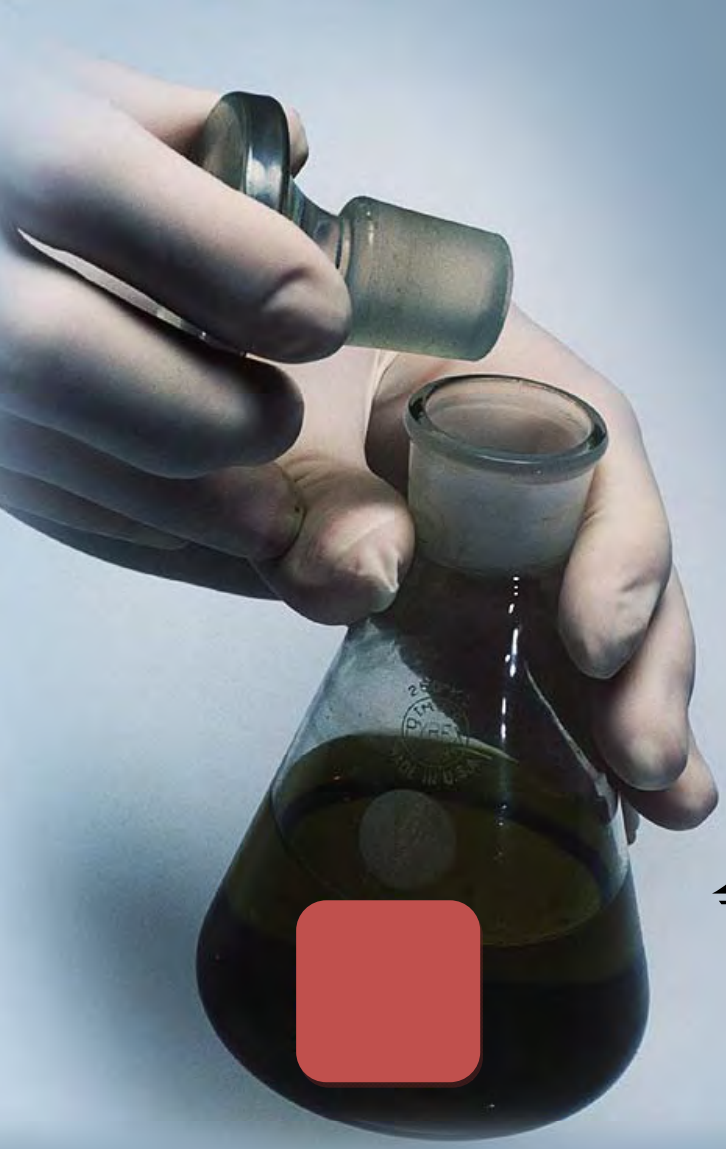
Declarations

I hereby declare that I have approved the articles/thesis and that my role in the study as indicated above is representative of my actual contribution. I give permission as author or co-author for submission of articles.

Prof. L.H. du Plessis	Prof. A.F. Kotzé
Prof. J.H. Steenekamp	J. van Heerden

I hereby agree to the above mentioned author contribution and give permission for the use in this thesis.

Dr. H.J.R. Lemmer



Abstract

Malaria is an infectious disease caused by various forms of the *Plasmodium* parasite. It is responsible for thousands of deaths yearly with 90 % of those deaths being in sub-Saharan Africa, thus making it a disease of global importance. The global burden of malaria is worsened by resistance to current treatment, a lack in funding and limited research outputs. More alternative ways of treatment must be explored and may include the co-formulation of antimalarial drug substances as well as alternative ways of drug delivery.

Antifolates are drugs which interfere with an organism's folate metabolism by inhibiting dihydropteroate synthase (DHPS) or dihydrofolate reductase (DHFR). Dapsone is a synthetic sulfone which has a mechanism of action that is very similar to that of sulphonamides. The mechanism of action is characterised by the inhibition of folic acid synthesis through the inhibition of dihydropteroate synthase (DHPS). Another antifolate drug, proguanil, is the prodrug of cycloguanil. Its mechanism involves the inhibition of dihydrofolate reductase (DHFR), thus inhibiting the malaria parasite to metabolise folates and therefore stunting its growth. Unfortunately, dapsone has a serious side-effect in people with a deficiency of the enzyme glucose-6-phosphate dehydrogenase (G6PD) causing oxidative stress on the red blood cells leading to the rupturing of these cells.

The main objective of this study was to formulate and characterise TMC-TPP microparticles loaded with the effective but toxic drug combination of dapsone and proguanil and to determine if these drug-containing microparticles had *in vivo* efficacy against malaria.

N-trimethyl chitosan chloride (TMC), a partially quaternised chitosan derivative, shows good water solubility across a wide pH range thus having mucoadhesive properties and excellent absorption enhancing effects even at neutral pH. A faster, more efficient microwave irradiation method was developed as an alternative to the conventional synthesising method of TMC. TMC with the same degree of quaternisation (DQ), ± 60 %, was obtained in a quarter of the reaction time (30 min) by using the newly developed method. The TMC synthesised with the microwave irradiation method also exhibited less degradation of the polymer structure, thus limiting the chance for the formation of any unwanted by-products (*O*-methylation, *N,N*-dimethylation and *N*-monomethylation).

The formation of complexes by ionotropic gelation between TMC and oppositely charged macromolecules, such as tripolyphosphate (TPP), has been utilised to prepare microparticles which are a suitable drug delivery system for the dapsone-proguanil combination. Both these drugs were successfully entrapped. These particles were characterised and the *in vivo* efficacy against the malaria parasites was determined. The microparticles with both the drugs, separately and in combination, displayed similar or better *in vivo* efficacy when compared to the drugs without the TMC microparticles.

An *in vitro* dissolution study was also performed by subjecting the dapsone and proguanil TMC formulations to 0.1N HCl dissolution medium. Samples were withdrawn after predetermined time points and the drug concentration was determined with HPLC. It was found that the TMC microparticles resulted in a sustained release profile since only 73.00 ± 1.70 % (dapsone) and 55.00 ± 1.90 % (proguanil) was released after 150 minutes. The *in vivo* bioavailability of the dapsone and proguanil TMC formulations was evaluated in mice by collecting blood samples at predetermined time points and analysing the samples with a sensitive and accurate LC-MS/MS method. The *in vivo* bioavailability of the dapsone TMC formulation relative to the normal dapsone formulation was found to be 244 % and 123 % for the proguanil TMC formulation relative to the normal proguanil formulation.

These TMC-TPP microparticles formulations showed better *in vivo* efficacy and bioavailability when compared to the normal formulation. Together with the sustained release, these formulations may be a promising cheaper and more effective treatment against malaria.

KEYWORDS

Dapsone
Proguanil
N-trimethyl chitosan chloride
Malaria
Tripolyphosphate
Microparticles
Microwave irradiation

Opsomming

Malaria is ’n aansteeklike siekte wat deur verskeie vorme van die *Plasmodium*-parasiet veroorsaak word. Dit is jaarliks verantwoordelik vir duisende sterftes met 90 % daarvan in sub-Sahara-Afrika, wat dit dus ’n siekte van wêreldwye belang maak. Die globale las van malaria word deur weerstand teen die huidige middels, ’n gebrek aan befondsing en beperkte navorsingsuitsette vererger. Verdere alternatiewe maniere vir behandeling moet ondersoek word en kan onder meer die gesamentlike formulering van malariamiddels sowel as alternatiewe maniere van geneesmiddelaflewering wees.

Antifolate is middels wat met ’n organisme se folaatmetabolisme inmeng deur dihidropteroaatsintase (DHPS) of dihidrofolaatreduktase (DHFR) te rem. Dapsoon is ’n sintetiese sulfoon met ’n werkingsmeganisme wat baie soortgelyk aan dié van die sulfoonamiede is. Die werkingsmeganisme word gekenmerk deur remming van foliensuursintese deur onderdrukking van dihidropteroaatsintase (DHPS). ’n Ander antifolaat, proguaniel, is die progeneesmiddel van sikloguaniel. Die meganisme daarvan behels die remming van dihidrofolaatreduktase (DHFR), en dit onderdruk dus die vermoë van die malariaparasiet om folaat te metaboliseer en sodoende belemmer dit groei. Ongelukkig het dapsoon ’n ernstige newe-effek in mense met ’n tekort aan die ensiem glukose-6-fosfaatdehidrogenase (G6PD) wat oksidatiewe stres van rooibloedselle veroorsaak wat tot ruptuur van hierdie selle lei.

Die hoofdoel van hierdie studie was om TMC-TPP wat met mikrodeeltjies van die doeltreffende, maar toksiese geneesmiddelkombinasie van dapsoon en proguaniel gelaai is, te formuleer en te karakteriseer om te bepaal of hierdie geneesmiddelbevattende mikrodeeltjies *in vivo* doeltreffendheid teen malaria het.

N-Trimetielkitosaanchloried (TMC), ’n gedeeltelik gekwaterniseerde kitosaanderivaat, toon goeie wateroplosbaarheid oor ’n wye pH-gebied en het dus selfs by neutrale pH mukokleefbare eienskappe en uitstekende vermoë om absorpsie te bevorder. ’n Vinniger en meer doeltreffende metode vir die sintese van TMC deur gebruik van mikrogolfbestraling is ontwikkel as ’n alternatief tot die konvensionele sintetiese metode. TMC met dieselfde mate van kwaternisering, ± 60 %, is in ’n kwart van die reaksietyd (30 min) deur die gebruik van die nuut ontwikkelde metode verkry. Die TMC gesintetiseer met mikrogolfbestraling het ook minder degradasie van die polimeerstruktuur gehad, en die kans vir die vorming van ongewenste neweprodukte (O-metilering, N,N-dimetilering en N-monometilering) is dus laer.

Die vorming van komplekse deur ionotropiese jelvorming tussen TMC en teenoorgesteld gelaaide makromolekule soos tripolifosfaat (TPP) is gebruik om mikrodeeltjies te maak wat geskik vir geneesmiddelaflewering van die dapsoon-proguanielkombinasie is. Albei hierdie geneesmiddels was suksesvol ingesluit. Hierdie deeltjies is gekarakteriseer en die *in vivo*-doeltreffendheid teen die malariaparasiete is bepaal. Die mikrodeeltjies met albei

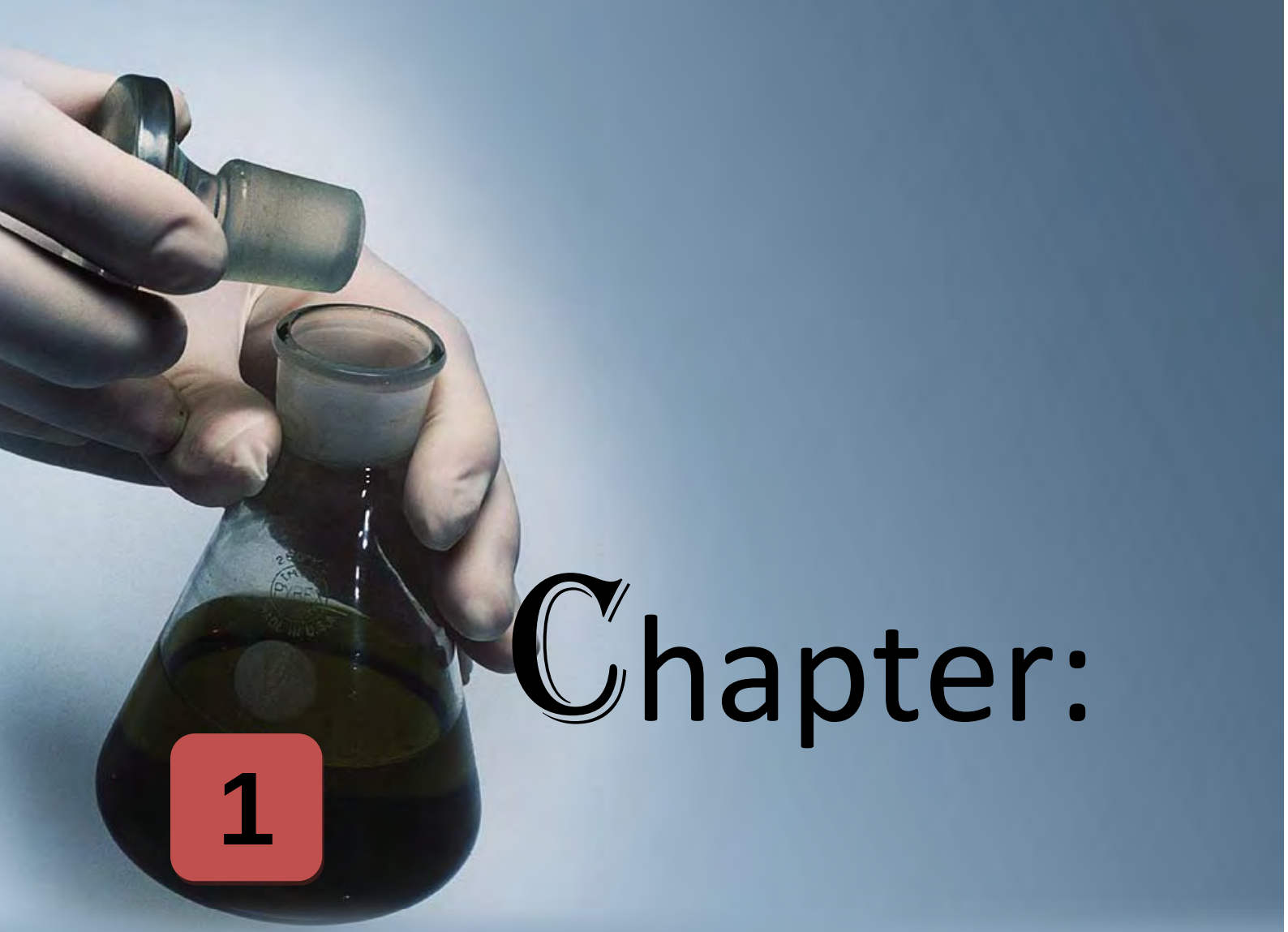
geneesmiddels, afsonderlik en in kombinasie, vertoon soortgelyke of beter *in vivo*-doeltreffendheid in vergelyking met die geneesmiddels sonder die TMC-mikrodeeltjies.

'n *In vitro*-dissolusiestudie van die formulerings van dapsoon en proguaniel in TMC in 0.1 N HCl dissolusiemedium is ook gedoen. Monsters is op voorafbepaalde tye geneem en die geneesmiddelkonsentrasie is met HDVC bepaal. Dit is gevind dat die TMC-mikrodeeltjies 'n volgehouevrystellingsprofiel vertoon het aangesien slegs 73.00 ± 1.70 % (dapsoon) en 55.00 ± 1.90 % (proguaniel) na 150 minute vrygestel is. Die *in vivo*-biobeskikbaarheid van die TMC-formulerings van dapsoon en proguaniel is in muise geëvalueer deur versameling van bloedmonsters op voorafbepaalde tye en die ontleding van die monsters met 'n sensitiewe en akkurate LC-MS/MS-metode. Dit is gevind dat die *in vivo*-biobeskikbaarheid van die TMC-formulering van dapsoon relatief tot die normale dapsoonformulering 244 % is en dié van die TMC-formulering van proguaniel 123 % van die normale proguanielformulering.

Hierdie TMC-TPP-mikrodeeltjiesformulerings het beter *in vivo*-doeltreffendheid en biobeskikbaarheid in vergelyking met die normale formulerings. Saam met die volgehoue vrystelling kan hierdie formulerings belowende goedkoper en meer doeltreffende middels teen malaria wees.

SLEUTELWOORDE

Dapsoon
Proguaniel
N-Trimetielkitosaanchloried
Malaria
Tripolifosfaat
Mikrodeeltjies
Mikrogolfbestraling



Chapter: 1

Introduction and aim of study focussing on the relevancy of the thesis. It gives a detailed problem statement with specific objective and aims. Reference style is a modified version of the North-West University Harvard style

1. Introduction

Malaria is an infectious disease caused by various forms of the Plasmodium parasite with *Plasmodium falciparum* being the most common and well-known form. It is responsible for hundreds of thousands of deaths yearly, with the majority of them being in third world countries. Most of these third world countries are located in Africa where the malaria death toll is the highest of any place in the world. According to the yearly report published by the WHO (2013) approximately 90 % of malaria deaths occur in sub-Saharan Africa where poverty and lack of treatment are the biggest contributing factors. This highlights the importance for the development of cheaper and more effective anti-malaria treatment in order to reduce malaria cases and deaths. Resistance to antimalarial drugs was first observed about fifty years ago with chloroquine being used as treatment. Since then, efforts to control the disease have been hindered by failed or failing drugs (Summers *et al.*, 2012) due to the malaria parasite's ability to evolve and mutate and in so doing, developing resistance to antimalarial treatments. Resistance of the malaria parasite can be attributed to the extensive utilisation of monotherapy treatment through the decades. This resulted in the parasite becoming resistant to all classes of medicine used in the treatment of malaria (White, 2004). Malaria parasites have acquired resistance between one and fifteen years after introduction (depending on the drug) to all classes of antimalarial drugs which have gone into widespread use (Mackinnon & Marsh, 2010), which suggests that most future new drugs will follow the same fate of rapidly losing efficacy. Another method will be to concentrate efforts on the reformulation of current or previous anti-malaria drug combinations. By understanding the life cycle of the malaria parasite researchers are able to develop drugs to target certain stages which are important for the development and reproduction of the parasite. The parasite's lifecycle consists of two phases that is alternated between the different hosts, namely the mosquito and a human. One of the asexual phases in malaria parasite's life cycle, schizogony, occurs in the human host and is divided into two distinguishable stages, namely the liver stage and blood stage. During the liver stage the parasite infects liver cells, creating schizonts. At the time these schizonts rupture, the parasites infect the erythrocytes (red blood cells) which leads to the erythrocytic cycle (Bogitsh *et al.*, 2013). Many of the drugs that are available for the treatment of malaria work when the parasite is in the blood stage (Gregson & Plowe, 2006).

Antifolates are one of the oldest malaria chemotherapy choices together with chloroquine. Antifolates, including the combination sulphadoxine-pyrimethamine, are still being used effectively in intermittent preventative treatment programmes. Antifolates interfere with the folate metabolism by inhibiting dihydropteroate synthase (DHPS) or dihydrofolate reductase (DHFR). The combination of the two inhibitors is synergistic and their recommended use is in combination for the treatment of malaria (Müller & Hyde, 2013; Nzila, 2006). However, resistance to antifolates are high and it is not considered first line therapies. Due to resistance of the parasites to the drugs and non-compliance of patients to the therapy, resulting in recrudescence, alternative ways of treatments must be explored (Nwaka *et al.*, 2004). Such alternatives may include the development of new compounds, synergistic drug combinations, co-formulation of these combinations and ways of drug delivery (Fidock & Wellems, 1997). The combination of pyrimethamine and sulfadoxine was for long a cheap and the only antifolate combination and effective

alternative to chloroquine-resistant malaria (Sudre *et al.*, 1992). However, starting in 1994, resistance to sulfadoxine could be observed because of its slow elimination rate, indicating that sulfadoxine efficacy is declining in eastern Africa emphasising the need for an alternative therapy to reduce the selective antifolate resistance (Mutabingwa *et al.*, 2001; Winstanley *et al.*, 1995).

Dapsone is a synthetic sulfone used to treat leprosy. It has a mechanism of action that is very similar to that of sulphonamides (Williams *et al.*, 2000) and has also been found to have schizonticidal (against shizonts in the liver) and gametocidal (gametocytes in the erythrocytes) activity when used to treat malaria (Kunal *et al.*, 2003). This mechanism of action involves the inhibition of folic acid synthesis. This is facilitated by the inhibition of dihydropteroate synthase (DHPS) (Williams *et al.*, 2000).

Proguanil, another antifolate, is a prodrug that is readily metabolised to its active metabolite cycloguanil, which is an inhibitor of dihydrofolate reductase (DHFR) (Carrington *et al.*, 1951). It is used as prophylaxis treatment against malaria, however, clinical failure rates have cast a shadow on the drug's further development (Murambiwa *et al.*, 2011).

In vitro analyses have demonstrated that cycloguanil, the active metabolite of proguanil, and dapsone are more potent than pyrimethamine and sulfadoxine, respectively. *In vivo*, chloroquine is efficacious in treating malaria and it retains activity against sulfadoxine/pyrimethamine-resistant parasites (Mutabingwa *et al.*, 2001). It was established that the combination of dapsone and proguanil, since being short acting drugs (half-lives of 20 and 12 hours, respectively) (Winstanley *et al.*, 1997), selects less efficiently for resistance than sulfadoxine/pyrimethamine (Kublin *et al.*, 2002; Nzila-Mounda *et al.*, 1998). Unfortunately, dapsone has a serious side-effect in people with a deficiency in the enzyme glucose-6-phosphate dehydrogenase (G6PD), causing red blood cells to rupture due to oxidative stress and resulting in potentially fatal haemolytic anaemia (Luzzatto, 2010). This caused the World Health Organisation (WHO) to withdraw dapsone as a treatment in 2008, opening the opportunity for new research to be done to possibly reduce the toxicity of this treatment.

A strategy to combat the malaria parasite's resistance, thus prolonging the activity and efficacy of future developed drugs is to design in advance drug delivery systems which consist of biomaterials (Movellan *et al.*, 2014). The development of novel delivery systems is not only less expensive than finding new drugs, but may also improve release of antimalarials at the desired rates thus reducing the possibility of toxicity (Murambiwa *et al.*, 2011). Colloidal drug delivery systems has for long been a keen focus area for drug delivery systems of various drugs. Colloidal drug delivery systems has the advantage of controlling drug release from the particles, thereby reduction of the incidence and severity of side effects related to high plasma peak drug concentrations (Attwood, 2007). The problems with liposome based formulations are that they possess poor modular chemical functionality and relatively weak stability. To overcome these problems, polymer based formulations can be used as alternative drug delivery systems. Polymer based drug delivery systems have various advantages over the lipid based drug delivery systems. The larger molecular mass of polymer chains over the lipid tails and versatility of the chemical functionality of the

polymer structures result in particles with more toughness, and permeability and surface functionality (Le Meins *et al.*, 2013).

Chitosan is natural cationic polysaccharide that has drawn increasing attention within pharmaceutical and biomedical applications because of its abundant availability, mucoadhesive and inherent pharmacological properties. Other beneficial biological properties such as biocompatibility, biodegradability, non-toxicity and low-immunogenicity also make it a favourable compound to work with (Felt *et al.*, 1998; Illum, 1998). The fact that chitosan can control the release and absorption of compounds, means that it can be used in different oral dosage forms like tablets and microparticles (Felt *et al.*, 1989; Singla & Chawla, 2001). A major drawback is its insolubility at physiological pH (7.4), whereas it is soluble and active as an absorption enhancer only in its protonated (uncoiled and positively charged) form in acidic environments. This problem was solved by synthesising *N*-trimethyl chitosan chloride (TMC), a partially quaternised chitosan derivative, which has good water solubility across a wide pH range thus having mucoadhesive properties and excellent absorption enhancing effects even at neutral pH (Amidi *et al.*, 2006; Hamman *et al.*, 2003; Kotzé *et al.*, 1998).

The formation of complexes between chitosan-based polymers (like TMC) and oppositely charged macromolecules has been studied extensively by many researchers and it was found that this property can be exploited to prepare micro/nanoparticles that are suitable for drug delivery (Chen *et al.*, 2008; Geçer *et al.*, 2010; Prego *et al.*, 2010; Sandri *et al.*, 2007; Sayin *et al.*, 2008). These particles are prepared by ionic cross-linking (ionotropic gelation) through self-assembly of chitosan or its derivatives, which is positively charged, and oppositely charged macromolecules by the addition of a low molecular weight anionic cross-linker, such as tripolyphosphate (TPP) (Amidi *et al.*, 2010).

2. Objectives

The objective of this study was the formulation and characterisation of TMC-TPP microparticles loaded with the effective but toxic drug combination of dapsone and proguanil. The strategy was followed by conducting a thorough literature study of chitosan, TMC and TMC based particle formulations. This was followed by the development phase including a literature study, dosage form selection and formulation. Afterwards entrapping of dapsone and proguanil, characterising those particles and determining their *in vivo* efficacy and bioavailability were done. An *in vitro* dissolution study was also performed to determine whether one can achieve a sustained release profile for the drugs to possibly reduce their toxicity.

The main objective of this study will be achieved through the following aims:

- The formulation of TMC-TPP microparticles.
- The entrapment of dapsone and proguanil in these microparticles.
- Optimization of methods to determine physicochemical properties.

- Determination of size, pH, entrapment efficacy and loading capacity of the formulations.
- *In vitro* dissolution study of the drugs entrapped in the TMC-TPP microparticles to determine the release profile.
- *In vivo* efficacy study to determine the efficacy of the drugs entrapped in the TMC-TPP microparticles when compared to the plain drug's efficacy.
- *In vivo* bioavailability (pharmacokinetic) study in mice to determine whether the drugs are released, absorbed and available in the body.

In addition to the main study objective, a new synthesis method for TMC was developed by using microwave irradiation to determine if the method can be optimised and whether it is more beneficial than the conventional, well known method.

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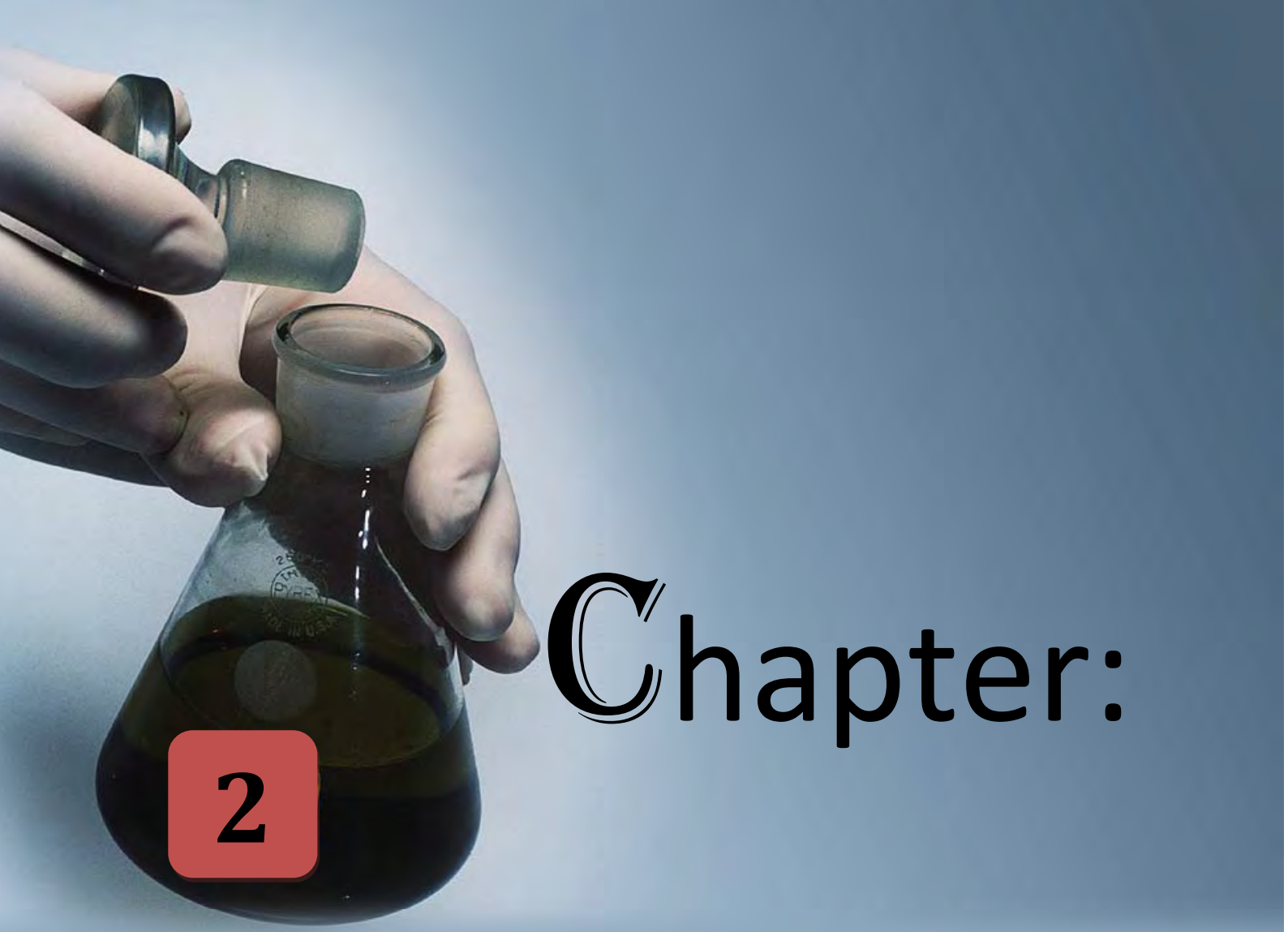

Chapter: 2

This chapter contains the literature study focusing on the combination of dapsone and proguanil and how they are incorporated into microparticles synthesised from trimethyl chitosan (TMC). Literature about the natural polymer, chitosan, and how to synthesise and characterise its derivative (TMC) is also included within this chapter. A biopharmaceutical evaluation including physiochemical properties and biological considerations to evaluate the microparticles is discussed in short. The reference style used in this chapter is a modified version of the Harvard style (as defined in Microsoft Word by the North-West University)

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List of Abbreviations

PABA	Para-amino benzoate
DHPS	Dihydropteroate synthase
G6PD	Glucose-6-phosphate dehydrogenase
DHFR	Dihydrofolate reductase
SP	Sulphadoxine-pyrimethamine
CD	Chlorproguanil-dapsone

WHO	World Health Organisation
MW	Molecular weight
SEC	Size exclusion chromatography
MALLS	Multi-angle laser light scattering
MHKS	Mark-Houwink-Kuhn-Sakurada
NSAID	Non-steroidal anti-inflammatory drug
ZO-1	Zona occludens
TMC	*N*-Trimethyl chitosan
NMR	Nuclear magnetic resonance spectroscopy
DQ	Degree of quaternisation
TPP	Tripolyphosphate
FD_4	Fluorescein isothiocyanate dextran
BSA	Bovine serum albumin
BHb	Bovine haemoglobin
FTIR	Fourier transform infrared
TEM	Transmission electron microscopy
DLS	Dynamic light scattering
HPLC	High performance liquid chromatography
WDI	World Drug Index
LS	Light scattering
PI	Polydispersity Index
LLD	Laser light diffraction
PSD	Particle size distribution

SEM	Scanning electron microscopy
EE	Entrapment efficiency
GI	Gastro intestinal
FITC	Fluorescein isothiocyanate conjugate
NALT	Nasal associated lymphoid tissue

1. Malaria

Malaria is an infectious disease caused by various forms of the Plasmodium parasite. It was responsible for approximately 627 000 deaths in 2012 and 90 % of those deaths were in sub-Saharan Africa (WHO, 2013), thus making it a disease of global importance. According to this report, a decrease in the pace of malaria related mortality rates, between the years 2011 and 2012, was observed due to a huge lack in funding. This highlights the importance for the development of cheaper and more effective anti-malaria treatment in order to reduce malaria cases and deaths. One such method will be to concentrate efforts on the reformulation of current or previous anti-malaria drug combinations. The parasite's life cycle consists of two phases that alternate between the different hosts, namely the mosquito and a human. The sexual phase, called gamogony, accompanied by an asexual phase, sporogony, occurs in the mosquito. Another asexual phase, schizogony, which occurs in the human host can be divided into two distinguishable stages, namely the liver stage and blood stage. During the liver stage the parasite infects liver cells, creating schizonts. At the time these schizonts rupture, the parasites infect the erythrocytes (red blood cells) and it is during this stage that the parasites cause the clinical symptoms to manifest. The erythrocytic cycle includes merozoites that develop into male and female gametocytes which are ingested by mosquitoes, completing the cycle (Bogitsh *et al.*, 2013; Mack, 2009). These symptoms mimic those of the common flu and usually include fever, malaise, headache, nausea, vomiting and diarrhoea. Many of the drugs that are available for the treatment of malaria work when the parasite is in the blood stage (Gregson & Plowe, 2006).

Resistance of the malaria parasite occurs when the parasite develops survival advantages against antimalarial drugs due to rare spontaneous mutations. This can be attributed to the extensive utilisation of monotherapy treatment through the decades. This resulted in the parasite becoming resistant to all classes of medicine used in the treatment of malaria (White, 2004). During the last decade the mainstay drug choice for treatment against malaria was the use of artemisinin and its derivatives due to the fact that it rapidly eliminates the asexual stages and early sexual forms of the parasite. Recently, resistance has been reported against artemisinins largely due to monotherapy. This resulted in the need to intensify actions to protect the therapeutic life of artemisinin-based combination therapy, since there is no alternative medicine which is ready to enter the market and replace artemisinin-based combination

therapy (WHO, 2014). Antifolates are one of the oldest malaria chemotherapy choices together with chloroquine. Antifolates, including the combination sulphadoxine-pyrimethamine, are still being used effectively in intermittent preventative treatment programmes (Müller & Hyde, 2013; Nzila, 2006). However, resistance to antifolates are high and it is not considered first line therapies. Due to resistance of the parasites to the drugs and non-compliance of patients to the therapy, resulting in recrudescence, alternative ways of treatments must be explored (Nwaka *et al.*, 2004). Such alternatives may include the development of new compounds, synergistic drug combinations, co-formulation of these combinations and ways of drug delivery (Fidock & Wellems, 1997).

2. Antifolates

Antifolates were originally developed for the treatment of leukaemia. The success in treating tumours led to the adaptation of this class of drugs to other rapidly dividing cells like parasites and bacteria. Antifolates interfere with the folate metabolism by inhibiting dihydropteroate synthase (DHPS) or dihydrofolate reductase (DHFR). The combination of the two inhibitors is synergistic and their recommended use is in combination for the treatment of malaria (Müller & Hyde, 2013; Nzila, 2006). Dapsone is a synthetic sulfone used to treat leprosy. Previous studies done on the structure-activity relationships have indicated the importance of the sulfone group for the pharmacological activity of dapsone (Colwell *et al.*, 1974; Saxena *et al.*, 1989; Wiese *et al.*, 1987). It has a mechanism of action that is very similar to that of sulphonamides (Williams *et al.*, 2000) and has also been found to have schizonticidal (against shizonts in the liver) and gametocidal (gametocytes in the erythrocytes) activity when used to treat malaria (Kunal *et al.*, 2003). This mechanism of action involves the inhibition of folic acid synthesis. This is facilitated by the inhibition of dihydropteroate synthase (DHPS) by competing with para-aminobenzoate (PABA) for the active binding site (Williams *et al.*, 2000). These kinds of drugs are known as class 1 antifolates. Adverse reactions of dapsone include nausea, headache and more commonly a rash. More serious adverse reactions are aplastic anaemia and agranulocytosis. The latter is very prominent when dapsone is used as prophylactic treatment of malaria in persons with a glucose-6-phosphate dehydrogenase (G6PD) deficiency (Degowin *et al.*, 1966; Nzila, 2006).

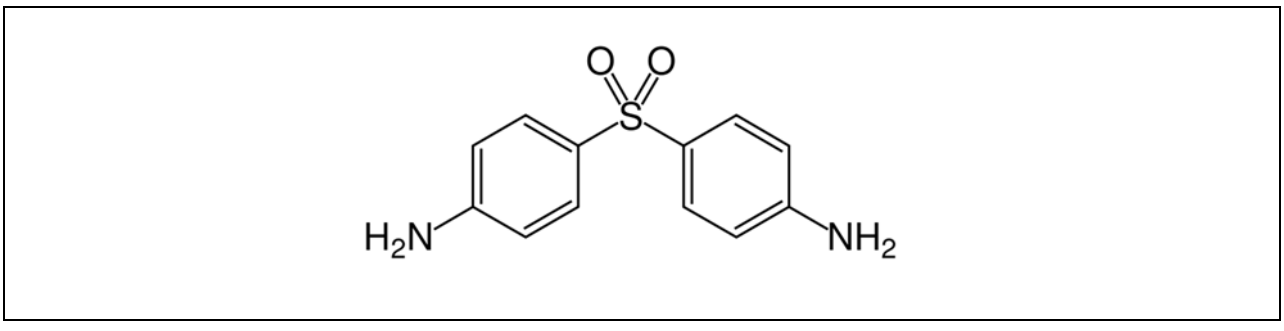

Figure 1: Chemical structure of dapsone: the sulfone group is responsible for the all the pharmacological activity.

According to Nzila (2006), proguanil was the first antifolate that was discovered through intensive research being done during the Second World War. It is a prodrug that is readily metabolised to its

triazine form cycloguanil, which is an inhibitor of dihydrofolate reductase (DHFR) (Carrington *et al.*, 1951). The 2,4-diamino scaffold is responsible for the pharmacological action by hydrogen bonding of the amino groups to the residues of the DHFR enzymes of the malaria parasite (Anderson, 2005). Due to the potency of proguanil, a search for new and more potent analogues had been launched. This led to the discovery of chlorproguanil, a product of the chlorination on the phenyl ring of proguanil that has more potent activity against malaria. Its active metabolite is known as chlorcycloguanil. These drugs belong to the class 2 antifolates and by inhibiting this enzyme the parasite cannot metabolise folates and thus cannot grow. There are no severe adverse effects with proguanil and some may include mouth ulcers, temporary hair loss and anxiety with prolonged use.

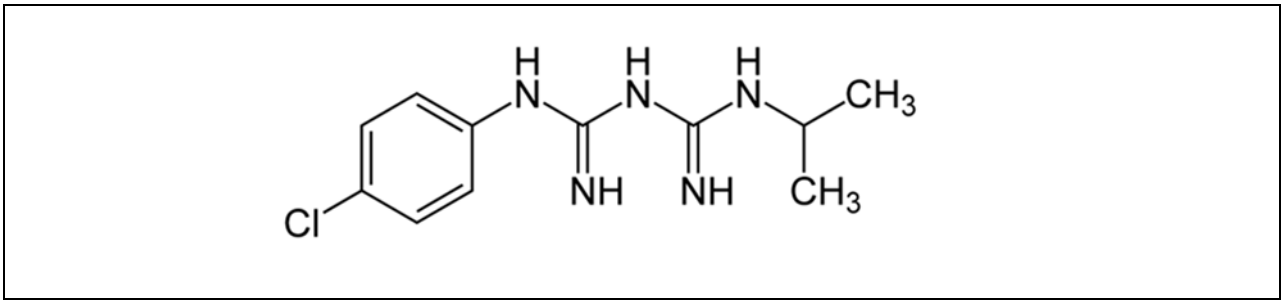

Figure 2: Chemical structure of proguanil. Proguanil is a prodrug metabolized in the body to its active form cycloguanil.

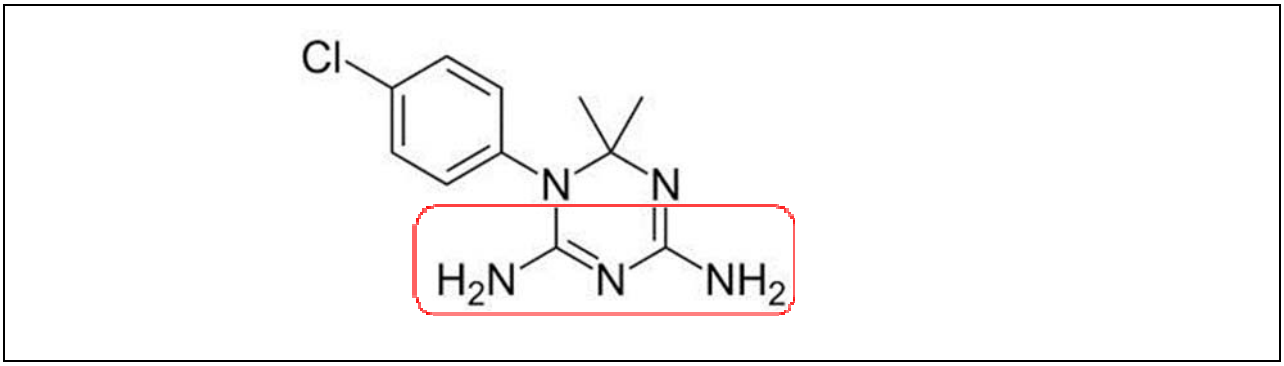

Figure 3: Chemical structure of cycloguanil. Due to the 2,4-diamino scaffold (highlighted in red), hydrogen bonding can take place to key catalytic residues in the DHFR active site.

Due to the increase in resistance to single drug therapy, combination therapy has been developed. One of these combination therapies is the chlorproguanil-dapsone combination. This antifolate combination known as Lapdap® has been shown to exhibit less saceptability to resistance than the other widely used antifolate combination of sulfadoxine and pyrimethamine (Nwaka *et al.*, 2004) and more potency against malaria (Nzila-Mounda *et al.*, 1998; Winstanley *et al.*, 1995). This is a synergistic combination, meaning that it interferes with the folate metabolism at two different biosynthetic stages to give an enhanced effect that is much better than the single drug therapy and it aims to prevent the development of resistance to the therapy (Luzzatto, 2010). For a long time there was only one antifolate combination for the treatment of malaria, namely sulphadoxine-pyrimethamine (SP) and it was known by the name of Fansidar®. Many countries in south and east Africa used this combination because of the resistance to chloroquine (Lang & Greenwood, 2003) and its affordability (Winstanley, 2001). Signs of resistance to this combination

started to appear in the early nineties due to the long half-life of pyrimethamine (>80 hours) (Watkins & Mosobo, 1993). The resistance became a severe problem to such an extent that it was estimated that pyrimethamine has had an effective lifespan of only five years where-as cycloguanil had a 3-year lifespan (Anderson, 2005; White, 1997). This exerted the strong selective pressure for mutations in its target gene, DHFR (Kublin *et al.*, 2002; Nzila *et al.*, 2000), and led to the search for a safe, effective and affordable treatment. Another criterion was also that such a combination should have the ability to withstand the ability of the malaria parasite to mutate and become resistant to treatment (Lang & Greenwood, 2003). To minimize this suseptability to resistance, the short acting antifolate combination chlorproguanil-dapsone (CD) was identified and developed as an antimalarial. In the mid-1980s Watkins and colleagues followed the approach where they took old antimalarial drugs and used it in combination with each other and decided on the combination of CD (Watkins *et al.*, 1987). This pioneered the way for the use of CD and the rise to the existence of Lapdap®. *In vitro* analyses have demonstrated that chlorcycloguanil, the active metabolite of chlorproguanil, and dapsone are more potent than pyrimethamine and sulfadoxine, respectively. *In vivo*, CD is efficacious in treating malaria and it retains activity against SP-resistant parasites (Mutabingwa *et al.*, 2001). It is well established that the combination of CD, because of its short acting drugs (half-lives of 12 and 20 hours respectively) (Winstanley *et al.*, 1997), is less suceptable to malaria resistance than SP (Kublin *et al.*, 2002; Nzila-Mounda *et al.*, 1998).

Unfortunately, this “perfect” combination was too good to be true due to the fact that dapsone had a serious side effect in people with a deficiency of the enzyme glucose-6-phosphate dheydrogenase (G6PD). About 20 % of people who received this combination developed haemolytic anaemia because of the G6PD deficiency. The red blood cells of people with this deficiency are much more susceptible to oxidative stress than that of the normal person and this causes the cell to rupture. And as misfortune should have it most people living in malaria areas have this deficiency (Luzzatto, 2010). At first the WHO set certain conditions for the use of Lapdap®. They determined that the use of Lapdap® is totally contraindicated in people which are G6PD-deficient. Certain recommendations were made and had to be implemented before Lapdap® could be used as prophylaxis against malaria. Firstly, haemolytic anaemia (haemoglobin < 50 g/L) and G6PD deficiency should be ruled out. A second condition was that if G6PD deficiency is prevalent in the area and no sufficient tests are available, an alternative antimalarial should be used. And finally, Lapdap® should only be used when there is no suitable alternative treatment (WHO, 2004). Eventually, four years later, in 2008 it was decided by GlaxoSmithKline to withdraw the treatment from the market. This created the opportunity for new research to be done to reduce the toxicity of this treatment by altering it in some way because of the significant potential that this combination has. Because of widespread resistance to most antimalarials, a failure for new compounds to reach the market and failure to control the spread of the disease resulting in a high number of deaths each year, the benefit of antifolates still outweigh the risks.

3. Drug delivery systems used in the treatment of malaria

Malaria parasites have developed resistance to all classes of antimalarial drugs that have been used over the years, which suggests that a new drug, developed in the future, will also rapidly lose efficacy and suffer the same fate. A strategy to combat the malaria parasite's resistance, thus prolonging the activity and efficacy of future developed drugs, is to design in advance drug delivery systems which consist of biomaterials (Movellan *et al.*, 2014). Colloidal drug delivery systems has for long been a keen focus area for drug delivery systems of various drugs. Colloids are a disperse system that consists of a desperse phase, dispersed as particles or droplets througout the continuous phase. Dispersions in which the particles range between 1 nm to 1 µm are termed colloidal systems. Colloidal drug delivery systems have the advantage of controlling drug release from the particles, thereby reducing the incidence and severity of side effects related to high peak plasma drug concentrations (Attwood, 2007). The most commonly used and extensively researched colloidal drug delivery system is liposomes (Slabbert *et al.*, 2011). Liposomes are used for controlled drug delivery of formulations. These formulations prevent rapid clearance of the drug in the body by controlling the size, charge and surface hydration of the drug (Murambiwa *et al.*, 2011). Slabbert and co-workers (2011) used liposomes and Pheroid™ to entrap mefloquine and studied the physical stability of these particles measuring the pH, size and entrapment efficacy. The problems with liposome based formulations are that they possess poor modular chemical functionality and relatively weak stability. To overcome these problems polymer based formulations can be used as alternative drug delivery systems. Polymer based drug delivery systems have various advantages over the lipid based drug delivery systems. The larger molecular mass of polymer chains over the lipid tails and versatility of the chemical functionality of the polymer structures result in particles with more toughness, and permeability and surface functionality (Le Meins et al., 2013).

4. Polymer chemistry of polysaccharides

Polymers are large molecules made of repeating units. The process by which these macromolecules are formed is polymerisation (Stevens, 1999). They have been used for many years in pharmaceutical chemistry, even before their behaviour was fully understood. It was only during the 1920s that a young scientist by the name of Hermann Staudinger systematically synthesised a variety of polymers and characterised the chemical nature thereof. Herman Mark and Linus Pauling pioneered the study of polymers by using X-ray studies of natural and synthetic materials to proof that polymers do exist. This led Mark to found the academic and communication basis that would allow polymer science grow to where it is today (Carraher, 2000).

There are different kinds of polymers that can be divided into two major groups, namely natural polymers (biopolymers) like polysaccharides, proteins, polynucleotides (DNA); and synthetic polymers, like plastics, fibers and films. In this study the focus is mainly on natural polymers, and more specifically polysaccharides. Polysaccharides are the most ubiquitous of all the biopolymers, with cellulose making up a third of all the solid matter in the plant kingdom (Teegarden, 2004). They are obtained by biosynthesis and have a variety of structures. The building blocks for polysaccharides are

monosaccharides like glucose or fructose, thus repeating these single units, bigger polysaccharides like cellulose, starch and glycogen are formed.

The much attracted attention that polysaccharides receive is due to the fact that they present many advantages, namely:

i. their biodegradability,
ii. their relatively low cost,
iii. the ease in which derivatives can be synthesised because of their reactivity to other organic molecules and
iv. their renewable character (Renaud *et al.*, 2005).

Polymers are very diverse due to the fact that they have different molecular weights, which in turn have an influence on the viscosity of the polymer when in a solution. All of these properties can be determined experimentally for natural and synthesized polymers with various methods.

4.1 Molecular weight (MW)

The physical properties of these polymers are largely influenced by the number of repeating units, hence making molecular weight an extremely important variable. One will seldom find a polymer with a monodispersed distribution of molecular weight due by the way that a polymer is formed. It is therefore important to express the molecular weight of a sample as a distribution and average. An optimum MW would really depend on what the polymer is intended to be used for. Very polar polymers, such as polyamides (chitin), may have MWs as low as 15000 to 20000 Da.

4.1.1 Molecular weight averages

A polymer sample is usually a mixture of molecules with the same structures but with different molecular weights. This heterogeneous mixture of molecular weights can be described by a constant known as the polydispersity of a sample. In any polymerisation reaction it is nearly impossible to obtain polymer chains with same length, thus they differ in molecular weight. It is thus necessary to deal with an average of molecular weights and a difference in distribution (Stevens, 1999). The type of average specified can be with respect to the number of molecules present with the specific molecular weight (number average molecular weight, M_n) or with respect to the concentrations of molecules with the specific molecular weight (weight average molecular weight, M_w) (Carraher, 2000; Tombs & Harding, 1998).

Methods that are depended on the colligative properties, like freezing-point depression, boiling-point elevation and osmotic pressure, usually give rise to M_n because the numbers of molecules of each weight in the sample are counted. This can be defined as (equation 1):

$$M_n = \frac{\sum N_i M_i}{\sum N_i} \qquad (1)$$

where N_i is the number of molecules, or the number of moles of those molecules, having a molecular weight of M_i.

Methods that depend on the mass or the polarisation of the species present, like light scattering and ultracentrifugation, are used to describe and determine M_w. These methods uses the sum of the weight fraction of each species times its molecular weight, thus the greater the mass, the greater the contribution will be to the measurement. This can be mathematically defined as (equation 2):

$$M_w = \frac{\sum W_i M_i}{\sum w_i} = \frac{\sum N_i M_i^2}{\sum N_i M_i} \qquad (2)$$

Stemming from the equations above, one will find that M_w is always greater than M_n. This is due to the fact that in measurements of colligative properties, each molecule contributes equally regardless of the weight, whereas with light scattering, the larger molecules contribute more due to more effective light scattering. It is for this reason that the ratio (equation 3)

$$I_n = \frac{M_w}{M_n} \qquad (3)$$

called the polydispersity index, may be used to describe the span of the molecular weight range in a polymer sample (Stevens, 1999; Carraher, 2000).

If $M_w = M_n$, then I_n will be equal to 1.0 thus indicating that the polymer sample is monodispersed, and meaning that all the molecules have the same molecular weight. An increase in molecular weight and subsequently in polydispersity yields a heterodispersed system, where polymers with different chain lengths and molecular weights are found.

4.1.2 Determination of molecular weight by using light scattering measurements

Apart from osmometry, light scattering by polymer molecules in a solution is the most widely used method of determining the absolute values of M_w. The method used to determine this is called size exclusion chromatography (SEC) coupled to a multi angle laser light scattering (MALLS) device.

Scattering is introduced in solutions by solvent molecules. The intensity of the scattered light is depended on the following factors of the molecules:

i. concentration,
ii. size and
iii. polarisability.

The refraction index is also dependable on the concentration of the solution and the amplitude or intensity of the molecules' vibrations. This intensity of scattered light is known as turbidity τ, which is related to concentration, *c*, by the following expression (equation 4):

$$\tau = HcM_w \qquad (4)$$

where

$$H = \frac{32\pi^3}{3}\frac{n_0^2(\frac{dn}{dc})^2}{\lambda^4 N_0} \qquad (5)$$

and n_0 is the solvent's refractive index, λ is the wavelength of the incident light, and N_0 is Avogadro's number. The d_n/d_c expression is referred to as the specific refractive increment. It is obtained by measuring the slope of the refractive index as a function of concentration and it remains constant for a given polymer, solvent and temperature.

In the determination of the weight-average molecular weight (M_w), measurements from the intensity of scattered light from a light source (mercury arc lamp or laser) at different concentrations and angles (θ) as seen in Figure 4, usually 0, 45, 90 and 135°, are taken.

To determine M_w, the expression for turbidity can be rewritten as

$$\frac{Hc}{\tau} = \frac{1}{M_w P(\theta)} + 2A_2 c \qquad (6)$$

where P(θ) is the function of the angle, θ, at which turbidity is measured, A_2 is second virial coefficient and c is the sample concentration. This angle is highly depended on the shape of the polymer molecules in the solution (Stevens, 1999; Carraher, 2000).

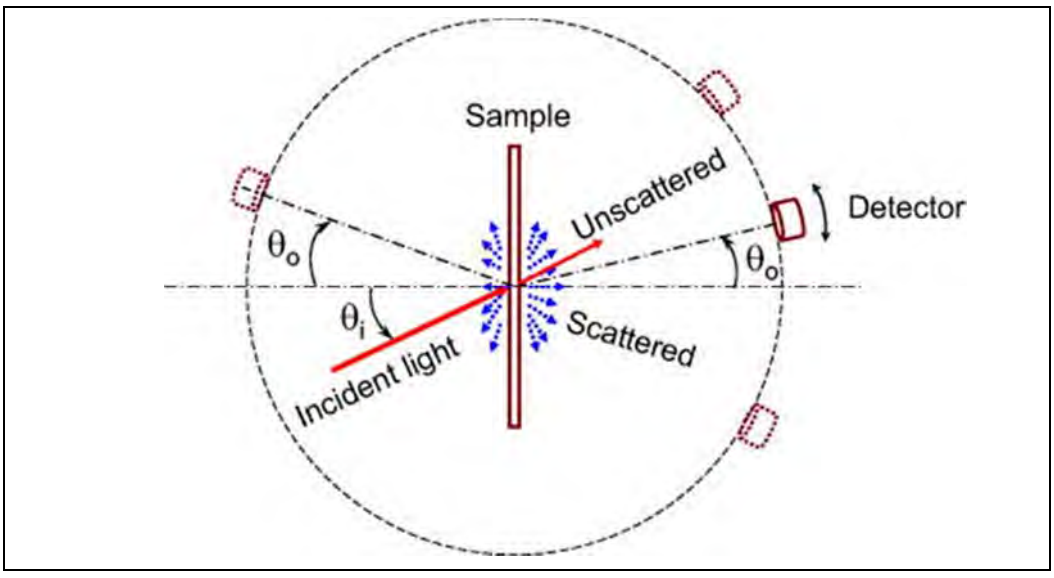

Figure 4: Light scattering by a particle. Detectors pick up the scattering and the angle (θ) is then determined.

4.2 Polymer structure and conformation

The polymer backbone usually consists of carbon (methylene groups, CH_2) and other atoms like oxygen and nitrogen. Around these atoms, a relatively large freedom of rotation exists giving the polymer its flexibility. In Martin (1993), this chain flexibility is illustrated by using an example of a four-carbon chain sequence (Figure 5). In the illustration carbon atoms C_1 and C_2 are in the plane of the paper, with C_3 rotating anywhere on the circle around the base of a cone. This rotation takes place around the C_2-C_3 bond at a fixed bond angle of 109° relative to the C_1-C_2 bond. The same principle can be applied to C_4's rotation relative to C_3, thus indicating that the number of possible conformations for a polymer is enormous. The chain flexibility is thus indicative of the multiple conformations that a polymer can have.

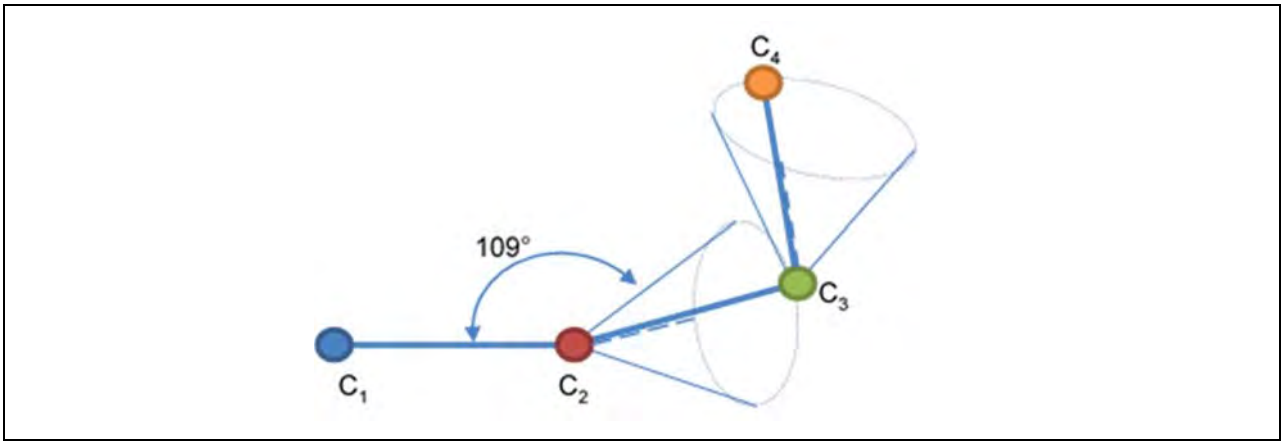

Figure 5: Rotational freedom around carbon atoms in a chain sequence as adapted from **Martin, 1993**.

Conformation studies are important if one wants control over the rheological properties of a polymer in solutions and the characteristics of the membrane or capsule prepared. By using the Mark-Houwink-Kuhn-Sakurada (MHKS) coefficients the conformation of polymers can be characterised. There are mainly four parameters that are used to determine the conformation:

i. intrinsic viscosity,
ii. sedimentation coefficient,
iii. radius of gyration and
iv. diffusion coefficient.

The parameter's log values are then plotted against the log of the molecular weight of the polymer. The slopes of these plots yield the polymer's conformation respective to what MHKS parameter (Table 1) has been used (Harding, 1995).

Table 1: Polymer conformation when the Mark-Houwink parameter, radius of gyration (Rg), is used. k is optical parameter, previously referred to as H. The MHKS exponent used in this equation is v (Tsaih & Chen, 1997).

MHKS type equation	Conformation		
	Sphere	Random coil	Rod
$R_g = kM^v$	0.3	0.5~0.6	1.0

This equation is important because of the use of SEC-MALLS to determine molecular weight, meaning that we can also determine the conformation of the polymer at the same time by plotting the log of R_g against the log of the molecular weight (Figure 6).

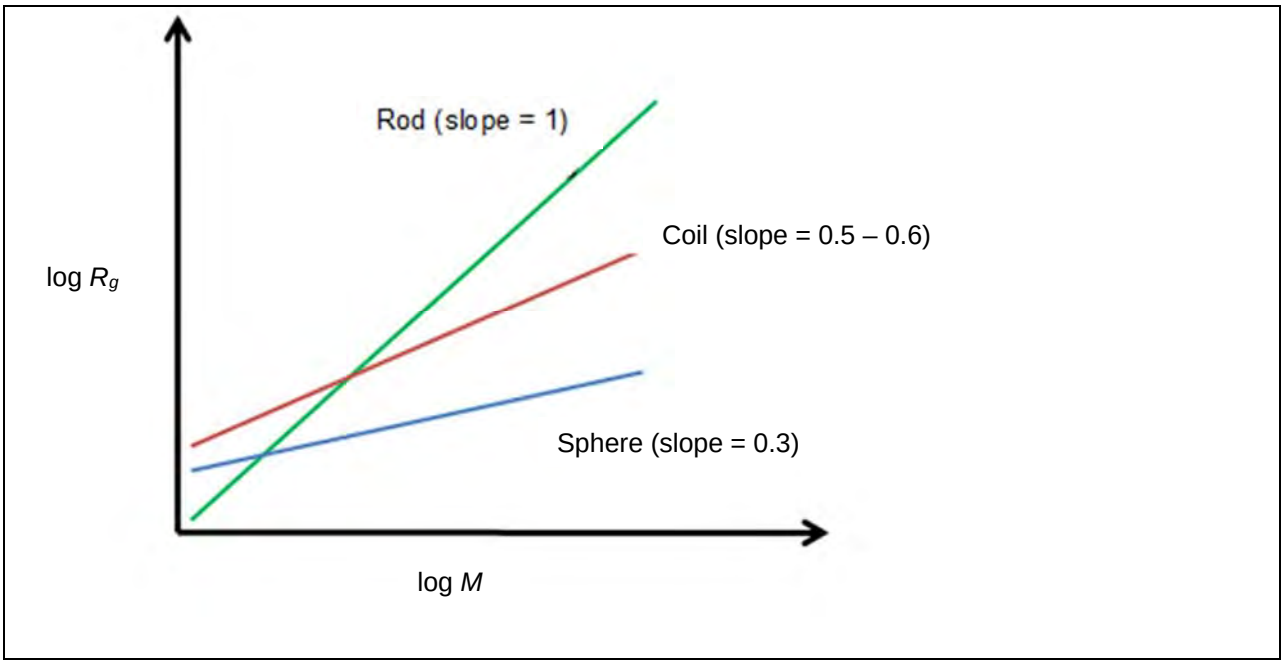

Figure 6: Standard plot of the log mean radius of gyration vs. log molecular weight for differently shaped polymers **(Carraher, 2000).**

5. Chitosan and its derivatives

5.1 Chitosan

Chitosan is a linear amino-polysaccharide madeof randomly distributed *N*-glucosamine and *N*-acetylglucosamine units that are linked together at β-1 to 4. It is obtained by the deacetylation of chitin, a widespread natural polysaccharide found in the exoskeleton of crustaceans such as crab and shrimp (Kumar *et al.*, 2004; Park *et al.*, 2010). This cationic polysaccharide has drawn increasing attention within pharmaceutical and biomedical applications because of its abundant availability, mucoadhesive and inherent pharmacological properties like hypocholesterolemic action, wound-healing properties and antiulcer activity. Other beneficial biological properties such as biocompatibility, biodegradability, non-

toxicity and low immunogenicity also make it a favourable compound to work with (Anitha *et al.*, 2014; Felt *et al.*, 1998; Illum, 1998:; Pillai *et al.*, 2009).

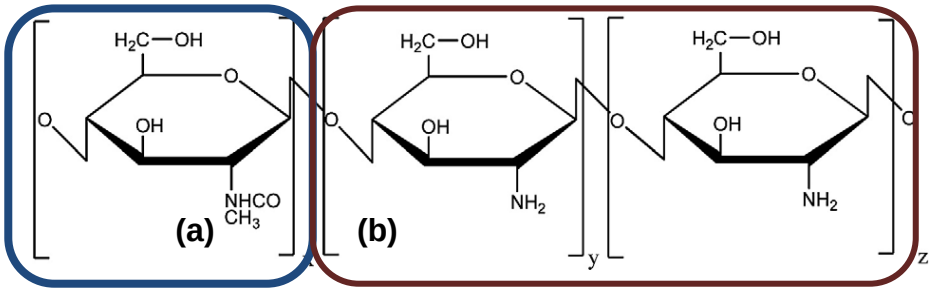

Figure 7: The different building units of chitosan namely a) N-acetylglucosamine and b) N-glucosamine (Amidi *et al.*, 2010)**.**

The chemical properties of chitosan mostly depend on the degree of acetylation (DA) of the chitin, referring to the introduction of an acetyl functional groups into the polymer. With a DA below 50 % chitin becomes soluble in an acidic medium and is then known as chitosan (Le Dung *et al.*, 1994). With a pK_a of 6.3, due to the amine group, chitosan is a primary aliphatic amine that is soluble in acidic solutions with a pH value below 6.0 (Kumar *et al.*, 2004; Sieval *et al.*, 1998). Aspden and co-workers (1995) described chitosan as a polysaccharide which can be individually characterised by the DA on the glucosamine and N-acetylglucosamine units as shown in Figure 7 and by its variable molecular weight. It has a primary amino group at C_2 and a hydroxyl group at the C_6 positions, thus creating the opportunity for chitosan to undergo a host of chemical reactions like acetylation (Singh & Ray, 2000).

Due to its favourable biological properties like biocompatibility, biodegradability and non-toxicity, chitosan is beneficial as a biological applicant for pharmaceutical use. This means that chitosan can easily be used in pharmaceutical products and administered orally, transdermally, nasally and by means of other routes. The oral route is the most favourable and the most practical way to administer a drug, especially from patient's point of view. However, it is not always the most suitable route for some active compounds, such as non-steroidal anti-inflammatory drugs (NSAID), which can damage the stomach's mucus membrane, for drugs poorly absorbed, such as peptides like insulin, or for drugs that undergo an extensive first-past effect (Felt *et al.*, 1998). Drug delivery is restricted by the physiological parameter called gastric emptying rate. Thus if one can control the residence time of the dosage form, one can control the therapeutic effect. Chitosan has been shown to control drug delivery by opening tight junctions between gastric epithelial cells to facilitate the paracellular transport of hydrophilic and macromolecular compounds, thus enhancing the absorption of these compounds (Boonyo *et al.*, 2007; Jonker *et al.*, 2002). This change takes place when the cationic chitosan interacts with the negatively charged cell membrane, which in turn causes the tight junctions to reorganise its structural proteins like zona occludens (ZO-1) and occuludin as illustrated in Figure 8 (Smith *et al.*, 2004). The reorganisation is brought about when the extracellular loops of occuludin (containing a COOH terminus) interacts with each other which then leads to the redistribution of the cytoskeletal F-actin. This redistribution causes the

ZO-1 proteins to reorganise and as a result the opening of the tight junctions (Boonyo *et al.*, 2007; Mitic & Anderson, 1998).

Since chitosan can control the release and absorption of compounds, it can be used in different oral dosage forms like tablets and microparticles (Felt *et al.*, 1998; Singla & Chawla, 2001).

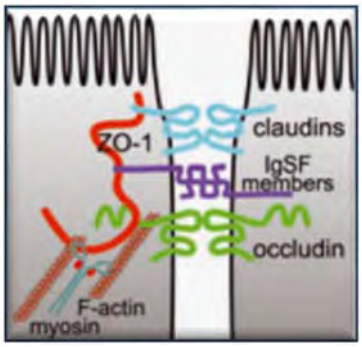

Figure 8: A tight junction is composed of multiple interacting transmembrane and cytoplasmic proteins that are linked to the actin cytoskeleton **(Shen, 2012:)**.

5.2 *N*-Trimethyl chitosan (TMC)

Although chitosan has many reported successes, a major drawback is its insolubility at physiological pH (7.4), where it is insoluble and active as an absorption enhancer only in its protonated (uncoiled and positively charged) form in acidic environments. On the other hand *N*-trimethyl chitosan chloride (TMC), a partially quaternised chitosan derivative, shows good water solubility across a wide pH range thus having mucoadhesive properties and excellent absorption enhancing effects even at neutral pH (Amidi *et al.*, 2010; Hamman *et al.*, 2003; Kotzé *et al.*, 1998). It is synthesised by the reductive methylation, also called acetylation, of the amino groups on the C_2 position of chitosan (Hamman *et al.*, 2003) and by doing so creates a positive charge on the amino group. By repeating the methylation step, during the reaction, a higher degree of trimethylation can occur). Unfortunately, trimethylation isn't the only reaction taking place because chitosan has more than one reactive group in its chemical structure. Significant *N,N*-dimethylation, *N*-monomethylation and *O*-methylation also occurs (Figure 9). With *O*-methylation a significant decrease in the water solubility could be observed, but the solubility will also decrease with an increase in molecular weight (Polnok *et al.*, 2004; Rúnarsson *et al.*, 2007; Sieval *et al.*, 1998).

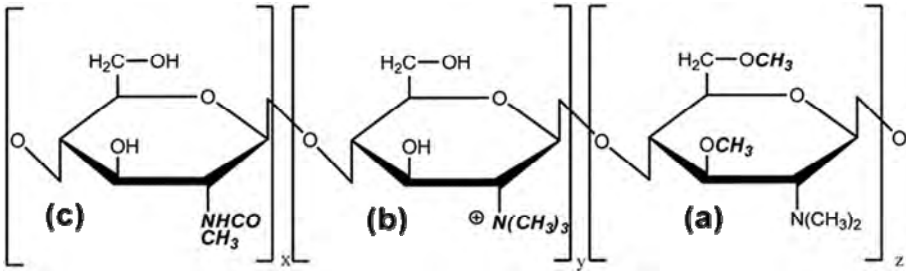

Figure 9: The chemical structure of TMC, showing di- and trimethylation (a and b) as well as O-methylation (a) **(Amidi *et al.*, 2010).**

This positive charge is spread along the chain of TMC and allows it to interact with the negative charges on cell membranes. By doing so, tight junctions in the epithelial membranes are opened and compounds can pass through more easily. The charge density of TMC plays an important role in the enhancement of absorption. This charge density can be determined by means of proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) and is called the degree of quaternization (DQ). An increase in DQ will then mean an increase in absorption-enhancing properties of TMC especially at environments with a neutral or alkaline pH (Hamman *et al.*, 2003; Jonker *et al.*, 2002; Kotzé *et al.*, 1999; Thanou *et al.*, 2000). Hamman and co-workers (2003) found that the relationship between the DQ and absorption enhancement effect reached a threshold value at about 48 %, even when TMC with higher DQ (59 %) was used. This was attributed to a couple of reasons including:

i. favourable chain flexibility and conformation at a DQ of 48 % (Hamman *et al.*, 2002),
ii. the number of attached methyl groups causes steric hindering at higher DQs and
iii. the saturation of the electrostatic interactions between the cationic TMC and the anionic cell membrane.

The above mentioned properties of TMC just emphasises the value of TMC as an excipient in pharmaceutical preparations as discussed below.

5.3 Pharmaceutical applications of chitosan and TMC

The formation of complexes between chitosan-based polymers (like TMC) and oppositely charged macromolecules has been studied extensively by many researchers and it was found that this property can be exploited to prepare micro- and nanoparticles that are suitable for drug delivery (Chen *et al.*, 2008; Geçer *et al.*, 2010; Prego *et al.*, 2010; Sandri *et al.*, 2007; Sayin *et al.*, 2008). These particles are prepared by ionic cross-linking (ionotropic gelation) through self-assembly of chitosan or its derivatives, which is positively charged, and oppositely charged macromolecules by the addition of a low molecular weight anionic cross-linker, such as tripolyphosphate (TPP) (Amidi *et al.*, 2010). Numerous drugs are incorporated in these TMC-TPP particles ranging from vaccines to lipophylic drugs, thus protecting the drug from enzymes and enhancing its absorption (Amidi *et al.*, 2010). This method of synthesising chitosan based particulates is suited for protein and vaccine delivery due to the mild conditions used during the synthesis process. This ensures that there is no degradation of the active ingredient taking place. Examples of these are provided in the summary of various studies in the following paragraphs.

Amidi *et al.* (2006) utilised these mild synthesis conditions to prepare ovalbumin-loaded TMC-TPP nanoparticles for the nasal delivery of proteins. They obtained particles with a small size and narrow size distribution that had an excellent protein-loading capacity (50 %) and loading efficacy (90 %). Furthermore, the particles had a positive charge that was conveniently suitable for the particle to attach to the nasal mucosal membrane. They also found that these particles showed no cytotoxicity on Calu-3 cells, cultured human airway epithelial cells which are used as a respiratory model for studying the effect on human airway epithelial cells, and the *in vivo* experiment showed that FITC-albumin loaded particles

were able to cross the nasal mucosal layer, taken up by rat epithelia and NALT cells and then transported to the underlying sub-mucosal layers. Due to this study, Amidi and co-workers (2007) continued their research using TMC nanoparticles to encapsulate and administer influenza antigen in the nasal cavity. They found that these TMC nanoparticles significantly enhanced the local and systemic immune response compared to the conventional intramuscular or intranasal administration of the influenza vaccine, thus concluding that these nanoparticles are a promising vehicle for nasal delivery of antigens.

In 2007, Sandri *et al.* also used the ionotropic gelation method to prepare TMC nanoparticles for the encapsulation of fluorescein isothiocyanate dextran (FD4). This active ingredient was used to serve as a model macromolecule that had a molecular weight comparable to that of proteins commonly used in therapy like insulin. They found that the *in vitro* data showed that the nanosytems were able to improve the permeability of FD4 across the nasal mucosal membrane and enhance the mucoadhesion. This made TMC-based nanosytems suitable carriers for the oral administration of macromolecules and, in particular, of peptides.

Chen and co-workers (2007; 2008) also cross-linked TMC with TPP to yield nanoparticles with a small particle size and a positive zeta potential. They encapsulated bovine serum albumin (BSA) and bovine haemoglobin (BHb) to determine loading and release profiles of the TMC nanoparticles. TMC with different DQs were used to see whether this factor had an influence on the release profile. Their results indicated that the TMC nanoparticles had a high loading efficiency (95 %) for BSA but a low one (30 %) for BHb and that the particle size and zeta potential were significantly influenced by the BSA but not by the BHb concentrations. Furthermore, nanoparticles of TMC with a low DQ showed an increase in particle size, a decrease in zeta potential and slower drug-release profile.

Geçer*et al.* (2010) synthesised TMC-TPP nanoparticles to improve the dissolution of the lipophylic drug candesartan-cilexetil. They characterised the nanoparticles by using Fourier transform infrared spectroscopy (FTIR), transmission electron microscopy (TEM) and dynamic light scattering analyses (DLS). High performance liquid chromatography (HPLC) was used to determine a dissolution profile of candesartan-cilexetil and it was found that the TMC nanoparticles had improved it. This means that TMC-TPP nanoparticles can be used to improve the dissolution of lipophylic drugs thus enhancing the absorption and therapeutic effect of the drug.

After taking all of this research in account, it is apparent that chitosan-based particles have a big role to play in drug delivery thus showing the potential and importance of these particles.

6. Biopharmaceutical considerations

Biopharmaceutics involves certain factors that influence the physicochemical properties of a drug, the dosage form and the route by which the drug is administered. Such factors include a) protecting drug activity in the product, b) the release of the drug from the product, c) the drug's dissolution rate at the

absorption site, and d) the systemic absorption of the drug (Shargel & Yu, 1999). This shows that biopharmaceutics is a major branch of the pharmaceutical sciences concerned with the relationship between the physicochemical properties of a drug in dosage forms and the pharmacologic, toxicological, or clinical response observed after its administration (Gibaldi, 1991). In short, biopharmaceutics mainly involves the drug absorption process (Panchagnula & Thomas, 2000:).

6.1 Physicochemical properties

The physicochemical properties of drugs are important variables that determine whether the systemic circulation can be reached. These properties must be kept in mind in order to optimize absorption, pharmacological activity, metabolism and to minimize possible toxic effects. In the period 1989 - 1990 considerable progress was achieved concerning the required physicochemical properties needed for oral absorption. Certain key properties of compounds in the World Drug Index (WDI) were analysed and this led to the discovery of the now well-established 'rule of five'. This rule predicts that poor absorption or permeation is more likely when there are more than 5 H-bond donors, 10 H-bond acceptors, the molecular weight is greater than 500 and the calculated Log P is greater than 5 (Lipinski *et al.*, 1997). The physical and chemical, called physicochemical, properties of solid drug particles affect not only the dissolution kinetics but are also important during the design of dosage forms. They determine the route of dosage and whether the drug will be stable (Shargel & Yu, 1999). The physicochemical properties that will be discussed in this section are the solubility, particle size and shape, and the entrapment efficacy.

6.1.1 Solubility

The first requirement for a drug to be absorbed into the systemic circulation is that it must be dissolved. This means that only a drug that has dissolved completely has the ability to permeate across the intestinal epithelial to have a therapeutic effect, thus making it one of the more important parameters. This parameter can be controlled by addition of different excipients like an acid or base. Controlled release can also be obtained with the addition of a buffering agent or slow dissolving excipient like certain polymers (methylcellulose) (Shargel & Yu, 1999).

Chitosan, in general, is known as a good candidate to be a convenient vehicle to improve the dissolution properties and bioavailability of a number of poorly water-soluble drugs, but due to its limited solubility at certain pH, its water-soluble derivative (TMC) is more favorable to work with. Geçer and co-workers formulated TMC nanoparticles of the water-insoluble drug, candesartan-cilexetil and found that the TMC enhanced the water-solubility sicnificantly (Geçer *et al.*, 2010).
water soluble

6.1.2 Particle size and shape

The interaction of microparticles with biological cells depends on the size, size distribution and functionality of the particles. If the size distribution of microparticles is narrower, physical and chemical properties are more uniform, it is easier, for example, to formulate more sophisticated drug delivery system (Ji *et al.*, 2009). The smaller the size of the particles, the larger is their surface area. For example, 1 g of microparticles having a diameter of 0.1 μm has a total functional surface area of 60 m^2. This enhances the chance of the particles of getting absorbed and undergoing chemical reactions. From a technological standpoint, polymeric particles can hardly be made smaller than 5 nm because in general a polymer has a molecular weight higher than 10 000 Da (Kawaguchi, 2000).

There are many different methods, described in the literature, being used to determine particle size and shape. The most commonly used is light scattering (LS), because of its rapid determination of the mean size, size distribution and polydispersity index (PI) of a sample. The basic principle of this method relies on the particle's interaction with light at an observation angle of 90°. The one setback that this method has is that the calculation model is based on the equivalent of a sphere, thus the presence of just a few aggregates will show a significant increase in the mean size determination. The accuracy of this method can be increased by utilising more measurement angles, like with the MALLS as previously mentioned (Gaumet *et al.*, 2008).

The method most commonly utilised with particles larger than 1 μm is laser light diffraction (LLD). In a strict sense, due to its operating principle, LDD is not a true particle size measurement technique, but rather a particulate system characterisation technique. In LDD the processing of light scattering data is based on the assumption of spherical particles, and the theoretical scattering pattern of a distribution of spherical particles is thus used to provide the best fit.

Some advantages of this method include the following (Tinke *et al.*, 2008):

i. universal applicability,
ii. broad dynamic size range (1 to 500 μm),
iii. small sample requirement,
iv. user friendliness, and
v. high robustness and precision.

The limitations of this method can be viewed from two different angles, namely the measurement point of view and the data processing point of view. The measurement can be influenced by:

i. limited angular resolution of the detectors,
ii. limited scattering information and intensity of smaller particles (< 500 μm),
iii. orientation of the particle, especially when it is not spherical.

When processing the data, the following factors may influence particle size distribution (PSD):

i. the assumption that all particles are spheres,

ii. the limitations of the algorithms used to determine the PSD, and
iii. the type of curve fitting (Gaumet *et al.*, 2008; Hong-Jian & Guan-Dong, 1992; Tinke *et al.*, 2008).

Scanning electron microscopy (SEM) allows the observation of the sample after drying and coating with a thin layer of gold. This method allows for a resolution between 3 and 5 nm, and even to 1 nm with some advanced microscopes. The limitations of this method are that it is extremely time consuming and the results are greatly influenced by the way in which the sample was prepared (Gaumet *et al.*, 2008).

6.1.3 Entrapment efficacy

To achieve desirable therapeutic efficacy, the drug should be efficiently incorporated into a drug carrier, be it microparticles or nanoparticles, which mainly depends on several preparation-associated parameters. Among these parameters, drug entrapment efficiency (EE) is of great significance to the screening of the preparation method and to the quality control of the product (Xu *et al.*, 2006).

Association (entrapment) of a drug with the micro/nanoparticles can, in principle, occur in two ways, as shown in Figure 10 (Alonso, 1996).

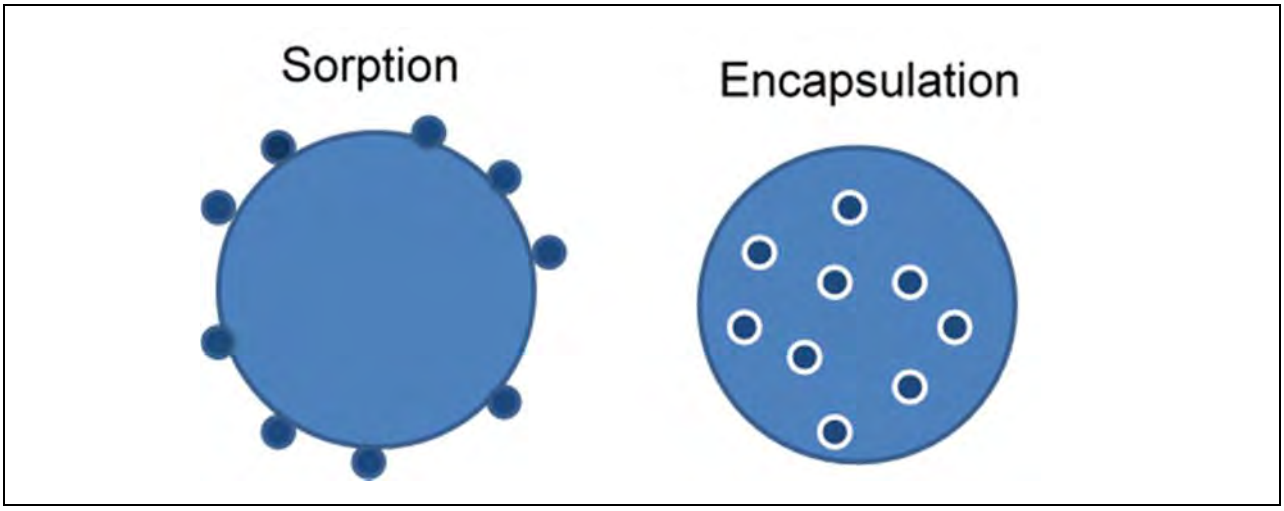

Figure 10: Entrapment methods. Sorption is characterised by drug clinging to the outside surface of the particle. Encapsulation is the entrapment of the drug inside the particle.

Firstly, the drug can be added prior to polymerisation, resulting in the encapsulation of the drug within the particles or on the other hand, the drug can be added after the polymerisation, resulting in sorption of the drug onto the surface of the particles. The extent of sorption depends on two factors, namely:

i. the affinity between the polymer and the drug, and
ii. the nature of the dispersion medium.

The sorption of drugs onto particles is believed to be due mainly to an ionic interaction (Pitaksuteepong *et al.*, 2002).

Drug entrapment can be influenced by a variety of factors such as:

i. polymer concentration,
ii. drug to polymer ratio,
iii. stirring rate,
iv. adding an emulsifier or cross linker, and
v. the solubility of the drug and polymer in their respective phases (Dhakar *et al.*, 2010).

Entrapment efficacy or loading efficacy, as it is sometimes called, can be determined by measuring the concentration of free drug in the supernatant. This can be done by making use of ultra-violet spectrometry. Once the concentration of the free drug has been determined, the following equation can be used to determine the entrapment efficacy:

$$\%\ Entrapment\ efficacy = \frac{(T-S)}{T} \times 100 \qquad (7)$$

where *T* is the total amount of drug used during entrapment and *S* is the amount of "free" drug that was present in the supernatant (Chen *et al.*, 2008; Mitra *et al.*, 2001).

6.2 Biological considerations

These considerations have to do with how effective the drug is in the body and what possible side-effects it might have. The most important consideration is that the finished product should achieve its therapeutic objective by delivering the drug with maximum bioavailability and minimal adverse reactions/side effects (Shargel & Yu, 1999). *In vitro-in vivo* correlation is important to predict and determine whether a drug will have the desired effect. It is also a great way to identify how the drug would react once in the body.

6.2.1 Physiological barriers (membranes)

Drugs are usually formulated so that they can be administered in the most non-invasive way possible, thus making the oral route the most favourable of all. But before a drug can have a therapeutic effect, the drug must cross biological membranes, which act as barriers, to enter the systemic circulation. The barrier effect is necessary for a cell to maintain a constant intracellular environment by allowing controlled passage of water, nutrients and waste products across the membranes. One of the important features of epithelial cells, namely tight junctions, makes transport across these cells possible (Brodin *et al.*, 2010). As previously mentioned, chitosan and TMC can open these tight junctions and allow the drug to cross the membrane and enter the systemic circulation to have its therapeutic effect (Kotzé *et al.*, 1998; Kotzé *et al.*, 1999).

6.2.2 *In vitro-in vivo* dissolution correlation

An important characteristic for an oral drug substance is the rate from which it goes into solution from the pharmaceutical formulation. This is called the rate of dissolution, and it is very relevant since generally only a dissolved drug will be absorbed in the gastrointestinal (GI) tract. These dissolution tests can be altered in such a way that they simulate GI fluids so that they can be used to elucidate the potential influence of GI conditions, like the effect of food, or to obtain a closer correlation with the *in vivo* situation. It is, however, important to remember that the relationship between *in vitro* dissolution and *in vivo* bioavailability/absorption is still far from being fully explored, and that there are limitations for the dissolution test when used as a predictor of *in vivo* performance (Pedersen & Müllertz, 2010). A successful correlation can only be obtained when the physicochemical properties as well as the uptake and elimination mechanism of the compounds are thoroughly understood (Wu, 2010).

6.2.3 Efficacy studies

Efforts to discover and develop new antimalarials or to improve the already existing drugs have increased dramatically due to the resistance of the malaria parasites to existing therapies. Selection of candidate drugs for clinical trials in man and the design of clinical protocols are based upon consideration of from a battery of preclinical test systems. As there is growing need for newer and more efficacious antimalarial drugs especially in tropical countries, more sensitive and economical screening models are needed (Kalra *et al.*, 2006). In 1948 the Belgian parasitologist, Ignace Vincke, made a discovery that is believed to be the greatest contribution to malaria chemotherapy research. He discovered the parasite, *P. berghei*, in wild rats which also proved to be infective in laboratory mice, rats and hamsters. This find has been exploited for the last fifty years in malaria chemotherapy and immunology. The fact that this parasite's life cycle is essentially the same as that of malaria parasites in humans makes it an invaluable model for the investigation of drugs against certain stages of the parasite's life cycle (Peters & Robinson, 1999). Efficacy studies are very important to determine whether a drug is effective or not. The method utilised these days is the Peters 4-day test.

The Peters 4-day test is most widely used preliminary test, in which the efficacy of a compound is assessed by comparison of blood parasitemia and mouse survival time in treated and untreated mice (Trager & Jensen, 1976). In short, mice are infected with *P. berghei* and dosed with the test compounds on day 0 to day 3. On day 4, 24 hours after the last dose (i.e. 96 h post-infection), blood smears from all animals are prepared with Giemsa stain and parasitemia is determined microscopically by counting 4 fields of approximately 100 erythrocytes per field. Untreated mice usually die within a week of being infected and if mice have no parasitemia on day 30 after infection, they are considered cured (Kalra *et al.*, 2006).

The choice of malaria model depends upon sensitivity, reproducibility and breadth of response to known antimalarial drug and also on practical considerations such as required rate of testing, technical complexity, quantity of test compound needed and cost per test.

7. Conclusion

Malaria remains one of the most important diseases in the world as we know it today. It cripples not only the physical well-being of man, but also the economy and with the shadow of resistance to existing drugs always lurking close by, just underscores the need to develop new strategies to combat this disease. One such strategy involves the combinations of already known drugs. By combining drugs with synergistic methods of action, one can accomplish the same or better therapeutic effect and decrease the possibility of resistance to develop. The dapsone and proguanil combination has been shown to have antimalarial activity against parasites that are resistant to chloroquine, a first line drug, with little resistance development. The only problem was the one side effect that dapsone has on patients with the G6PD deficiency, causing potentially fatal aplastic anaemia.

This led to the hypothesis that if one can achieve the same therapeutic effect with a lower dose, one can possibly minimise the risk of the side effects being so severe. Another method to minimise the side effects is to achieve sustained release of the drug. In this way the drug can be released into the body at a controlled rate, thus achieving the same therapeutic effect but with far less side effects. This can be achieved by entrapping the therapeutic agent in microparticles synthesised from a natural polymer like chitosan.

Physicochemical properties of the particles like size, shape, zeta potential and entrapment efficacy had to be performed before efficacy studies could be conducted. Only after the formulation study has been done, efficacy studies could be conducted to determine the efficacy of the drug's entrapped particles. To determine the best formulation, a factorial design had to be implemented, followed by the characterisation of the optimal formula. After this had been done, the drugs were entrapped in the particles and used during an *in vivo* efficacy study on malaria infected mice. The release profile of the particles was also investigated by means of dissolution studies. Additionally the *in vivo* bioavailability of the drug entrapped in particles was determined (Chapter 3).

Another part of the study was to synthesise TMC with a faster, more environmentally friendly method. The result was then to use microwave irradiation, because it lowers the reaction time and uses less electricity. A factorial design was implemented to determine what effect some factors like reaction time, reaction steps and reaction energy would have on the synthesis of the TMC. The synthesised TMC polymers where then characterised by using ^{1}H-NMR, FTIR and SEC-MALLS (Chapter 4).

By doing the study as mentioned above, it could be determine whether TMC-TPP microparticles enhanced the therapeutic effect and reduced toxicity (possible sustained release). A new improved method of synthesising TMC could also be developed.

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Chapter: 3

Chapter 3 contains the manuscript of an article to be submitted to the Journal of Controlled Release. The article contains the background, aims, all the experimental details and results of this study on the particles, including the physicochemical properties and *in vivo* efficacy results of the dapsone and proguanil particles. The article is prepared according to the Guide for Authors that can be found on the website of this journal (http://www.elsevier.com/journals/journal-of-controlled-release/0168-3659/guide-for-authors#68000), except that for easy reading figures, schemes and tables are inserted at their logical places as they would appear in the printed version.

Formulation, characterisation and *in vivo* efficacy of dapsone and proguanil in trimethylated chitosan microparticles

J. van Heerden, R. Lemmer, A.F. Kotzé, J.H. Steenekamp, L. Wiesner, L.H. du Plessis

Unit for Drug Research and Development, North-West University, Hoffman Street, Potchefstroom, 2531, South Africa

* Corresponding author: Lissinda.DuPlessis@nwu.ac.za, Tel: +27 18 299 4246,
Fax: +27 18 299 2251

ABSTRACT

Each year, malaria causes millions of deaths worldwide. The combination of two antifolates, dapsone and proguanil (DP), has previously been used to treat and prevent malaria. Dapsone, however, caused aplastic anaemia in patients with glucose-6-phosphate dehydrogenase (G6PD) that is potentially fatal, causing it to be withdrawn by the World Health Organisation (WHO) as a treatment in 2008. In the present study, DP was entrapped in trimethyl chitosan-tripolyphosphate (TMC-TPP) microparticles to determine the *in vivo* efficacy. Several physicochemical characteristics of dapsone and proguanil-TMC-TPP particles (size, zeta-potential, entrapment and loading efficiency and drug release profile) were determined and assessed. A Peters 4-day suppression test was performed on mice to determine the efficacy of DP/TMC-TPP microparticles. The bioavailability of the formulations was assessed in mice.

The optimal formulation's particle size ranged from 18.56 µm ± 0.72 to 74.56 µm ± 3.81 when drug was entrapped and with zeta potentials between 7.17 mV ± 2.26 and 21.83 mV ± 0.55. The *in vivo* efficacy study showed that the DP/TMC-TPP particles had the same efficacy when compared to the normal drug, but also showed indications of sustained release.

In conclusion, DP loaded particles have the potential to be used again as treatment and prevention of malaria, due to sustained release that can minimise toxic side effects.

KEYWORDS

Dapsone
Proguanil
N-trimethyl chitosan
Malaria
Peters 4-day suppression test
Microparticles

1. Introduction

The prevention and treatment of malaria is heavily depended on the use of antimalarial drugs. However, due to the *Plasmodium falciparum* parasites' resistance to chloroquine fifty years ago, efforts to control the disease have been hindered by failed or failing drugs [1]. More than 80 % of worldwide malarial deaths occur in sub-Saharan Africa where poverty and access to treatment contributes to the already existing problem [2]. Due to the increasing resistance, researchers started looking at combination therapies. The rationale behind this was that two drugs that worked synergistically at two different sites could lower the possibility of resistance developing. The combination of pyrimethamine and sulfadoxine was for long a cheap and the only antifolate combination and effective alternative to chloroquine-resistant malaria [3]. However, starting in 1994, resistance to sulfadoxine could be observed because of its slow elimination rate, indicating that sulfadoxine efficacy is declining in eastern Africa emphasising the need for an alternative therapy to reduce the selective antifolate resistance [4,5]. This led to the general belief that malaria chemotherapy should consist of combinations of drugs with independent modes of action. In the mid-1980s Watkins and colleagues followed the approach where they took existing antimalarial drugs and used it in combination with each other and decided on the combination of proguanil (PG) and dapsone (DDS), a combination that seemed to meet the essential criteria of affordability, efficacy and availability [4,6]. PG inhibits the enzyme dihydrofolate reductase (DHFR) and DDS inhibits dihydropteroate synthase (DHPS), together working synergistically to inhibit the parasite's growth [7-9]. *In vitro* analyses have demonstrated that cycloguanil, the active metabolite of proguanil, and dapsone are more potent than pyrimethamine and sulfadoxine, respectively. *In vivo*, chloroquine is efficacious in treating malaria and it retains activity against sulfadoxine/pyrimethamine-resistant parasites [4]. It was established that the combination of DDS and PG, because of its short acting drugs (half-lives of 20 and 12 hours respectively) [10], selects less efficiently for resistance than sulfadoxine/pyrimethamine [11, 12]. Unfortunately, this combination had one big kink in its armour, due to the fact that dapsone had a serious side effect in people with a deficiency in the enzyme glucose-6-phosphate dehydrogenase (G6PD). In people, living with this inherit deficiency, red blood cells are much more susceptible to oxidative stress than in the normal person and this causes the cell to rupture leading to potentially fatal haemolytic anaemia [13]. This

caused the World Health Organisation (WHO) to withdraw DP as a treatment in 2008, opening the opportunity for new research to be done to possibly reduce the toxicity of this treatment.

Chitosan is natural cationic polysaccharide that has drawn increasing attention within pharmaceutical and biomedical applications because of its abundant availability, mucoadhesive and inherent pharmacological properties like hypocholesterolemic action, wound-healing properties and antiulcer activity. Other beneficial biological properties such as biocompatibility, biodegradability, non-toxicity and low-immunogenicity also make it a favourable compound to work with [14,15]. Although chitosan has many reported successes, a major drawback is its insolubility at physiological pH (7.4), whereas it is soluble and active as an absorption enhancer only in its protonated (uncoiled and positively charged) form in acidic environments. This is where *N*-trimethyl chitosan chloride (TMC), a partially quaternised chitosan derivative, came in and showed good water-solubility across a wide pH range thus having mucoadhesive properties and excellent absorption enhancing effects even at neutral pH [16-18]. The formation of complexes between chitosan-based polymers (like TMC) and oppositely charged macromolecules has been studied extensively by many researchers and it was found that this property can be exploited to prepare micro/nanoparticles that are suitable for drug delivery [19-23]. These particles are prepared by ionic cross-linking (ionotropic gelation) through self-assembly of chitosan or its derivatives, which is positively charged, and oppositely charged macromolecules by the addition of a low molecular weight anionic cross-linker, such as tripolyphosphate (TPP) [24]. Numerous drugs, ranging from vaccines to lipophylic drugs, are incorporated in these TMC-TPP particles thus protecting the drug from enzymes and improving its absorption. In some instances these particles can increase the aqueous solubility of poorly water-soluble drugs [22,25,26].

The aim of this study was to optimise the formulation for the TMC-TPP microparticles and to characterise these particles. After entrapping DDS and PG, characterising those particles and determining their *in vivo* efficacy and bioavailability, an *in vitro* dissolution study was also performed to determine whether one can achieve a sustained release profile for the drugs to possibly reduce their toxicity.

2. Materials and methods

2.1 *Materials*

Dapsone (batch number: 20100128) and proguanil (batch number: PDG/0911B/RD03) were purchased from DB Fine Chemicals (South Africa). *N*-trimethyl chitosan with a degree of quaternization (DQ) of 60 % was synthesised from 95 % deacetylated (MW 120 kDa) chitosan (Primex, Avaldsnes, Norway) and characterized by NMR, as described by Rúnarsson et al. [27]. The DQ was calculated using the following equation, DQ (%) = [(∫TM/∫H)×6/9]×100, where ∫TM is the integral of the trimethyl amino group (quaternary amino group) peak at 3.3 ppm and ∫H is the integral of the six ^{1}H peaks from 3.6 to 4.5 ppm [27]. Sodium tripolyphosphate (TPP), phosphate buffer solution (PBS) with a pH of 7.4, Tween 80 and Giemsa stain solution were obtained from Sigma-Aldrich (Steinheim, Germany). Absolute ethanol and methanol were

bought from Rochelle Chemicals (South Africa). All the chemicals (Merck®, Johannesburg, South Africa) used for the dissolution were of analytical grade or HPLC grade (where applicable).

2.2 *Preparation of TMC microparticles*

A factorial experimental design was utilised to determine the optimal reaction conditions that will yield the smallest and most uniform particles. TMC/TPP microparticles were synthesised by using the ionic gelation technique as described by Amidi and co-workers [18, 24]. Ionic gelation is achieved when the cationic TMC is crosslinked with the anionic TPP. Briefly, TMC was dissolved in 50 ml Tween 80 (0.1 %) to yield an aqueous solution of TMC (2 mg/ml). Under continuous stirring (1000 rpm) 20 ml of TPP (1.66 mg/ml) was added slowly drop-wise by means of a Watson-Marlow 205S peristaltic pump (30 rmp) to the TMC solution, inducing ionic complexation into microparticles. During stirring, the TMC-TPP solution was also subject to ultrasonification using an ultrasonic rod (100 watt amplitude). The TMC-TPP microparticles were collected by centrifugation (60 min, 5,500 *g*), resuspended in water and lyophilised under vacuum.
DDS and PG loaded particles were prepared using a similar method as mentioned above, the only difference was that the drug, in a ratio 2:1 (TMC:drug), was dissolved in methanol and added drop-wise simultaneously together with the TPP solution to the TMC solution. This happens whilst the TMC solution is subjected to continuous stirring and ultrasonification. After the microparticles have been collected by centrifugation, the particles were washed with methanol to remove any non-entrapped drug from the surface of the particles, whereafter the particles were lyophilised. The supernatants and the methanol used to wash the particles were stored for determining the loading efficiency of the particles.

2.3 *Particle size and zeta-potential*

Particle suspensions were diluted in absolute ethanol until a slightly opalescent dispersion was achieved. The mean particle size, distribution and uniformity of the formulations were measured by dynamic light scattering analysis using a Malvern Mastersizer 2000 (Malvern instruments, Malvern, UK) equipped with a blue solid light laser. Samples was analysed at an obscuration rate of 10 % to 20 % at a scattering angle of 90 ° in duplicate. Samples were stirred continuously to obtain a homogenous dispersion of the samples. Zeta-potential was measured using a Malvern Zetasizer 2000 (Malvern instruments, Malvern, UK). Measurements were taken at 25 °C without sample dilution and results were reported as the mean of three measurements ± SD.

2.4 *Morphological analysis*

The shape and surface characteristics of the microparticles were observed by environmental scanning electron microscopy (ESEM). The microparticles sample was thinly sprinkled onto a metal stub and vacuum coated with a thin layer of gold palladium to a thickness of 20 nm in an argon atmosphere. The

coated samples were examined at an acceleration voltage of 10 kV (FEI Quanta 200 ESEM, FEI Company, Hillsboro, OR, USA).

2.5 *Loading efficiency and loading capacity*

Loading efficiency (LE) and loading capacity (LC) of DDS and PG in TMC-TPP microparticles were analysed by ultraviolet spectrophotometry (Shimazu, Kyota, Japan) in a 1 cm^3 quartz cell. An empty microparticles suspension was used as blank to correct for interferences by TMC, TPP and Tween 80. The stored supernatant and methanol, used to wash the particles where diluted and analysed to determine the DDS and PG that was not entrapped in the particles. Samples were compared to standard curves of DDS and PG. Standard solutions with known concentrations of DDS and PG in a 50 % *v/v* methanol were prepared and the absorbance were measured at 260.6 and 295.6 nm (DDS) and 259.0 nm (PG). The calibration curve was determined by plotting the peak absorbance against concentration and fitted with linear regression.

Loading efficiency (LE) and loading capacity (LC) for both protein and fluorescence assay were calculated as follows [18, 28]. The experiments were performed in triplicate.

$$\text{LE (\%)} = \frac{\text{Initial drug load - Free drug}}{\text{Initial drug load}} \times 100 \qquad (1)$$

$$\text{LC (\%)} = \frac{\text{Initial drug load - Free drug}}{\text{Microparticles dry weight}} \times 100 \qquad (2)$$

2.6 *Dissolution*

Dissolution tests were performed in an Erweka D700 (Erweka, Heustenstamm, Germany) six station paddle-method dissolution apparatus. The paddles rotated at a speed of 75 rpm for both the dapsone and proguanil dissolution. The dissolution medium used was 500 ml of 0.1N HCl containing 0.2 % *w/v* NaCl. The temperature was regulated with a thermostat at 37 ± 0.5ºC. The dissolution studies were done based on the method by Lötter and co-workers [29]. In short, the powdered samples (binary mixture of powdered sample equivalent to ± 60 mg of dapsone and ± 60 mg of proguanil per sample) were accurately transferred into test tubes. Glass beads, ± 50 mg (Sigma Aldrich, Johannesburg, South Africa) with a diameter of 0.1 mm were added to each test tube in order to minimize possible agglomeration of particles. Thereafter 10 ml of dissolution medium was withdrawn from each vessel and transferred into the respective allocated test tube. The suspended samples were agitated for 30 seconds using a Vortex Genie shaker (Scientific Industries Inc., Bohemia, New York), before being rinsed into the respective dissolution vessels. Samples were withdrawn after 7.5, 15, 22.5, 30, 45, 60, 90, 120 and 150 minutes. The samples were filtered using in-line Millipore 0.45 µm filters (Microsep, Sandton, South Africa) and immediately after sampling the withdrawn volume was replaced with new dissolution medium at the same

temperature. Samples were transferred to glass HPLC-vials to be analysed using an HPLC method as described in section 2.7.

2.7 *HPLC methods*

An HPLC technique based on the artesunate tablets assay method (A) of the 2011 International Pharmacopeia [30] was adapted and used to quantitatively determine proguanil and dapsone from assay and dissolution samples.

Analytical chromatographic separations were achieved using an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA) equipped with a Novapak C18, 3 µm, 4.6 mm x 100 mm column (Phenomenex, Torrance, CA) held at 30ºC. The software Rev. B 02.01-SR2 (260) Chemstation for LC 3D (Agilent Technologies, Santa Clara, CA) was used. The mobile phase consisted of 440 ml acetonitrile and 560 ml buffer (pH 3.0). The buffer contained potassium dihydrogen phosphate (1.36 g in 900 ml water) and the pH was adjusted to 3.0 with phosphoric acid and diluted to 1000 ml with distilled water. The flow was set at 1 ml/min for all the analytical tests and the wavelength was set at 216 nm. The injection volume for PG and DDS was 10 µl. The solvent consisted of acetonitrile (assay) and the relevant dissolution medium (dissolution). The evaluation of specificity, linearity, range, accuracy, repeatability, limit of detection (LOD) and limit of quantification (LOQ) was based on the ICH guideline on validation of analytical procedures [31]

2.8 In vivo *pharmacodynamic evaluation: antimalarial efficacy studies*

Animal experiments were executed in compliance with the guidelines of Ethics Committee on the premises of North-West University Animal Research Centre. The experimental protocol was approved by the North-West University's Ethics Committee (protocol number: NWU-00042-10-S5). Healthy male C57 BL8 mice weighing 30 ± 5 g were used for the study. The mice were kept in a closed, controlled environment which ensured ideal growth and exposure to the minimal pathogens as depicted. The mice's diet consisted out of Epol® mice cubes [Epol (Pty) Ltd., Pretoria, South Africa].

In vivo antimalarial efficacy studies were performed on the rodent malaria parasite, *Plasmodium berghei* ANKA strain which is infective in mice. This strain's life cycle is in the essence similar to that of the human malaria parasite. It is lethal in mice, causing high mortality rates and its sensitivity to all currently used antimalarial drugs provides a good model to estimate the efficacy and survival. The parasite was revived from frozen stocks and infection was initiated in the donor mice with intraperitoneal injection of 10^6 parasitized erythrocytes (pRBCs). Parasites were maintained in two host mice until the parasitemia reached a level of 25 % [32].

Blood taken from infected donor mice with approximately 25 % parasitemia was diluted suitably in phosphate buffered saline to contain approximately 10^6 parasitized erythrocytes. Experimental animals were infected intraperitoneally with this blood (200 µl) on day 0 to introduce 2 x 10^6 pRBCs to each. Mice

were randomly divided into fourteen groups (n = 8). Two hours post infection; mice were treated orally with single dose of the various formulations (A – N). Formula A and Formula B were the control groups containing DMSO/PBS and plain TMC-TPP microparticles, respectively. Dapsone and proguanil formulations (normal and TMC) were prepared with dosage strengths of 0.03 mg/kg and 16 mg/kg respectively. The combination formulations contained the same concentration of each drug as those of the mono-therapy formulations. Further, on days 1, 2, and 3, mice were treated again similarly as on day 0. Then, throughout the study, blood was withdrawn from tail vein from day 1 onwards at regular time intervals for the assessment of parasitemia [33, 34].

2.8.1 *Assessment of parasitemia in the infected mice*

Peripheral blood smears were prepared on glass slides by using blood obtained from tail veins of infected mice. The thin films were fixed in methanol for 5 min and stained with Giemsa stain solution (one part giemsa and four parts phosphate buffer). Blood smears were examined at a magnification of 100×/oil immersion lens using a Nikon DXM1200 digital camera on a Nikon ECLIPSE TE300 confocal microscope with ACT-1 software. Parasitemia was determined by counting 700 to 900 erythrocytes covering 5 random microscope fields of view. Parasitemia was calculated using equation 3.

$$\% \text{ Parasitemia} = \frac{\text{Total amount of infected erythrocytes}}{\text{Total amount of erythrocytes}} \times 100 \qquad (3)$$

2.9 In vivo *pharmacokinetic study*

The bioavailability of dapsone and proguanil formulated with N-trimethyl chitosan chloride (TMC) microparticles in a 1:1 (*w/w*) ratio, relative to free drug was evaluated in male C57/BL6 mice, weighing approximately 20 - 25g. The formulations were administered orally (N = 5) at 3 mg/kg (dapsone) and 16mg/kg (proguanil) in a mixture of PBS and DMSO (90:10; v/v). The dose was prepared according to the average weight of the animals and an assumed volume of 200 µl of vehicle. Volumes were adjusted to dose the compound at 3 mg/kg or 16 mg/kg for each animal's individual weight.

Whole mouse blood sample was collected at predetermined sampling time points (0.17, 0.5, 1, 2, 4, 8, and 24 h) *via* tail vein bleeding into 1.5 ml heparinised microfuge tubes and were kept on ice. Blood samples were transferred to a - 80 °C freezer within 60 minutes after collection for storage until analysis was carried out.

A sensitive and accurate LC-MS/MS method was developed to analyse the study samples. Concentration vs. time graphs were constructed and the bioavailability of dapsone-TMC was determined relative to the free dapsone using the following formula.

$$Relative\ bioavailability = \frac{AUC_{test}}{AUC_{ref}} \times \frac{Dose_{ref}}{Dose\ _{test}};$$

where test = drug-TMC and ref = free drug.

2.10 *Statistical analysis*

All results are expressed as mean ± standard deviation (SD). Results were graphically depicted by using GraphPad Prism Version 5. A two-way analysis of variance (ANOVA) was performed on each treatment. The number of formulations and the duration of the experiment (days) were the considered factors. As missing values occurred throughout the data these ANOVAs were performed by implementing mixed models. For these models the MIXED procedure of SAS (SAS Institute, Inc. 2003) was used. The significance of the treatment outcome with formulations, together with their respective interactions, were determined from these ANOVAs, while estimates of the least square mean values of the percentages for each combination of formulation and day were also calculated from the fitted models. A Dunnett's test was done to determine which of the least square mean parasitemia values of the formulations differed statistically significantly.

3. Results and discussion

3.1 *Microparticle characterisation*

The microparticles were successfully synthesised by cross-linking TMC with TPP. This synthesis method was optimised by making use of a factorial experimental design as depicted in Table 1.

The optimal formula was chosen on the basis of which method produced the smallest particles and this was achieved when the TMC and TPP was cross-linked in a fix ratio of 3:1 (TMC:TPP), in the presence of Tween 80 and subjected to ultrasonification using a tip sonicator. As a rule of thumb, suspensions with zeta potential above 30 mV (absolute value) are physically stable. Suspensions with a potential above 60 mV show excellent stability but suspensions below 20 mV are of limited stability; whereas below 5 mV they undergo pronounced aggregation. Adsorption of a steric stabiliser layer leads to a reduction of the measured zeta potential, which is however not an indication of a reduced electrostatic repulsion. The adsorption layer of the stabiliser shifts the plain of shear, at which the zeta potential is measured, to a larger distance from the particle surface resulting in a lower zeta potential being measured. In such cases zeta potentials of about 20 mV are still sufficient to fully stabilise the system in combination with steric stabilisation [35].

Table 1. Summary of the optimisation on TMC-TPP microparticles, including characteristics of the particles including size, zeta potential and pH.

Ratio of TMC:TPP	No Tween 80	Tween 80	With Tween 80 and tip sonification
2:1	Avg. particle size: 57.12 µm ± 5.51 Zeta potential: 13.17 mV ± 0.45 pH: 6.11	Avg. particle size: 158.35 µm ± 9.49 Zeta potential: 15.3 mV ± 0.72 pH: 6.74	Avg. particle size: 53.92 µm ± 2.75 Zeta potential: 11.33 mV ± 0.93 pH: 6.34
3:1	Avg. particle size: 20.29 µm ± 1.69 Zeta potential: 16.3 mV ± 5.4 pH: 5.61	Avg. particle size: 21.03 µm ± 0.18 Zeta potential: 25.53 mV ± 4.43 pH: 5.44	Avg. particle size: 18.56 µm ± 0.72 Zeta potential: 21.83 mV ± 0.55 pH: 5.45
5:1	Avg. particle size: 97.26 µm ± 12.89 Zeta potential: 35.56 mV ± 1.42 pH: 6.21	Avg. particle size: 62.88 µm ± 0.14 Zeta potential: 35.7 mV ± 2.33 pH: 6.11	Avg. particle size: 87.12 µm ± 3.11 Zeta potential: 34.33 mV ± 1.04 pH: 6.00

Chitosan and subsequently TMC are compounds that combines the electrostatic stabilisation due to the positive charge they carry, and the steric stabilisation due to their polymeric nature. It also has mucoadhesive properties [16-18]. Theoretically this is indicative of an ideal stabiliser due to its combination of electrostatic and steric stabilisation, together with ability to enhance bioavailability by adhering to the mucus membrane and opening the tight junctions. Thus the zeta potential (21.83 mV ± 0.55) of the chosen formulation will be sufficient to produce a stable suspension. Both the drugs were entrapped in the TMC-TPP particles using the method as previously described. Their particle size and zeta potential were measured for both dapsone (46.21 µm ± 0.86, 7.17 mV ± 2.26) and proguanil (74.56 µm ± 3.81, 15.70 mV ± 0.70) TMC microparticles. This indicated that the particle size increased due to the drug that was incorporated with the TMC particles. The significant decrease in zeta potential can be attributed to adsorption of the drugs on the particle surface, thus masking the surface charge of the TMC particle [36].

ESEM was also used to determine the morphology and size of the particles as depicted in Figures 1 A to C.

As clearly indicated in the ESEM photos the particles have an amorphous shape. The drugs appear to be entrapped inside the particles, but as mentioned earlier due to the decrease in zeta potential, it is indicative that some of the drug is adsorbed on the surface of the particles.

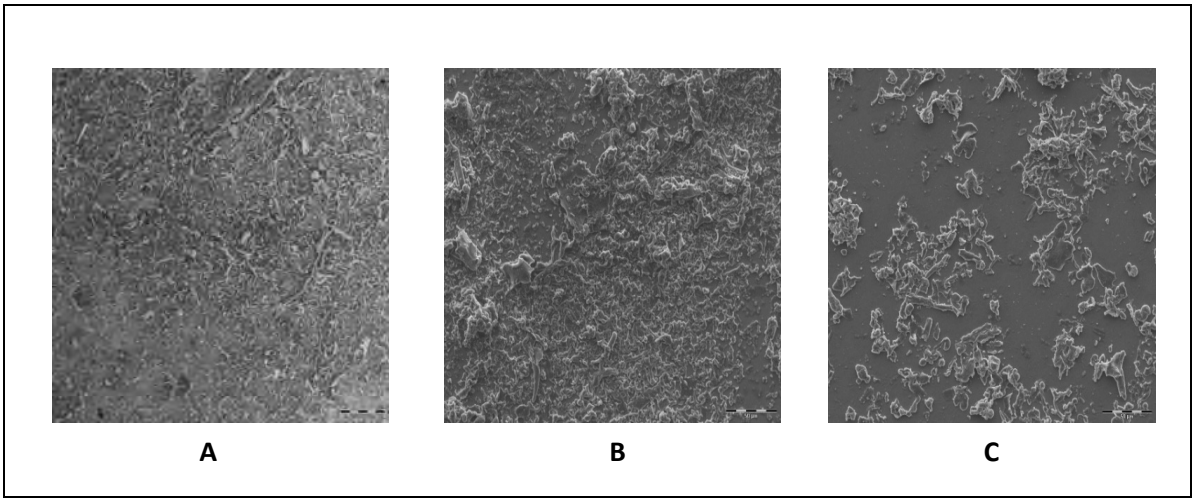

Fig. 1 ESEM micrographs of **A)** TMC-TPP microparticles (scale bar = 500 µm), **B)** dapsone-TMC-TPP microparticles (scale bar = 50 µm) and **C)** proguanil-TMC-TPP particles (scale bar = 50 µm).

3.2 *Loading efficiency and loading capacity*

The loading efficiency and loading capacity was determined for both the dapsone-TMC and the proguanil-TMC particles using the method described earlier. The dapsone-TMC particles had a loading efficiency of 50.72 ± 0.14 % and a loading capacity of 58.57 ± 0.16 % whereas the proguanil-TMC particles had a loading efficiency of 44.20 ± 1.45 % and a loading capacity of 50.16 ± 1.64 %. Previous work done by Chen and co-workers [20] on oral protein delivery by entrapping bovine serum albumin (BSA) in TMC-TPP particles has almost the same loading capacity (44.77 ± 0.76 %) as the particles in this study. The loading efficacy differs a lot with the particles in Chen's study having a loading efficacy of 94.08 ± 1.88 %. This is a common occurrence due to vast variety of compounds having different charges on their chemical structures. Thus the negatively charged BSA would have more affinity for the positively charged TMC than the mostly neutral structures of dapsone and proguanil thus resulting in a higher entrapment efficacy than the particles in this study.

3.3 In vitro *drug release*

The dissolution profiles for the dapsone-TMC and proguanil-TMC microparticles are shown in Figure 2. The mean AUC values for the two formulations are reported in Table 2.

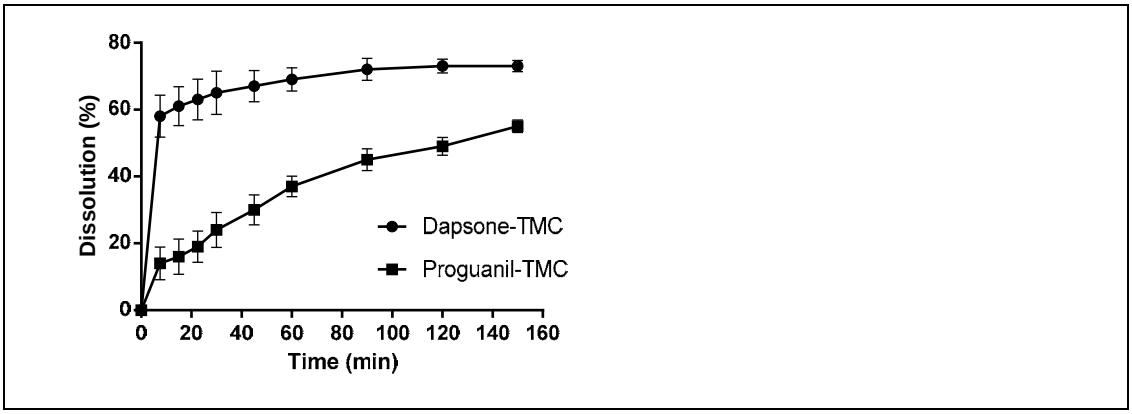

Fig. 2 *In vitro* release profiles of dapsone and proguanil from TMC-TPP microparticles (n = 6).

It is clear when observing Figure 2 that the two formulations released their drug content at a pH of 3 with different release profiles. The difference in release profiles may be attributed to the different zeta potential of the formulations. As mentioned earlier, a decrease in zeta potential can be an indicator that drug particles were adsorbed to the surface of the TMC micro-particle. Thus the higher dissolution rate in the first ten minutes of dapsone-TMC microparticles could be indicative that most of the dapsone was on or near the surface of the microparticles. The formulations seem to have a sustained release since only 73.00 ± 1.70 % (dapsone) and 55.00 ± 1.90 % (proguanil) was released after 150 minutes.

Table 2. Mean AUC-values (± SD) for both the formulations.

Formulation	$AUC_{0\text{-}150}$ (µg/ml.min)
Dapsone-TMC	12146.40 ± 386.27
Proguanil-TMC	6696.28 ± 159.79

In Table 2 the mean AUC-values of the dissolution profiles are reported. Form these results it can be concluded that dapsone is released easier from the TMC microparticles than proguanil. The area under the curve (AUC) is a parameter frequently used in assessing bioavailability. In terms of plasma-time profiles, area under curve values are a measurement of the extent of drug bioavailability. The AUC reflects the total amount of drug that reaches the systemic circulation. Likewise, AUC can also be used in dissolution studies as a measurement of the extent of drug dissolution by calculating the AUC of the dissolution-time profile. The AUC can be approximated by the trapezoidal rule (see equation 4). This method is fairly accurate when sufficient data points are used [37].

$$[AUC]\frac{t_n}{t_{n-1}} = \frac{C_{n-1} + C_n}{2} t_n - t_{n-1} \qquad (4)$$

where: AUC = Area under curve from time, t_n to time t_{n-1},

C_n = concentration at time n, and

C_{n-1} = concentration at time, n-1.

3.4 In vivo *efficacy study*

The results of the normal drug formulations and the TMC formulations are given in Tables 3 and 4 and depicted in Figures 3 A) and B). The least square means of the parasitaemia percentages were calculated. These values are the within-group means which are appropriately adjusted for the other effects in the model. More precisely, they estimate the marginal means for a balanced population (as opposed to the unbalanced design) [38].

When compared to the controls, both the normal and TMC formulations indicated better *in vivo* efficacy. The parasitaemia was kept under control up until day 11 whereafter it increased significantly. The normal dapsone and proguanil formulations reflected parasitaemia which ranged from 0.00 % - 45.48 % and 0.00 % - 50.29 %, respectively. The normal combination resulted in parasitemia of 0.00 % - 5.41 %. The control formulation's (DMSO/PBS) parasitaemia ranged from 0.00 – 51.47 % over the duration of the study and reflected a mean parasitaemia of 24.06 %. The dapsone-TMC and proguanil-TMC formulations reflected parasitaemia which ranged from 0.00 % - 39.46 % and 0.00 % - 37.93 % respectively. The combination TMC formulation resulted in parasitemia of 0.00 % - 42.10 %. The other control formulation's (TMC) parasitaemia ranged from 0.00 – 41.34 % over the duration of the study and reflected a mean parasitaemia of 23.43 %.

The plain dapsone formulations showed a sharp increase in parasitaemia on day 3 compared to the more gradual increase in parasitaemia of the dapsone-TMC formulation. Overall the dapsone-TMC formulation (13.51 %) indicated a better reduction in the mean parasitaemia than the normal dapsone formulation (17.14 %). Both these dapsone formulations exhibited a significant statistical difference ($p < 0.0001$) when compared to the control formulation.

The proguanil formulations reflected almost identical parasitaemia values up until day 18 where the proguanil-TMC formulation had a significantly lower parasitaemia (37.93 %) than its normal formulation counterpart (50.29 %). Overall the proguanil-TMC formulation (18.11 %) indicated a slightly better reduction in the mean parasitaemia than the normal proguanil formulation (19.30 %). The proguanil formulation ($p = 0.0232$) did not show any statistically significant difference but the proguanil-TMC formulation ($p < 0.0001$) showed statistically significant differences when compared to the control formulation (TMC).

Both the combination formulations showed better efficacy when compared to the control formulations. During the study, all of the test subjects that received the normal combination formulation died after 11 days but the test subjects that received the combination TMC formulation survived until day 18, thus no mean parasitaemia could be determined for the subjects that received the normal combination formulation. The mean parasitaemia for the combination TMC formulation was 16.85 % which is lower than the control formulation's (TMC) 23.43 % mean parasitaemia, thus indicating to have an *in vivo*

efficacy. The combination TMC formulation indicated statistically significant differences ($p < 0.0001$) when compared to control formulation.

These results clearly indicated that the TMC formulations were more effective against malaria than their plain formulation counter-parts. Thus it can be concluded that the TMC microparticles enhances the efficacy of the antimalarial drugs dapsone and proguanil.

Previous studies, utilising polymeric nanoparticles, had similar results as demonstrated in this study. Polymeric nanoparticles are among the drug delivery systems which have been studied in comparison to liposomes for many applications [39-41]. Polymeric nanoparticles themselves and drugs encapsulated within them are more stable in biological fluids and under harsh preparation, processing and storage conditions compared to other delivery systems [42].

Akhtar and co-workers produced chitosan nanoparticles by electrostatic interaction between chitosan and penta sodium tri-polyphosphate. They encapsulated curcumin and tested the *in vivo* efficacy in mice. An enhanced antimalarial effect was achieved [43]. Föger et al. [44] demonstrated the susceptibility of *P. falciparum* to chitosan antisense nanoparticles by encapsulating phosphorothioate antisense oligodeoxynucleotides (ODNs) for silencing malarial topoisomerase II gene.

Taking all these previous studies in account, it can be concluded that chitosan based particles have an enhanced efficacy against malaria parasites.

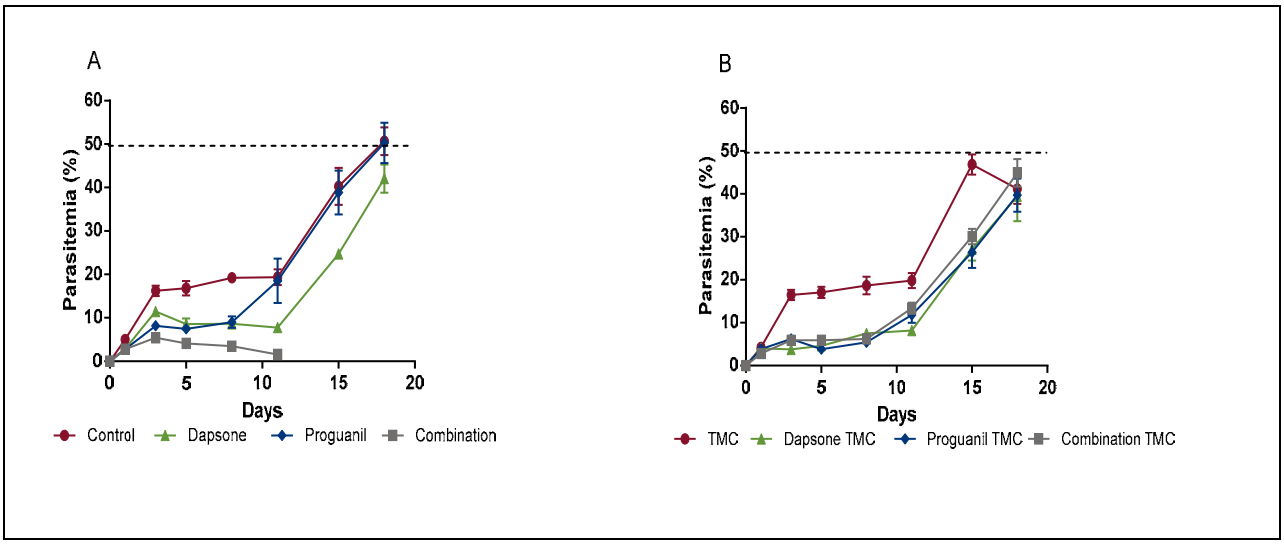

Fig. 3 *In vivo* mean parasitaemia values for A) The normal drug formulations and B) the TMC formulations plotted against the control formulations.

Table 3. Least square means parasitaemia values, including the standard error of the mean (SEM), for the normal drug formulations for each day.

Normal drug and combination formulations compared to the control																
Formulation	**Least Square Means**															
	Day 0	**SEM**	**Day 1**	**SEM**	**Day 3**	**SEM**	**Day 5**	**SEM**	**Day 8**	**SEM**	**Day 11**	**SEM**	**Day 15**	**SEM**	**Day 18**	**SEM**
Control (DMSO/PBS)	0	0	5.05	1.71	16.25	1.71	16.84	1.71	19.20	1.71	19.36	1.71	40.29	1.71	51.47	1.81
Dapsone (0.03 mg/kg)	0	0	3.06	1.71	11.80	1.71	6.08	1.71	9.36	1.71	11.58	1.71	33.34	1.71	45.48	1.71
Proguanil (16.00 mg/kg)	0	0	2.80	1.83	8.03	1.94	7.53	2.59	9.03	2.77	18.51	2.79	38.89	2.79	50.30	2.79
Combination	0	0	2.80	1.71	5.41	1.71	4.26	1.80	3.55	1.82	1.60	1.95	N/D	N/D	N/D	N/D

Table 4. Least square means parasitaemia values, including the standard error of the mean (SEM), for the TMC formulations for each day.

TMC formulations compared to the control																
Formulation	**Least Square Means**															
	Day 0	**SEM**	**Day 1**	**SEM**	**Day 3**	**SEM**	**Day 5**	**SEM**	**Day 8**	**SEM**	**Day 11**	**SEM**	**Day 15**	**SEM**	**Day 18**	**SEM**
Control (TMC)	0	0	4.29	1.65	4.29	1.65	17.09	1.65	18.67	1.65	19.38	1.74	46.74	1.76	41.34	1.88
Dapsone-TMC (0.03 mg/kg)	0	0	4.03	1.71	3.96	1.80	4.72	1.82	7.23	2.12	8.08	2.16	27.10	2.16	39.46	2.16
Proguanil-TMC (16.00 mg/kg)	0	0	4.43	1.65	4.28	1.65	5.68	1.65	11.11	1.65	23.40	1.74	40.04	1.76	37.93	1.76
Combination TMC	0	0	1.30	1.65	5.35	1.65	5.93	1.65	6.93	1.65	16.06	1.74	40.30	1.76	42.10	1.76

3.5 *In vivo bioavailability*

The *in vivo* bioavailability of the TMC formulation is plotted against the normal formulation in Figure 4. The *in vivo* bioavailability of the dapsone-TMC formulation (3 mg/kg oral dose; equivalent to 1.5 mg/kg dapsone) relative to the normal dapsone formulation (3 mg/kg oral dose) was found to be 244 % (n = 5). The dapsone-TMC microparticles were absorbed faster, i.e. the maximum concentration was attained 1 hour after the administration of the dose relative to that of the normal dapsone formulation that reached its maximum concentration after 2 hours.

For the proguanil-TMC formulation (16 mg/kg oral dose; equivalent to 8 mg/kg proguanil) relative to the proguanil formulation (16 mg/kg oral dose) bioavailability was found to be 123 % (n = 5); whereas the bioavailability of cycloguanil obtained from 16 mg/kg of the proguanil-TMC formulation oral dose, was found to be 156 %. Both the proguanil formulations were absorbed quickly, with both formulations attaining the maximum concentration at 2 hours after dose administration.

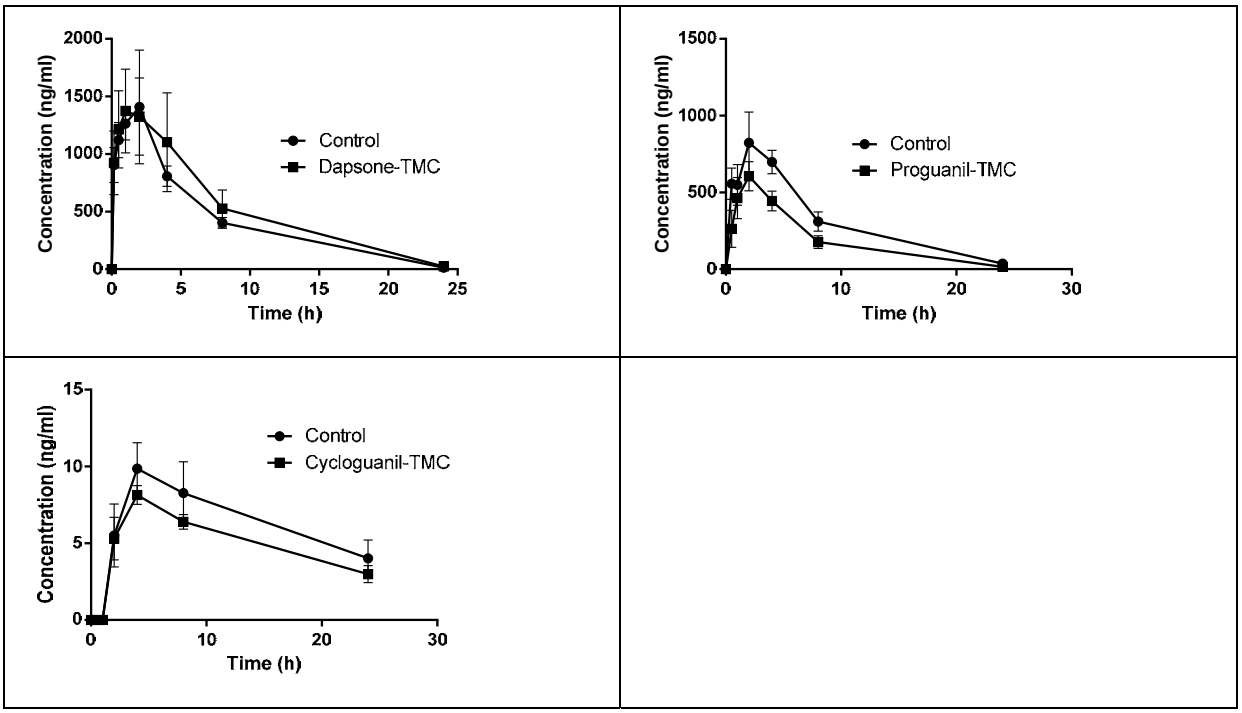

Fig. 4 *In vivo* bioavailability of the TMC formulation plotted against the normal formulations indicating the enhanced bioavailability of the dapsone-TMC and proguanil-TMC formulations. The bioavailability of active metabolite of proguanil, namely cycloguanil, was also determined.

4. Conclusion

In this study, TMC-TPP microparticles were successfully synthesised and characterised. Dapsone and proguanil were entrapped and the loading efficiency and capacity were successfully determined. The *in vitro* release profiles were determined by performing powder dissolutions. These studies revealed that the

TMC-TPP microparticles exhibited some degree of sustained release. To determine the efficacy of the formulations an *in vivo* study was performed on mice using the Peters 4-day method. All of the formulations showed better activity than the control formulations. The dapsone-TMC formulation (0.03 mg/kg) illustrated better efficacy by obtaining a mean parasitaemia of 13.51 % in comparison with the normal dapsone formulation which has a mean parasitaemia of 17.14 %. Both the proguanil formulations had very similar *in vivo* efficacy. Overall the proguanil-TMC formulation (18.11 %) indicated a slightly better reduction in the mean parasitaemia than the normal proguanil formulation (19.30 %). The normal combination formulation caused toxicity in the subject mice resulting in the death of all the mice by day 11. The combination TMC formulation prevented this and the subjects survived until day 18. This formulation also had better *in vivo* efficacy when compared to the control formulation. The *in vivo* bioavailability study indicated that both the dapsone and proguanil-TMC formulations were more bioavailable when compared to the corresponding normal formulations. It can be concluded that by formulating dapsone and proguanil in TMC-TPP microparticles has enhanced the absorption of the drugs, thus resulting in better bioavailability. Therefore the possibility is there to incorporate a higher dose in the TMC-TPP microparticles to enhance the efficacy. These TMC-TPP microparticles formulations may constitute a promising approach for the treatment of malaria.

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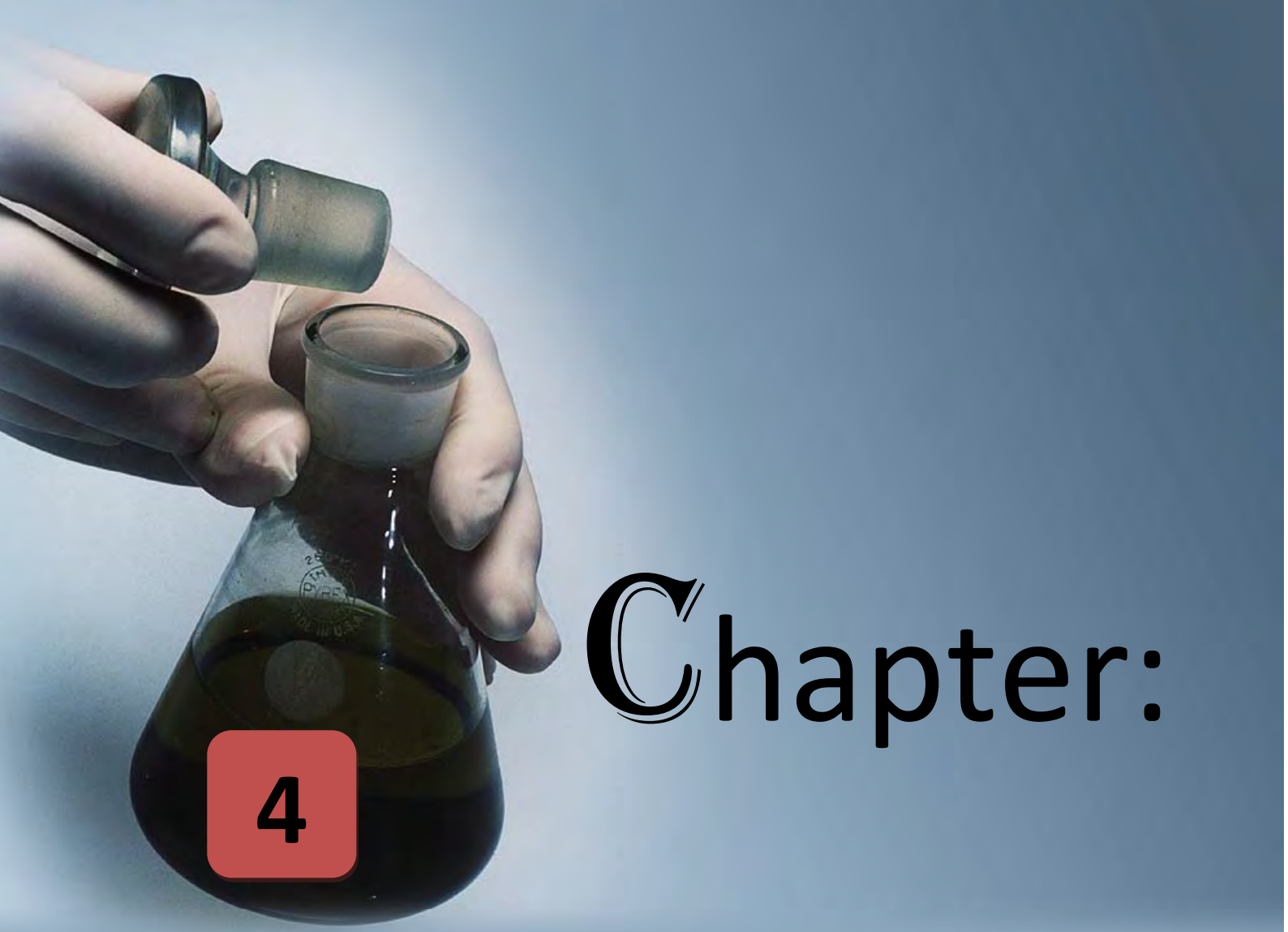

Chapter: 4

Chapter 4 contains the manuscript of an article to be submitted to Carbohydrate Polymers. The article contains the background, aims, all the experimental details and results of this study on the synthesis of TMC by microwave irradiation. The article is prepared according to the Guide for Authors that can be found on the website of this journal (http://www.elsevier.com/journals/carbohydrate-polymers/0144-8617/guide-for-authors), except that for easy reading figures, schemes and tables are inserted at their logical places as they would appear in the printed version.

Preparation and characterisation of quaternised *N*-trimethyl chitosan chloride by microwave irradiation compared to the conventional method

J. van Heerden,* A.F. Kotzé, J.H. Steenekamp, L.H. du Plessis

Centre of Excellence for Pharmaceutical Sciences (Pharmacen), North-West University, Hoffman Street, Potchefstroom, 2531, South Africa

* Corresponding author: Lissinda.DuPlessis@nwu.ac.za, Tel: +27 18 299 4246,
Fax: +27 18 299 2248

ABSTRACT

N-Trimethyl chitosan chloride (TMC) has long been studied and used as an absorption enhancer and drug carrier in the pharmaceutical environment. TMC has a fixed positive charge on the quaternary amino-group which allows it to be highly soluble in the physiological pH range which is mainly neutral and basic environments. It is an improved derivative of chitosan and has better aqueous solubility and thus more pharmaceutical applications. Conventionally TMC is synthesised by reductive methylation in the presence of methyl iodide (MeI) and a base which is commonly sodium hydroxide (NaOH). This method is however very time consuming, technical and not *N*-selective which allows *O*–methylation to take place. *O*-methylation significantly reduces the solubility of TMC especially with highly *N*-quaternised products. Here we report a method where microwave irradiation is utilised in the place of conventional heating, thus allowing the reaction to take place quicker and minimising the formation of *O*-methylation.

KEYWORDS

N-trimethyl chitosan chloride (TMC)
Chitosan
Microwave irradiation
O-methylation
Degree of quaternisation
Conventional heating

1. Introduction

Chitosan is a linear amino-polysaccharide made of randomly distributed *N*-glucosamine and *N*-acetylglucosamine units that are linked together at β-1 to 4 (Kumar, et al., 2004, Park, et al., 2010). This cationic polysaccharide has drawn increasing attention within pharmaceutical and biomedical applications because of its abundant availability, mucoadhesive and inherent pharmacological properties like hypocholesterolemic action, wound-healing properties and antiulcer activity. Other beneficial biological properties such as biocompatibility, biodegradability, non-toxicity and low-immunogenicity also make it a favourable compound to work with (Felt, et al., 1998, Illum, 1998, Mourya and Inamdar, 2009). Although chitosan has many reported successes, a major drawback is its insolubility at physiological pH (7.4), whereas it is insoluble and active as an absorption enhancer only in its protonated (uncoiled and positively charged) form in acidic environments. On the other hand *N*-trimethyl chitosan chloride (TMC), a partially quaternised chitosan derivative, shows good water-solubility across a wide pH range thus having mucoadhesive properties and excellent absorption enhancing effects even at neutral pH (Amidi, et al., 2006, Hamman, et al., 2003, Kotzé, et al., 1998, Snyman, et al., 2002). It is synthesised by the reductive methylation, also called acetylation, of the amino groups on the C_2 position of chitosan (Hamman, et al., 2003) and by doing so creates a positive charge on the amino group. By repeating the methylation step, during the reaction, a higher degree of trimethylation can occur (Snyman, et al., 2002). This is also referred to as the degree of quaternisation (DQ). Unfortunately, trimethylation isn't the only reaction taking place because chitosan has more than one reactive group in its chemical structure. Significant *N,N*-dimethylation, *N*-monomethylation and *O*-methylation also occurs. With *O*-methylation a significant decrease in the water solubility could be observed, but the solubility will also decrease with an increase in molecular weight (Polnok, et al., 2004, Rúnarsson, et al., 2007, Sieval, et al., 1998). Thus it will be more desirable to prepare TMC polymers with a high DQ but with a low degree of O-methylation.

TMC is usually prepared by a conventional heating method, which is time-consuming (45 min – 2 days) (Domard, et al., 1986, Kotzé, et al., 1999, Rúnarsson, et al., 2008, Thanou, et al., 2000). Compared with routine heating treatment, microwave irradiation technology, as a more efficient heating method due to its rapid heating and energy penetration, is quickly emerging as an alternative energy source that is powerful enough to perform chemical reactions in minutes, instead of hours or even days (Luo, et al., 2010,

Tharun, et al., 2013). The ever so increasing interest in clean and green environment friendly chemistry has been the driving force behind microwave irradiation technology being utilised in the grafting and modification of polysaccharides. Some of the advantages that microwave irradiation has over conventional methods are by significantly reducing the reaction time and the use of toxic solvents. This in turn leads to higher yields, product selectivity and clean product formations (Liu, et al., 2013, Singh, et al., 2012). Herein, a newly developed microwave-assisted method was developed to prepare TMC. Utilising a factorial design with three changing factors namely microwave power (watt), reaction time (minutes) and number of reaction steps TMC was prepared within a fraction of time compared to the conventional method. This newly microwave-assisted synthesised TMC was characterised using NMR, FTIR and SEC-MALLS.

2. Experimental

2.1 *Materials*

ChitoClear® (95 % deacetylated chitosan) was a purchased from Primex ehf (Singlufjordur, Iceland). Iodomethane, sodium hydroxide pellets, sodium iodide, sodium chloride, acetic acid, ammonium acetate, absolute ethanol, diethylether and N-methyl-2-pyrrolidinone (Sigma-Aldrich, South Africa) were used as received. All chemicals used in the synthesis process were of analytical grade.

2.2 *Synthesis of N-trimethyl chitosan chloride (TMC)*

2.2.1 *Conventional Heating Method*

N-Trimethyl chitosan chloride (TMC) with a DQ of 60 % was synthesised by reductive methylation of chitosan by a chemical reaction between chitosan and iodomethane in the presence of sodium hydroxide based on the method previously described (Kotzé, et al., 1998, Kotzé, et al., 1999, Sieval, et al., 1998, Snyman, et al., 2002) with some modifications. The methylation of chitosan was successively repeated (two times) with the polymer obtained from the preceding step. These repeated steps are called “additional steps”. The reaction conditions of each step of the synthesis of the TMC polymers were as follows:

2.2.1.1 *Reaction step*

A mixture of 1 g of chitosan and 2.4 g of sodium iodide (NaI) was mixed in 40 ml N-methyl-2-pyrrolidinone (NMP) in a water bath at 70 °C for 60 min while stirring vigorously. Subsequently, 5 ml of methyl iodide (MeI) and 5 ml of 20 % *w/v* aqueous sodium hydroxide (NaOH) was added to the mixture and the reaction was carried out for 30 min at 60 °C in the presence of a Liebig’s condenser to prevent any evaporation of

methyl iodide. The polymer was collected by precipitation from solution using ethanol and diethyl ether. The product obtained from this step (*N*-trimethyl chitosan iodide) was washed twice with diethyl ether on a sintered glass filter and dried under vacuum.

2.2.1.2 *Additional step*

Prior to precipitation of the product from the solution mixture at the end of the previous reaction step, an additional 5 ml of methyl iodide and 5 ml of 20 % *w/v* sodium hydroxide were added. The reaction was further continued for another 30 min at 60 °C. The product (*N*-trimethyl chitosan iodide) was precipitated from solution using ethanol and diethyl ether, washed twice with diethyl ether on a sintered glass filter, and finally dried in a vacuum chamber.

2.2.1.3 *Ion-exchange step*

The products prepared as described above were dissolved in 50 ml of 10 % (w/v) sodium chloride solution to exchange the iodide ion with chloride, and were subsequently precipitated by using ethanol and diethyl ether. The products were repeatedly dissolved in 40 ml of water and precipitated with the use of ethanol and diethyl ether to remove the remaining sodium chloride. The final products were dried in the vacuum chamber for at least 12 h prior to further characterization.

2.2.2 *Microwave irradiation method*

TMC polymers with different DQ were synthesised by a similar method as described above. The major difference was that the conventional heating was replaced with microwave irradiation. Microwave radiation was conducted using a CEM Discover™ open vessel microwave synthesis system. The instrument consisted of a continuous focused microwave power delivery system with operator selectable power output from 0 to 300 W, a maximum current of 6.3 amps and a frequency of 50/60 Hz. The temperature of the contents of the vessel was monitored using an infrared (IR) sensor located underneath the reaction vessel. The contents of the vessel was stirred by means of a rotating magnetic plate located below the floor of the microwave cavity and a teflon coated magnetic stirring bar in the vessel. The vessel was kept cooled during the reaction by means of cooled air and MeI was kept in the reaction by using a Liebig condenser. To develop this new method various reaction parameters like microwave power (*A*), number of additional reaction steps (*B*) and reaction time (*C*) were investigated. For each parameter, two levels (values) were investigated as listed in Table 1.

Table 1. Factorial design: Investigated variables and their levels.

Levels of each variable	***A*: microwave power (W)**	***B*: number of additional reaction steps**	***C*: reaction time (min)**
1	100	0	15
2	130	1	30

The reaction conditions of each step of the newly microwave irradiation assisted synthesis method were as follows:

2.2.2.1 *Dissolving step*

At the beginning of each reaction, special care was taken to ensure that the 1 g chitosan and 2.4 g of sodium iodide were dissolved in 50 ml NMP. The reaction mixture was subjected to 300 W of microwave irradiation for 8 minutes (4 min ramp and 4 min hold). Afterwards the mixture was cooled down in a freezer.

2.2.2.2 *Reaction step*

To the cooled reaction mixture, 12 ml MeI and 5 ml NaOH (20 % *w/v*) were added and then subjected to 100 or 130 W microwave irradiation for 15 min (2 min ramp and 13 min hold) or 30 min (2 min ramp and 28 min hold). This step may be repeated. Before each repeated step, the mixture had to be cooled.

2.3 *Characterisation of the synthesised TMC polymers*

2.3.1 *Degree of quaternisation*

All ^{1}H-NMR spectra were obtained with a Bruker AVANCE III 600 (Bruker Biospin GmbH, Karlsruhe, Germany) operated at 600.17 and 150.91 MHz, respectively at 298 °K. The *N*-acetyl peak was used as internal reference with D_2O as solvent. The measurements were done with water suppression. The degree of substitution for the TMC derivatives was calculated using the combined integrals, in the ^{1}H-NMR spectra (D_2O as solvent), for H-2 (GluNAc), H-3, H-4, H-5, H-6, and H-6′ (6H) peaks at δ 3.6 – 4.5 and H-2 (GluN) peak at 3.10 ppm. This integral ([H-2, H-3, H-4, H-5, H-6, H-6′]) represented six protons. The following equations were used to estimate the degree of substitution:

% *N,N,N*-Trimethylation = [[$N(CH_3)_3$]/[H-2, H-3, H-4, H-5, H-6, H-6′] x 6/9] x 100
% *N,N*-Dimethylation = [[$N(CH_3)_2$]/[H-2, H-3, H-4, H-5, H-6, H-6′] x 6/6] x 100
% *N*-Monomethylation = [[$N(CH_3)$]/[H-2, H-3, H-4, H-5, H-6, H-6′] x 6/3] x 100
% *O*-Methylation = [[$O(CH_3)$]/[H-2, H-3, H-4, H-5, H-6, H-6′] x 6/6] x 100

where [$N(CH_3)_3$], [$N(CH_3)_2$], and [$N(CH_3)$] were the integrals of the *N,N,N*-trimethyl- (δ 3.30 ppm), *N,N*-dimethyl- (δ 2.87 ppm), and *N*-monomethylamino (δ 2.77 ppm) singlet peaks, respectively. [$O(CH_3)$] and [$C=O(CH_3)$] were the integrals of the *O*-methyl (δ 3.35 ppm, for O^3-CH_3 and 3.43 for O^6-CH_3) singlet peaks, respectively (Rúnarsson, et al., 2007). The degree of substitution is given in percentage.

2.3.2 *Fourier transformed infrared (FTIR) spectroscopy*

Fourier transformed infrared (FTIR) spectra were acquired over the range of 500 – 4000 cm^1 on a Shimadzu IRPrestige-21 spectrophotometer (Shimadzu, Japan) with Shimadzu IRsolution software version 1.40. For IR analysis, the samples were dispersed in a matrix of powdered potassium bromide (KBr) by gentle grinding using a mortar and pestle.

2.3.3 *Molecular weight*

The absolute molecular weights of the synthesised TMC polymers were measured with a size exclusion chromatography (SEC) (Hewlett Packard 1100, USA) connected to a multi-angle laser light scattering (MALLS) detector that consisted of a laser photometer (Dawn DSP, Wyatt Technology Corporation, USA) coupled to a refracting index detector (ERC 7515A, Japan).

The TMC polymers were dried in a vacuum oven (at 40 °C) for 24 h and prepared in solutions of 5 mg/ml from which 0.8 ml samples were filtered through 0.2 µm membrane filters and collected in chromatographic sample vials. The mobile phase consisted of a 0.2 M ammonium acetate solution and the pH was adjusted to 4.50 with acetic acid. The experimental set-up consisted of an HP 1100 vacuum degasser, isocratic pump and auto-sampler connected to an inline TSK-guard PWH (Tosoh, Japan) inline column. The size exclusion columns included a TSKgel GMPWxl (Tosoh, Japan; inside diameter = 7.8 mm, length = 30 cm, particle size 13 µm, pore size 100 - 1000 Å) column connected in series with a TSKgel G5000PWxl (Tosoh, Japan; inside diameter = 7.8 mm, length = 30 cm, particle size = 10 mm, pore size = 1000 Å) column. Samples of the TMC solutions (100 µl) were injected at a flow rate of 1.0 ml/min and were analysed with the DAWN® DSP laser photometer (Wyatt Corporation, Santa Barnara, CA) and a refracting index detector. The data from the detector were interpreted with a computer using Astra® for Windows version 4.73 (Wyatt Technology Corporation, USA).

This intensity of scattered light is known as turbidity, τ, which is related to concentration, *c*, by the following expression (equation 1)

$$\tau = HcM_w \qquad (1)$$

where

$$H = \frac{32\pi^3}{3}\frac{n_0^2(\frac{dn}{dc})^2}{\lambda^4 N_0} \qquad (2)$$

and n_0 is the solvent's refractive index, λ is the wavelength of the incident light, and N_0 is Avogadro's number. The d_n/d_c expression is referred to as the specific refractive increment. It is obtained by measuring the slope of the refractive index as a function of concentration and it remains constant for a given polymer, solvent and temperature.

In the determination of the weight-average molecular weight (M_w), measurements from the intensity of scattered light from a light source (mercury arc lamp or laser) at different concentrations and angles (θ), usually 0, 45, 90 and 135 °, are taken.
To determine M_w, the expression for turbidity can be rewritten as

$$\frac{Hc}{\tau} = \frac{1}{M_w P(\theta)} + 2A_2 c \qquad (3)$$

where P(θ) is the function of the angle, θ, at which turbidity is measured, A_2 is second virial coefficient and c is the sample concentration. This angle is highly depended on the shape of the polymer molecules in the solution (Carraher, 2000, Stevens, 1999).

3. Results and discussion

3.1 Degree of quaternisation

Nuclear magnetic resonance (NMR) spectroscopy is the most accurate and effective method to determine the structure of chitosan derivatives (Loubaki, et al., 1991, Luo, et al., 2010). The degree of substitution was determined using the equations as described by Rúnarsson and co-workers (2007). Figure 1 shows the ^{1}H-NMR spectrum of the TMC (TMCCM) synthesised utilising the conventional method (Hamman, et al., 2002, Hamman, et al., 2003, Kotzé, et al., 1998, Kotzé, et al., 1999, Sieval, et al., 1998, Snyman, et al., 2002, Thanou, et al., 2000). It reflected a 62.86 % degree of *N,N,N*-trimethylation, 14.28 % *N,N*-dimethylation, 9.52 % *N*-monomethylation and 33.33 % *O*-methylation. The TMC (TMCMWR) synthesised by means of microwave irradiation, showed degrees of quaternisation which ranged from 2.90 % to 64.07 % as reflected in Table 1. When comparing the TMCCM with the TMCMWR polymer (Figure 2) with almost the same degree of quaternisation, it was observed that the TMCMWR had a slightly higher degree of *O*-methylation (45.45 %), but a lower degree of *N,N*-dimethylation (2.06 %) and virtually no *N*-monomethylation.

3.2 FTIR

The presence of methylation is the most evident in the region 1200 – 1700 cm^{-1} of the TMC IR spectra. The regions of interest are as follows:

I. At about 1475 cm^{-1}: TMC exhibits a peak which is not present in the IR spectra of chitosan. This is attributed to the asymmetric angular deformation of the C-H bonds of the methyl groups.
II. The chitosan indicated a band which stretched from 1500 – 1620 cm^{-1}. This is due to the angular deformation of the N-H bond of the amino groups. Both the TMCCM and TMCMWR also exhibited this band, but it is weaker due to the occurrence of N-methylation.

Table 1: Synthesised polymers

Polymer	Reaction time (min.)[#]	Reaction Steps*	Energy (Watt)[$]	TM (%)	DM (%)	MM (%)	OM (%)	M_w (SD %)
Chitosan	N/A	N/A	N/A	N/A	N/A	N/A	N/A	2.25E+05 (1.28 %)
TMCCM	120	2	----	62.86	14.28	9.52	33.33	8.52E+04 (1.57 %)
TMCMWR1	15	1	100	6.67	10.91	65.45	2.73	1.88E+05 (0.87 %)
TMCMWR2	30	1	100	2.90	11.30	8.70	1.74	5.37E+04 (2.55 %)
TMCMWR3	15 + 15 = 30	2	100	64.07	2.60	0.00	45.45	2.04E+05 (0.87 %)
TMCMWR4	30 + 30 = 60	2	100	31.44	44.34	18.87	14.15	1.42E+05 (1.43 %)
TMCMWR5	30 + 30 = 60	2	130	14.17	45.06	10.08	14.80	7.73E+04 (1.47 %)
TMCMWR6	15	1	130	17.70	48.58	22.01	10.05	7.19E+04 (1.87 %)
TMCMWR7	30	1	130	23.30	85.69	29.49	34.95	2.95E+04 (0.40 %)
TMCMWR8	15+ 15 = 30	2	130	13.84	32.08	7.54	5.66	2.48E+05 (1.42 %)

#Reaction time: The time required to obtain the specific TMC, indicated in minutes.

***Reaction steps**: The amount of steps required to obtain the specific TMC.

$Energy: The energy used during the microwave reaction method to obtain the specific TMC, indicated in watts. This is only applicable to the microwave irradiation method.

TM: *N,N,N*-trimethylation

DM: *N,N*-dimethylation

MM: *N*-monomethylation

OM: *O*-methylation

M_w: Weight-average molecular weight of the respective TMC polymers in g/mol.

SD %: Standard deviation percentage

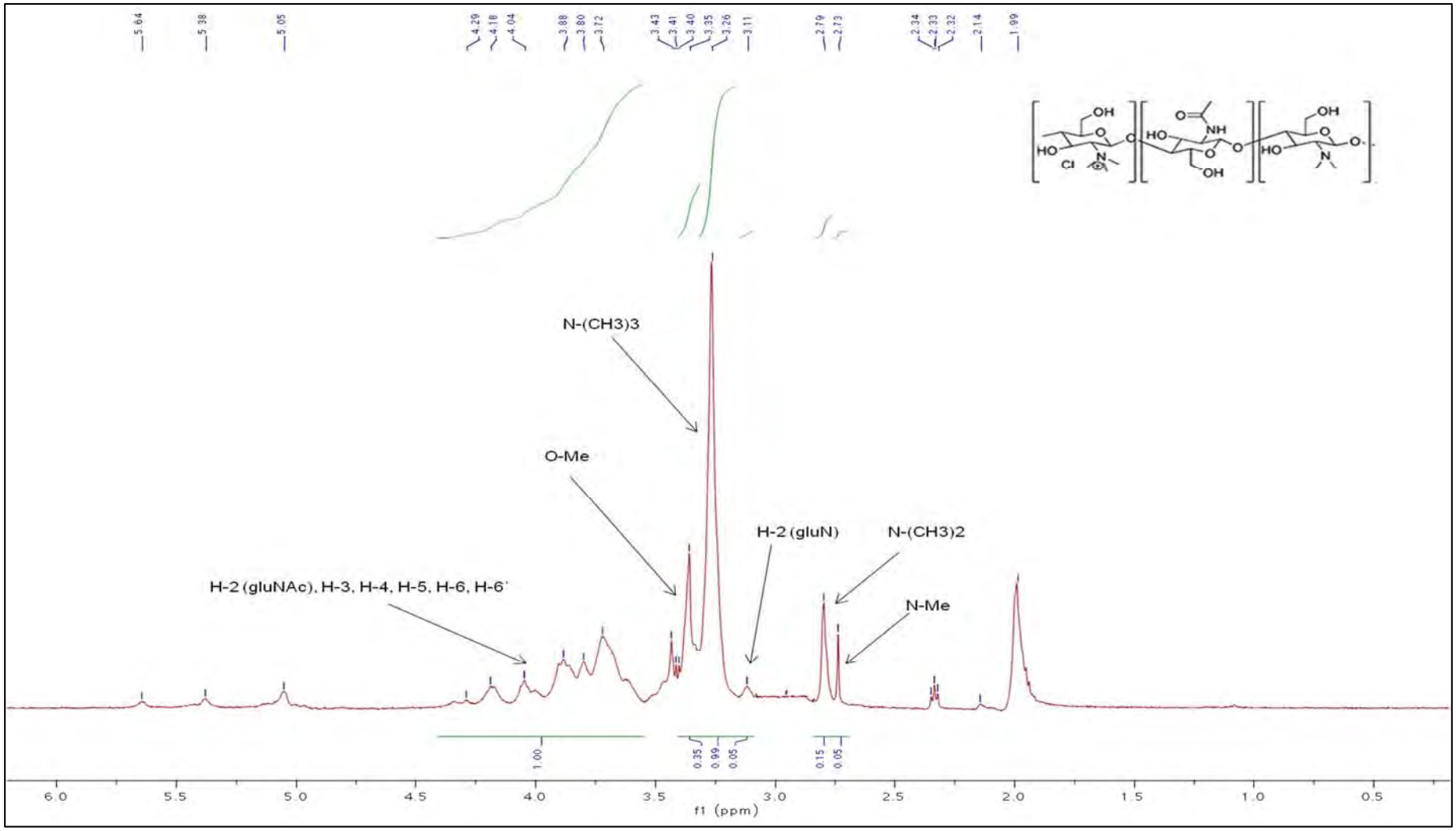

Figure 1: ^{1}H-NMR spectrum of the TMC (TMCCM) synthesised utilising the conventional method with a 62.86 % degree of quaternisation.

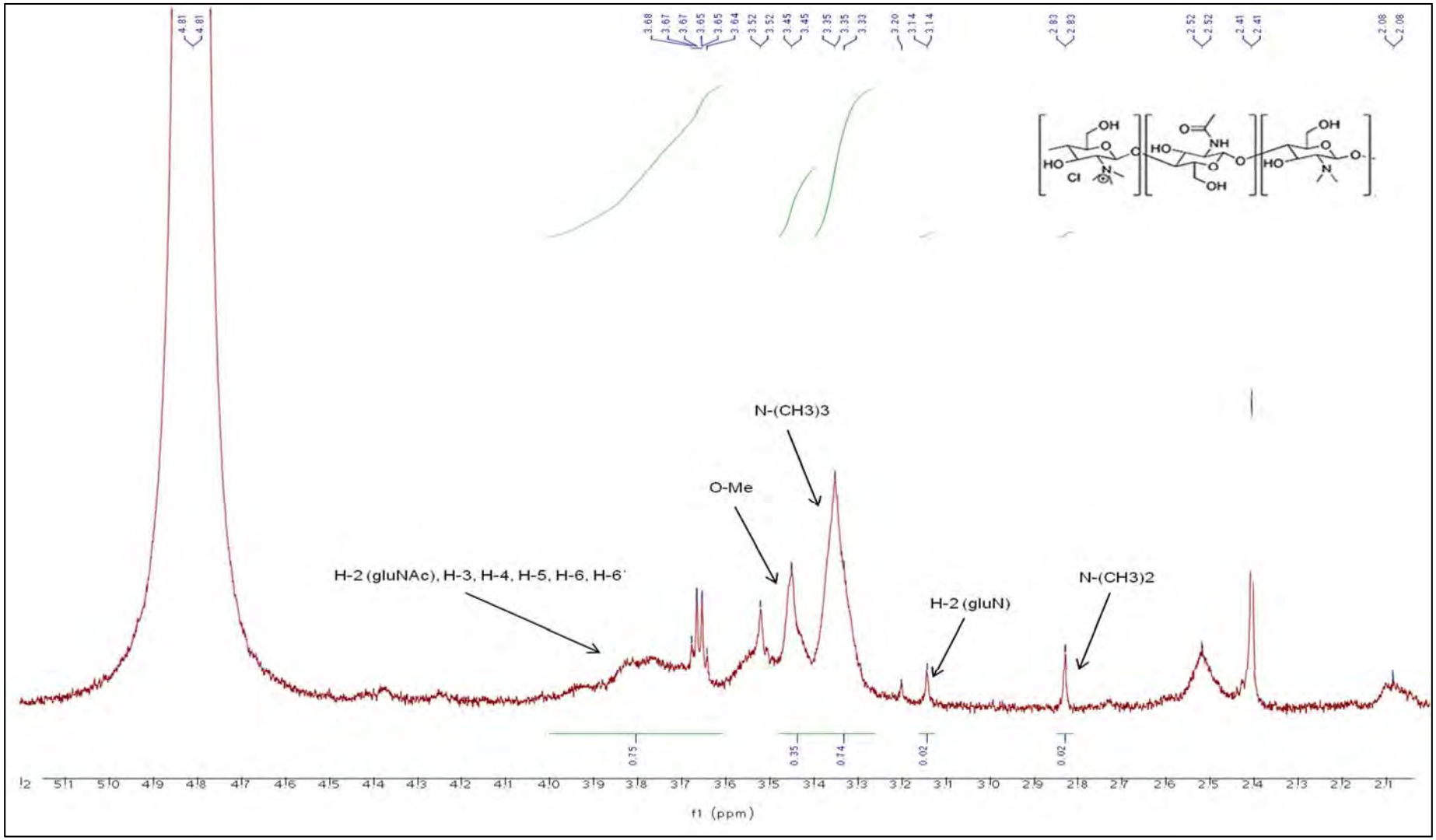

Figure 2: ^{1}H-NMR spectrum of the TMC (TMCMWR3) synthesised utilising the microwave irradiation method with a 64.07 % degree of quaternisation.

The alcohol groups of chitosan have characteristic peaks between 1030 and 1160 cm^{-1}. When a change occurs in these peaks, it is indicative of the introduction of alkyl groups at C-3 and C-6 (Geisberger, et al., 2013, Mourya and Inamdar, 2009). This is also known as *O*-methylation. Both the TMCCM and TMCMWR displayed a change in the aforementioned peak.

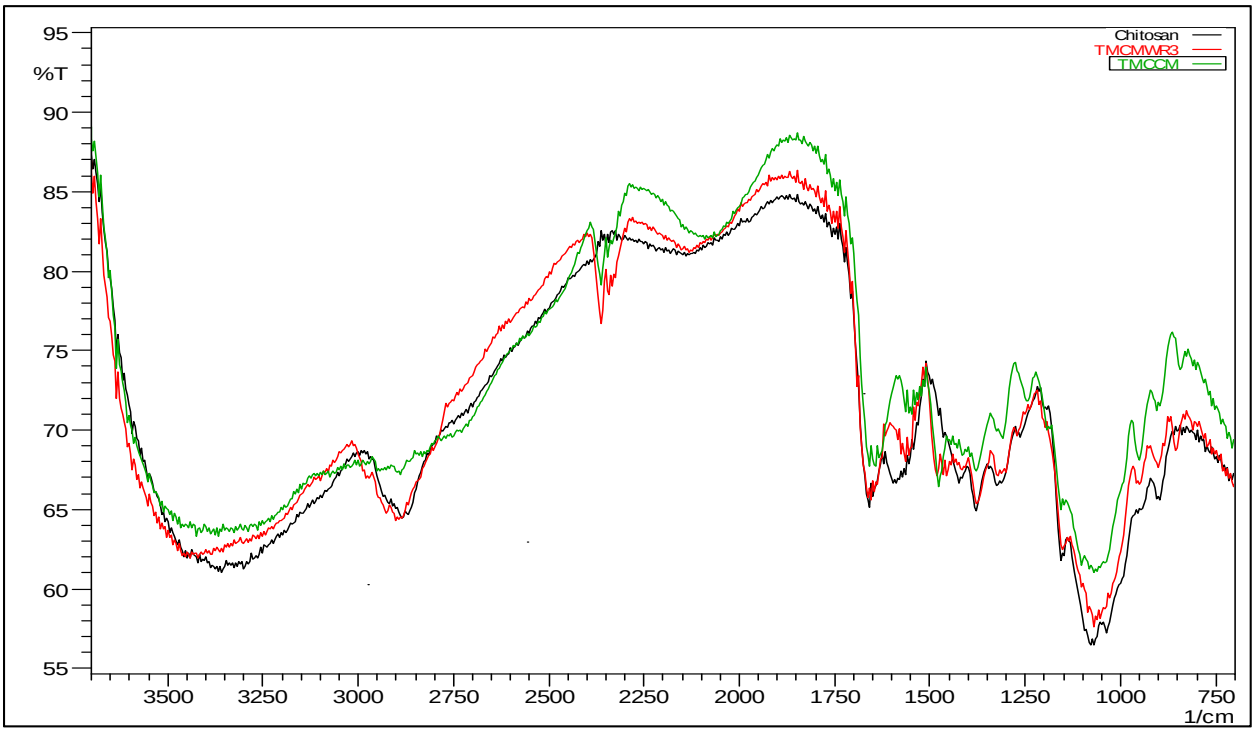

Figure 3: Complete overlay FTIR spectra of chitosan, TMCCM and TMCMWR3.

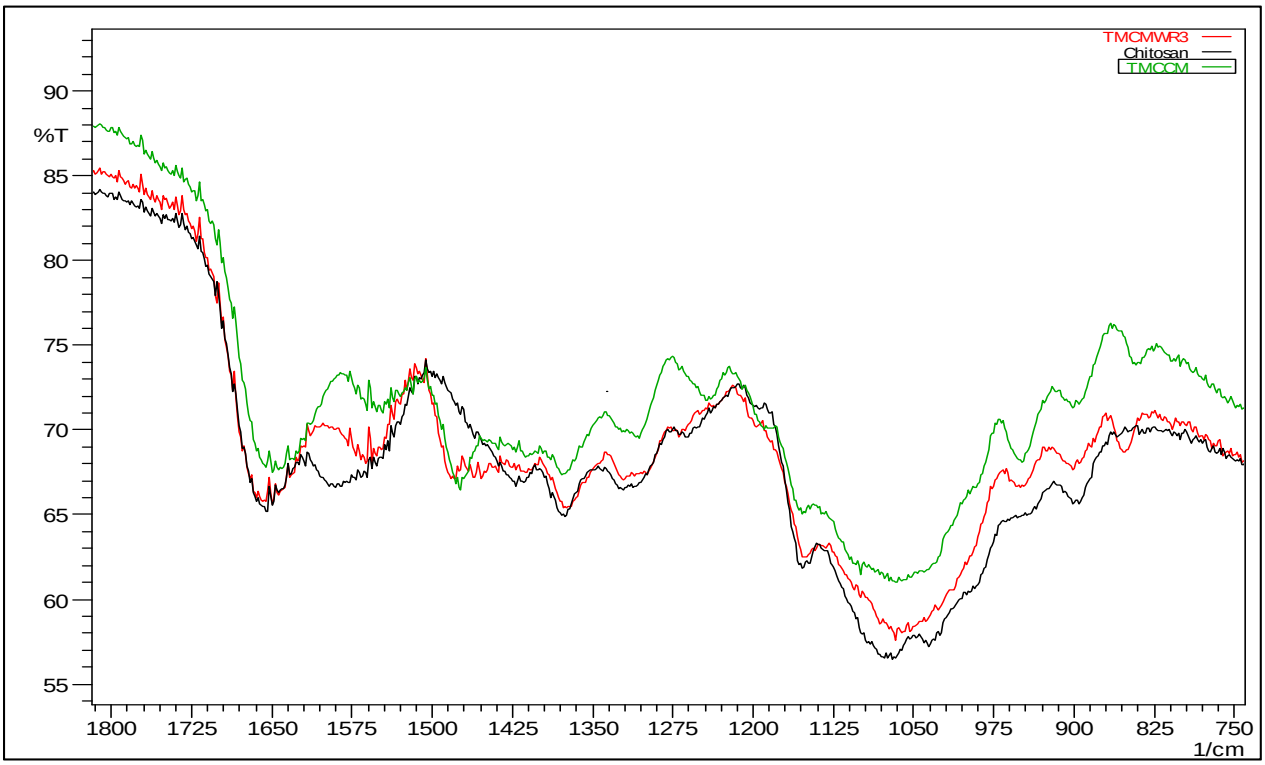

Figure 4: Condensed overlay FTIR spectra of chitosan, TMCCM and TMCMWR3 to better illustrate the importantchanges in the different regions.

3.3 Molecular weight

A summary of the results obtained with the SEC/MALLS characterisation of the TMC polymers is presented in Table 1. As proven in previous research (Snyman, et al., 2002), the weight-average molecular weight of the TMCCM polymer, as determined with SEC/MALLS, decreased significantly from the weight-average molecular weight of the starting material (chitosan). Interestingly, the expected trend of a decrease in weight-average molecular weight with an increase in the degree of quaternisation was not observed with the TMCMWR polymers due to possible less degradation of the polymer chain. The TMCMWR3 polymer, which exhibited a similar degree of quaternisation (64.07 %) as the TMCCM polymer (62.82 %), showed remarkably less degradation (52.8 %) of the weight-average molecular weight than the TMCCM polymer. Based on the results in Table 1 it is evident that the energy of the reaction and exposure time had the most prolific effect on the weight-average molecular weight. The TMCMWR polymers exposed to the 130 watt irradiation energy exhibited a greater decrease in weight-average molecular weight than the polymers that were synthesised with 100 watt irradiation energy. From the results in Table 1, it is obvious that the reactions with 2 steps, 15 minutes each (30 minutes), showed the least amount of weight-average molecular weight degradation. TMCMWR8 showed an increase in weight-average molecular weight. This could be attributed to very little polymer chain degradation and more substitution to these bigger polymer units.

4. Conclusion

Various TMC polymers were successfully synthesised by utilising microwave irradiation with different reaction conditions. These TMC polymers were characterised by means of NMR and FTIR to determine if the structure reflected similar character traits as those of TMC polymers which were synthesised by the conventional heating method. The TMCMWR polymers reflected similar spectra compared to the spectra of TMCCM, which are widely published in various journals. Furthermore, a 64.07 % degree of quaternisation was obtained for one of the TMCMWR polymers in only 30 minutes compared to the 120 minutes required to obtain 62.86 % when using the conventional heating method. The TMCMWR3 polymer showed a slight increase in O-methylation when compared to the TMC prepared by means of the conventional heating method. On the other hand, the TMCMWR3 polymer reflected more selectivity for *N,N,N*-trimethylation than the corresponding TMCCM due to the fact that TMCCM exhibited considerately more *N,N*-dimethylation and *N*-monomethylation than the TMCMWR3 polymer. The weight-average molecular weight was determined by the SEC/MALLS characterisation. The TMCCM showed far more degradation in the molecular weight as the corresponding TMCMWR polymers. This can be attributed to the heat exposure during the reaction over a prolonged period of time, whereas the TMCMWR polymers are exposed to reaction energy for a much shorter period of time. The reaction energy proved to be an important factor which contributed to the degradation of the molecular weight of the TMCMWR polymers. As with the conventional method, TMCMWR polymers synthesised at 100 watt reaction energy, showed an increase in the degree of quaternisation with an increase in the number of reaction steps. This trend was not observed with the TMCMWR polymers which was synthesised at 130 watt irradiation energy.

Microwave irradiation energy proved to be a better alternative to the conventional heating method when synthesising TMC. The same degree of quaternisation was obtained with this newly developed method in a quarter of the time that was needed when using the conventional method. The TMCMWR polymers also exhibited less degradation of the polymer structure, thus limiting the chance for the formation of any unwanted by-products. This method is also easy to tailor to achieve the desired degree of quaternisation. In these modern times where time is money and saving the environment is of the at most importance, this method of TMC synthesis might hold the key by drastically reducing reaction time and energy used. At the moment only small quantities could be synthesised by utilising the microwave irradiation method. To make it a practical substitution for the conventional method, the method needs to have the capability to be scaled up in order to be used in mass production of TMC. Thus, further future research will be needed to determine the possibility of up-scaling and to better the method even more.

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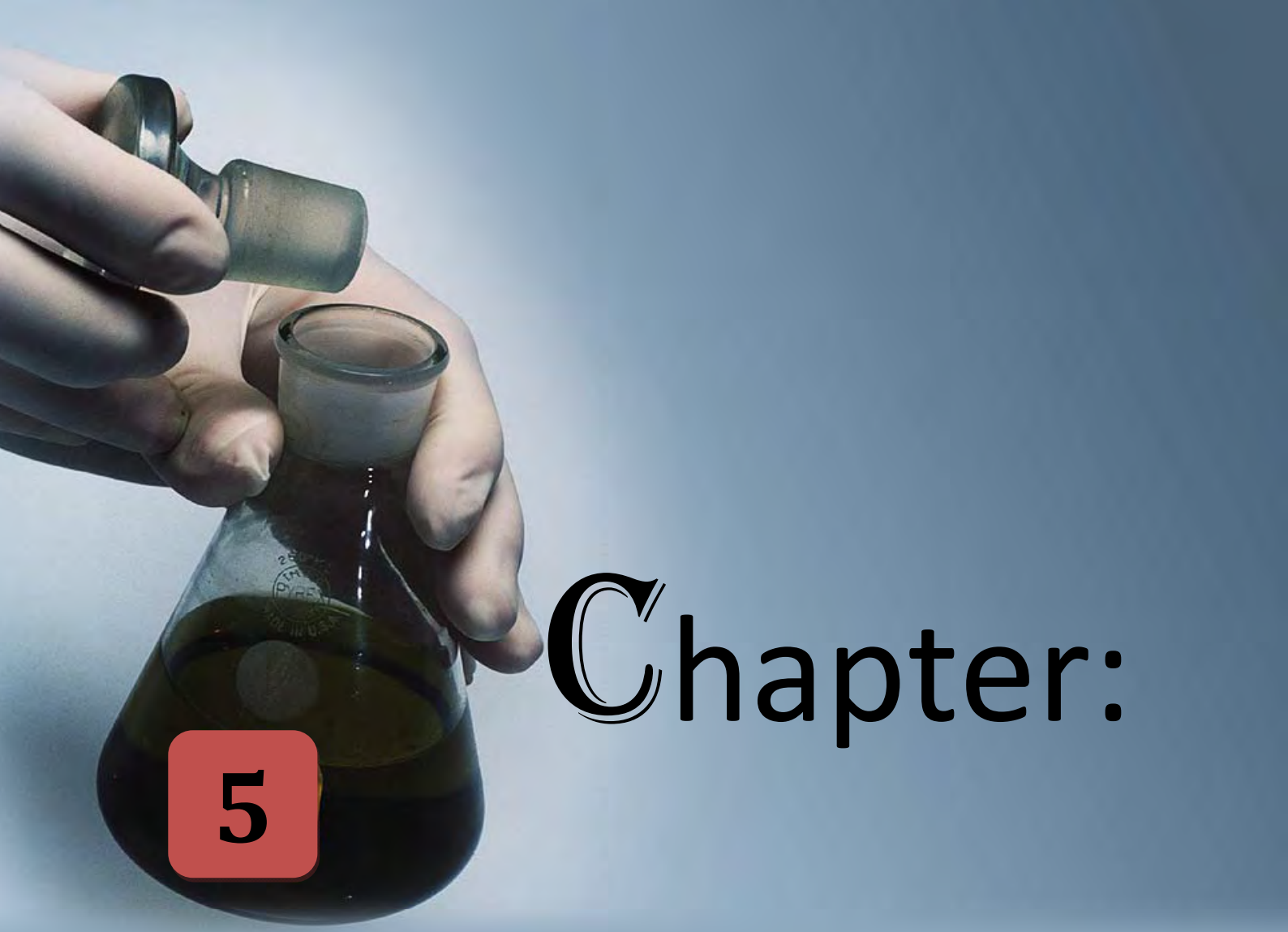

Chapter: 5

This chapter is the conclusion as concluded from the all research done during this study as well as possible further investigation that can also be conducted is mentioned.

1. Conclusion

The burden of malaria is growing every year with it being the cause of hundreds and thousands of deaths (WHO, 2013). With the lack in global funding and the increase of the malaria parasite's resistance to current treatment, emphasis is placed on the need for better, safer and cheaper treatment. One such method will be to concentrate efforts on the reformulation of current or previous anti-malarial drug combinations. Antifolates are one of the oldest malaria chemotherapy choices together with chloroquine. Antifolates, including the combination sulphadoxine-pyrimethamine, are still being used effectively in intermittent preventative treatment programmes (Müller & Hyde, 2013; Nzila, 2006). However, resistance to antifolates are high and it is not considered first line therapies. Due to the increase in resistance to single drug therapy, combination therapy has been developed. Dapsone and chlorproguanil were previously used in a combination that was known as Lapdap®. Lapdap® has been shown to exhibit less selection to resistance than the other widely used antifolate combinations of sulfadoxine and pyrimethamine (Nwaka *et al.*, 2004) and more potency against malaria (Nzila-Mounda *et al.*, 1998; Winstanley *et al.*, 1995), but was wathdrawn from the market in 2008 due to a serious and lethal side effect in people with a deficiency of the enzyme glucose-6-phosphate dheydrogenase (G6PD). This creates the opportunity for new and alternative research to be done to reduce the toxicity of this treatment by altering it in some way because of the significant potential that this combination has.

A strategy to combat the malaria parasite's resistance, thus prolonging the activity and efficacy of future developed drugs, is to design in advance drug delivery systems that consist of biomaterials (Movellan *et al.*, 2014). Previous such stratagies included incorporating antimalarial drugs like mefloquine in lipid based formulations such as liposomes and Pheroid™ (Slabbert *et al.*, 2011). However, the liposomes posed poor modular chemical functionality and relatively weak stability. To overcome these challenges, polymer based formulations were identidied as alternative drug delivery systems. Their larger molecular mass and structural chemical functionality give them advantages over lipid based drug delivery systems (Le Meins *et al.*, 2013).

Chitosan is a amino-polysaccharide that is obtained when chitin, found in the crustaceans' exoskeletons, is deacetylated (Kumar *et al.*, 2004; Park *et al.*, 2010). Chitosan's practicality is, however, limited due to its insolubility at physiological pH (7.4) thus limiting its uses in pharmaceutical and biomedical applications. To overcome this problem, *N*-trimethyl chitosan chloride (TMC), was synthesised by reductive methylation (acetylation) of the amino groups on the C_2 position of chitosan (Hamman *et al.*, 2003). TMC shows good water-solubility across a wide pH range thus having mucoadhesive properties and excellent absorption enhancing effects even at neutral pH (Amidi *et al.*, 2010; Hamman *et al.*, 2003; Kotzé *et al.*, 1998). The formation of complexes between chitosan-based polymers (like TMC) and oppositely charged macromolecules has been studied extensively by many researchers and it was found that this property can be exploited to prepare micro- and nanoparticles that are suitable for drug delivery (Chen *et al.*, 2008; Geçer *et al.*, 2010; Prego *et al.*, 2010; Sandri *et al.*, 2007; Sayin *et al.*, 2008). These particles are prepared by ionic cross-linking (ionotropic gelation) through self-assembly of chitosan or its

derivatives, which is positively charged, and oppositely charged macromolecules by the addition of a low molecular weight anionic cross-linker, such as tripolyphosphate (TPP) (Amidi *et al.*, 2010). Taking all of this research into account, it is becoming apparent that chitosan-based particles have a big role to play in drug delivery thus showing the potential and importance of these particles.

In this study, a specific research strategy was followed to reach certain aims. These aims included:

- The formulation of TMC-TPP microparticles.
- The entrapment of dapsone and proguanil in these microparticles.
- Optimization of methods to determine physicochemical properties.
- Determination of size, pH, entrapment efficacy and loading capacity of the formulations.
- *In vitro* dissolution study of the drugs entrapped in the TMC-TPP microparticles to determine the release profile.
- *In vivo* efficacy study to determine the efficacy of the drugs entrapped in the TMC-TPP microparticles when compared to the plain drug's efficacy.
- *In vivo* bioavailability (pharmacokinetic) study to determine whether the drugs are released, absorbed and available in the body.

In the succeeding paragraphs, the aims will be discussed with results as obtained during the study, as well as possible further investigation.

There are many challenges remaining today, not the least of which is creating particles that can enhance gastrointestinal uptake. The particle size dictates the types of applications for which particles can be used. Microparticles have some advantages over nanoparticles. Some nanoparticles may cause toxic effects on living systems (Bell *et al.*, 2013) and they are more difficult to prepare. Larger particles, like microparticles, will tend to stay where placed and have a local effect, while smaller ones, like nanoparticles, may be taken away by white blood cells or travel away by themselves (Kohane *et al.*, 2002; Kohane *et al.*, 2006). Therefore, microparticles might theoretically have longer contact with the intestinal wall. Even though microparticles will not cross most biological barriers, TMC is a proven absorption enhancer and which will allow the entrapped drug to pass through the epithelial membrane and enter the systemic circulation.

TMC-TPP microparticles were successfully synthesised and characterised as described by previous researchers (Amidi *et al.*, 2006; Chen *et al.*, 2007; Chen *et al.*, 2008; Geçer *et al.*, 2010; Sandri *et al.*, 2007). The synthesis method was optimised by making use of a factorial experimental design. The TMC:TPP ratio was changed for the different formulations by using three different ratios namely, 2:1, 3:1 and 5:1. Other variables were the absence or addition of Tween 80 and whether the formulations were to be subjected to sonification or not. Results varied with the average particle size ranging from 18.56 µm ± 0.72 to 158.35 µm ± 9.49. The optimal formula was chosen on the basis of which method produced the smallest particles and this was achieved when the TMC and TPP were cross-linked in a fixed ratio of 3:1 (TMC:TPP), in the presence of Tween 80 and subjected to ultrasonification using a tip sonicator. This

formulation had zeta potential of 21.83 mV ± 0.55 which is still sufficient to fully stabilise the system in combination with steric stabilisation (Müller, 1996) and produce a stable suspension. Their particle size and zeta potential were measured for both dapsone (46.21 µm ± 0.86, 7.17 mV ± 2.26) and proguanil (74.56 µm ± 3.81, 15.70 mV ± 0.70) TMC microparticles. This indicated that the particle size increased due to the drug that was incorporated with the TMC particles. The significant decrease in zeta potential can be attributed to adsorption of the drugs on the particle surface, thus masking the surface charge of the TMC particle (Harivardhan Reddy & Murthy, 2004). ESEM micrographs were taken of the microparticles and it could clearly be seen that the particles have an amorphous shape as opposed to spherical particles found in other studies (Geçer *et al.*, 2010; Mitra *et al.*, 2001; Prego *et al.*, 2010; Sandri *et al.*, 2007; Xu *et al.*, 2003). The drugs appeared to be entrapped inside the particles, but due to the decrease in zeta potential, it is indicative that some of the drug is adsorbed to the surface of the particles.

Loading efficiency (LE) and loading capacity (LC) of dapsone and proguanil in TMC-TPP microparticles were analysed by ultraviolet spectrophotometry. Samples were compared to standard curves of dapsone and proguanil. Standard solutions with known concentrations of dapsone and proguanil in a 50 % *v/v* methanol were prepared and the absorbance was measured at 260.6 and 295.6 nm (dapsone) and 259.0 nm (proguanil). The calibration curve was determined by plotting the peak absorbance against concentration and fitted with linear regression. Loading efficiency (LE) and loading capacity (LC) for both drugs were calculated as described by the referenced equations (Amidi *et al.*, 2006; Chen *et al.*, 2007). The experiments were performed in triplicate. The dapsone TMC particles had a loading efficiency of 50.72 ± 0.14 % and a loading capacity of 58.57 ± 0.16 % whereas the proguanil TMC particles had a loading efficiency of 44.20 ± 1.45 % and a loading capacity of 50.16 ± 1.64 %. This correlated with previous research done by Chen and co-workers (Chen *et al.*, 2008) where they entrapped bovine serum albumin (BSA) in TMC-TPP particles, through which nearly the same loading capacity (44.77 ± 0.76 %) was obtained when compared to this study.

In vitro dissolution study of the drugs entrapped in the TMC-TPP microparticles was performed to determine the release profiles. The dissolution profiles for the dapsone-TMC and proguanil-TMC microparticles were plotted against each other and it was clear when observing that the two formulations released their drug content at a pH of 3 with different release profiles. The difference in release profiles may be attributed to the different zeta potential for both formulations. The formulations seem to have a sustained release since only 73.00 ± 1.70 % (dapsone) and 55.00 ± 1.90 % (proguanil) were released after 150 minutes. Previous studies utilising chitosan-TPP based particles also indicated sustained release (Prego *et al.*, 2010; Xu *et al.*, 2003).

In vivo efficacy study to determine the efficacy of the drugs entrapped in the TMC-TPP microparticles when compared to the plain drug's efficacy was performed. When compared to the controls, both the normal and TMC formulations indicated better *in vivo* efficacy. The parasitaemia was kept under control up until day 11 where after it increased significantly. The normal dapsone and proguanil formulations reflected parasitaemia which ranged from 0.00 % - 45.48 % and 0.00 % - 50.29 % respectively. The

normal combination resulted in parasitemia of 0.00 % - 5.41 %. The control formulation's (DMSO/PBS) parasitaemia ranged from to 0.00 – 51.47 % over the duration of the study and reflected a mean parasitaemia of 24.06 %. The dapsone-TMC and proguanil-TMC formulations reflected parasitaemia which ranged from 0.00 % - 39.46 % and 0.00 % - 37.93 % respectively. The combination TMC formulation resulted in parasitemia of 0.00 % - 42.10 %. The other control formulation's (TMC) parasitaemia ranged from 0.00 – 41.34 % over the duration of the study and reflected a mean parasitaemia of 23.43 %. As far as we know, this is the first antimalarial combination which was entrapped in TMC-TPP microparticles and of which the *in vivo* efficacy was tested.

The plain dapsone formulations showed a sharp increase in parasitaemia on day 3 when compared with the more gradual increase in parasitaemia of the dapsone TMC formulation. Overall the dapsone TMC formulation (13.51 %) indicated a better reduction in the mean parasitaemia than the normal dapsone formulation (17.14 %). Both these dapsone formulations exhibited a statistically significant difference ($p < 0.0001$) when compared to the control formulation.

Both the proguanil formulations reflected almost identical parasitaemia values up until day 18 where the proguanil TMC formulation had a significantly lower parasitaemia (37.93 %) than its normal formulation counterpart (50.29 %). Overall the proguanil-TMC formulation (18.11 %) indicated a slightly better reduction in the mean parasitaemia than the normal proguanil formulation (19.30 %). The proguanil formulation ($p = 0.0232$) did not show any statistically significant difference but the proguanil-TMC formulation ($p < 0.0001$) showed statistically significant differences when compared to the control formulation (TMC).

Both the combination formulations showed better efficacy when compared to the control formulations. During the study, all of the test subjects that received the normal combination formulation died after 11 days but the test subjects that received the combination TMC formulation survived until day 18, and thus no mean parasitaemia could be determined for the subjects that received the normal combination formulation. The mean parasitaemia for the combination TMC formulation was 16.85 % which is lower than the control formulation's (TMC) 23.43 % mean parasitaemia, thus indicating to have an *in vivo* efficacy. The combination TMC formulation indicated statistically significant differences ($p < 0.0001$) when compared to control formulation. These results clearly indicated that the TMC formulations were more effective against malaria than their plain formulation counter-parts. Thus it can be concluded that the TMC microparticles enhance the efficacy of the antimalarial drugs dapsone and proguanil.

The pharmacokinetic properties of these TMC microparticles were determined by performing an *in vivo* bioavailability study. The *in vivo* bioavailability of the dapsone TMC formulation (3 mg/kg oral dose; equivalent to 1.5 mg/kg dapsone) relative to the normal dapsone formulation (3 mg/kg oral dose) was found to be 244 % (n=5). The dapsone TMC microparticles were absorbed faster, i.e. the maximum concentration was attained 1 hour after the administration of the dose relative to that of the normal dapsone formulation that reached its maximum concentration after 2 hours. For the proguanil TMC

formulation (16 mg/kg oral dose; equivalent to 8 mg/kg proguanil) relative to the proguanil formulation (16 mg/kg oral dose) bioavailability was found to be 123 % (n=5); whereas the bioavailability of cycloguanil obtained from 16 mg/kg of the proguanil-TMC formulation oral dose, was found to be 156 %. Both the proguanil formulations were absorbed quickly, with both formulations attaining the maximum formulation at 2 hours after dose administration. The *in vivo* bioavailability study indicated that both the dapsone- and proguanil-TMC formulations were more bioavailable when compared to the corresponding normal formulations. It can be concluded that by formulating dapsone and proguanil in TMC-TPP microparticles has enhanced the absorption of the drugs, thus resulting in better bioavailability. This was the first anti-folate drug combination, entrapped in TMC-TPP microparticles, of which the *in vivo* bioavailability was determined. A previous study where pyrimethamine, an anti-folate antimalarial drug, was entrapped in chitosan-TPP microparticles also indicated better *in vivo* bioavailability when compared to plain pyrimethamine (Tripathy & Roy, 2014).

In addition to the main study objective, a new synthesis method for TMC was developed by using microwave irradiation to determine whether the method can be optimised and if it is more beneficial than the conventional, well known method. Compared with routine heating treatment, microwave irradiation technology, as a more efficient heating method due to its rapid heating and energy penetration, is quickly emerging as an alternative energy source that is powerful enough to perform chemical reactions in minutes, instead of hours or even days (Luo *et al.*, 2010; Tharun *et al.*, 2013). Utilising a factorial design with three changing factors, namely microwave power (watt), reaction time (minutes) and number of reaction steps, various TMC polymers were prepared within a fraction of time compared to the conventional method. These newly microwave-assisted synthesised TMC was characterised using NMR, FTIR and SEC-MALLS. The TMC polymers synthesised by using microwave irradiation reflected the same spectra as those of TMCCM, which are widely published in various journals. Furthermore, a 64.07 % degree of quaternisation was obtained for one of the microwave irradiation synthesised TMC polymers in only 30 minutes compared to the 120 minutes required to obtain 62.86 % when using the conventional heating method. Microwave irradiation energy proved to be a better alternative to the conventional heating method when synthesising TMC. The same degree of quaternisation was obtained with this newly developed method in a quarter of time that was needed when using the conventional method. The microwave irradiation synthesised TMC polymers also exhibited less degradation of the polymer structure, thus limiting the chance for the formation of any unwanted by-products. This method is also easy to tailor to achieve the desired degree of quaternisation.

2. Future prospects

Recommendations to consider for future studies on dapsone-proguanil TMC-TPP particles:

- Further studies will be needed to determine the optimal reaction parameters when scaling up the TMC-TPP particles synthesis.
- The possibility to synthesise nanoparticles utilising the same method should be explored.

- Different polymer based particles, utilising TMC, should be synthesised, characterised and compared to the TMC-TPP microparticles.
- A structural stabilising agent like PEG could be introduced to form micro/nano spheres.

Recommendations to consider for future studies on the microwave assisted TMC synthesis method:

- At the moment only small quantities could be synthesised by utilising the microwave irradiation method. To make it a practical substitution for the conventional method, the method needs to have the capability to be scaled up in order to be used in mass production of TMC.
- To include more variables in the method in order to perfect and develop it further.
- Experiment with different solvents during the reactions, like using DMF in the place of NMP.

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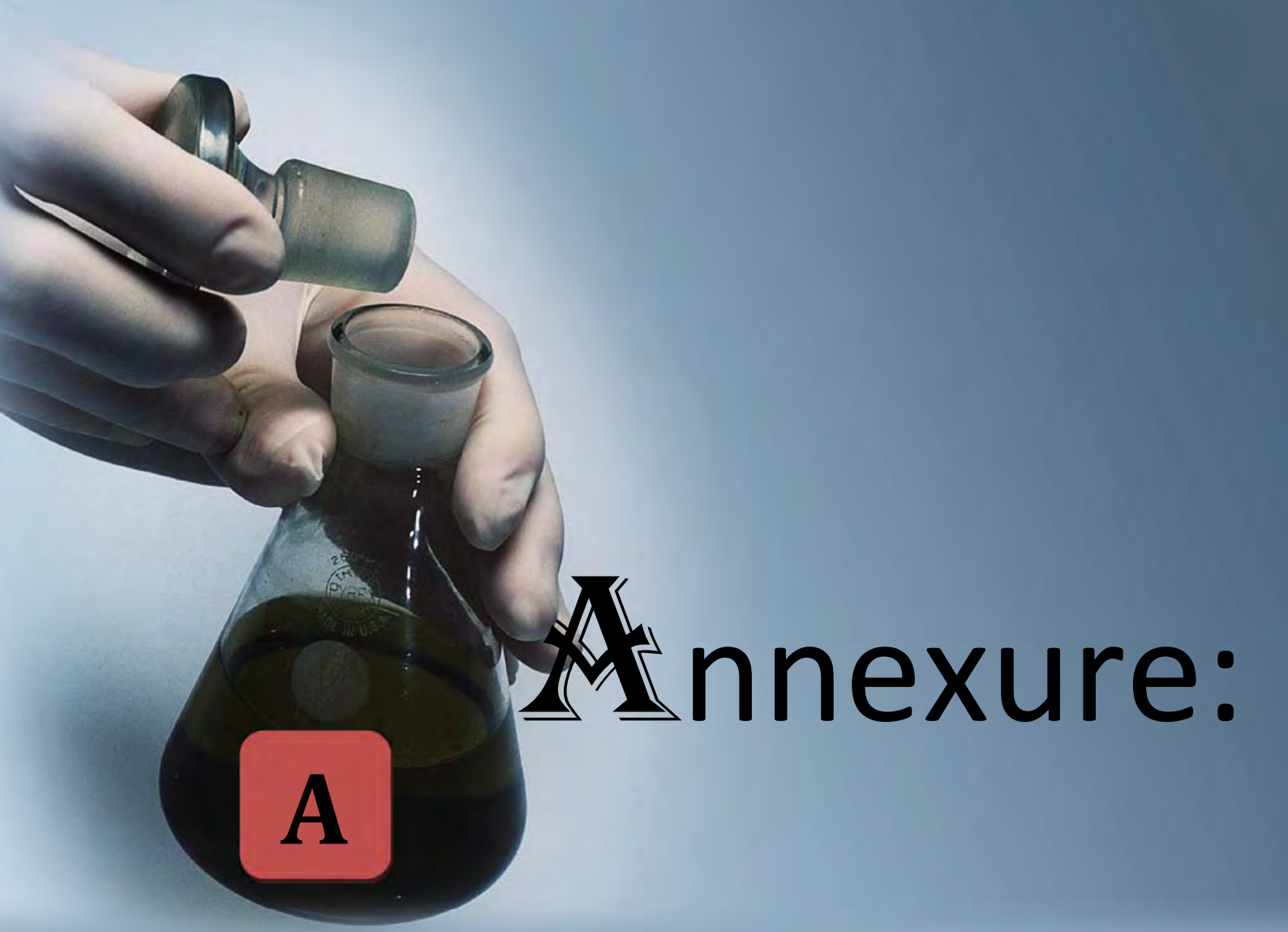


Annexure:

This annexure contains the calculations of the standard curves for dapsone and proguanil for analysis by UV.

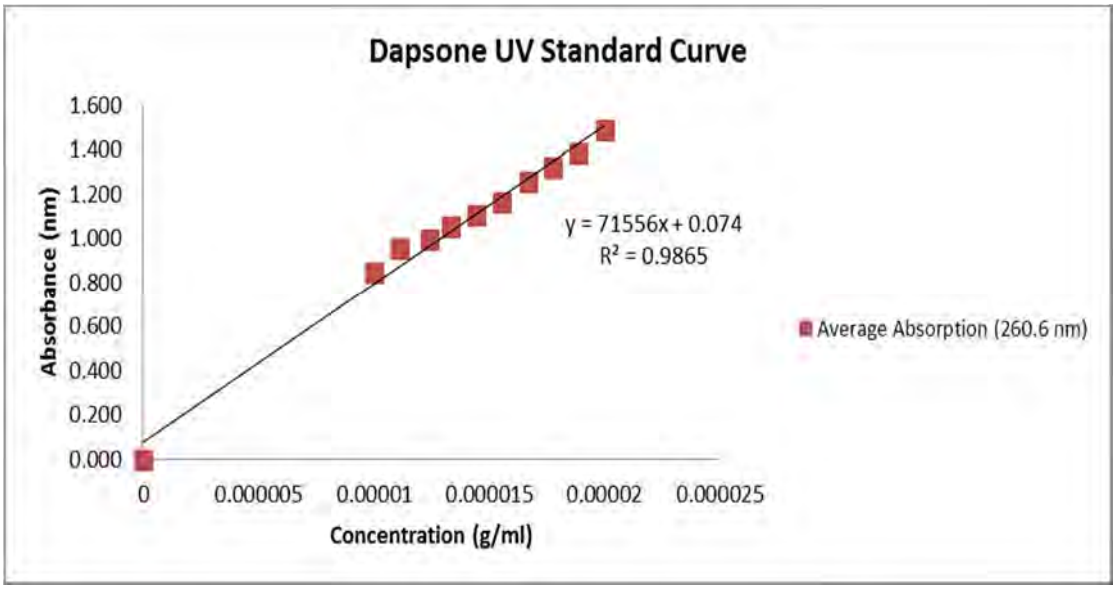
1. UV standard curve: Dapsone

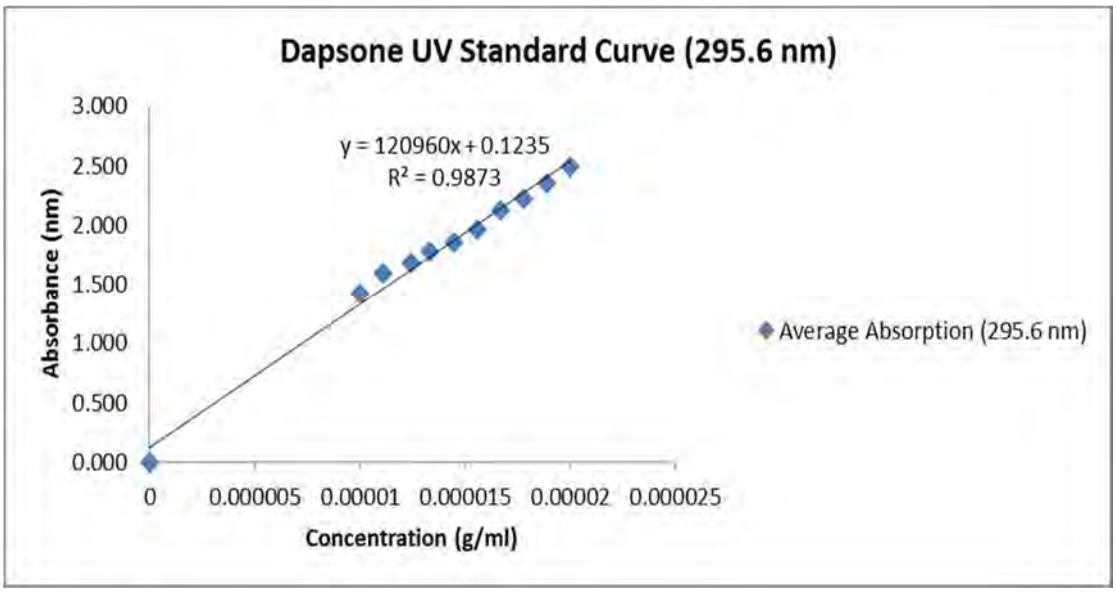
Stock solutions with different known concentrations.

Solution name	Description	Quantity to be dissolved	Unit	50% MeOH (ml)	Concentration (g/ml)
Stock solution	200 mg Dapsone in 50% MeOH	0.20	g	100	0.002
Dillution 1	50 µl of "stock solution" in 5ml 50% MeOH	50.00	µl	5	0.00002
Dillution 2	47.2 µl of "stock solution" in 5ml 50% MeOH	47.20	µl	5	0.00001888
Dillution 3	44.4 µl of "stock solution" in 5ml 50% MeOH	44.40	µl	5	0.00001776
Dillution 4	41.7 µl of "stock solution" in 5ml 50% MeOH	41.70	µl	5	0.00001668
Dillution 5	38.9 µl of "stock solution" in 5ml 50% MeOH	38.90	µl	5	0.00001556
Dillution 6	36.1 µl of "stock solution" in 5ml 50% MeOH	36.10	µl	5	0.00001444
Dillution 7	33.3 µl of "stock solution" in 5ml 50% MeOH	33.30	µl	5	0.00001332
Dillution 8	31.0 µl of "stock solution" in 5ml 50% MeOH	31.00	µl	5	0.0000124
Dillution 9	27.8 µl of "stock solution" in 5ml 50% MeOH	27.80	µl	5	0.00001112
Dillution 10	25.0 µl of "stock solution" in 5ml 50% MeOH	25.00	µl	5	0.00001

UV absorbance of the stock solutions at wavelengths of 260.6 nm and 295.6 nm.

Solution name	Absorption at 295.6 nm			Absorption at 260.6 nm			Average Absorption (295.6 nm)	Average Absorption (260.6 nm)	Concentration (g/ml)
	1	2	3	1	2	3			
Dillution 1	2.484	2.434	2.576	1.480	1.447	1.529	2.498	1.485	0.00002
Dillution 2	2.367	2.392	2.286	1.399	1.412	1.335	2.348	1.382	0.00001888
Dillution 3	2.259	2.138	2.286	1.339	1.266	1.353	2.228	1.319	0.00001776
Dillution 4	2.108	2.102	2.157	1.245	1.246	1.278	2.122	1.256	0.00001668
Dillution 5	1.929	1.204	2.005	1.139	0.710	1.186	1.967	1.163	0.00001556
Dillution 6	1.870	1.551	1.852	1.102	0.913	1.106	1.861	1.104	0.00001444
Dillution 7	1.823	1.721	1.810	1.078	1.017	1.070	1.785	1.055	0.00001332
Dillution 8	1.738	1.635	1.674	1.026	0.965	0.988	1.682	0.993	0.0000124
Dillution 9	1.594	1.573	1.637	0.943	0..931	0.969	1.601	0.956	0.00001112
Dillution 10	1.439	1.409	1.438	0.852	0.833	0.851	1.429	0.845	0.00001
Zero Concentration							0	0	0





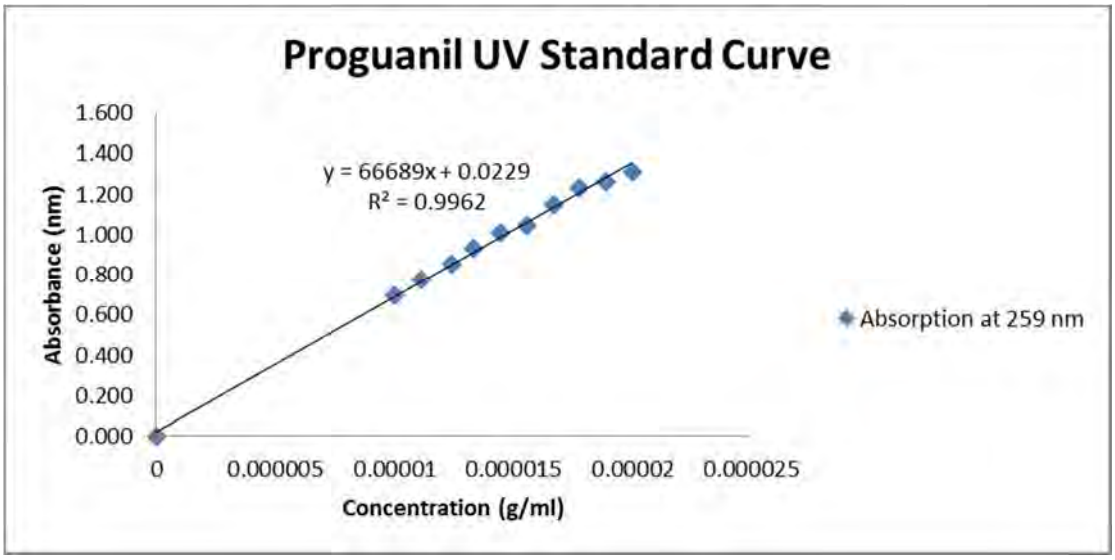
2. UV standard curve: Proguanil

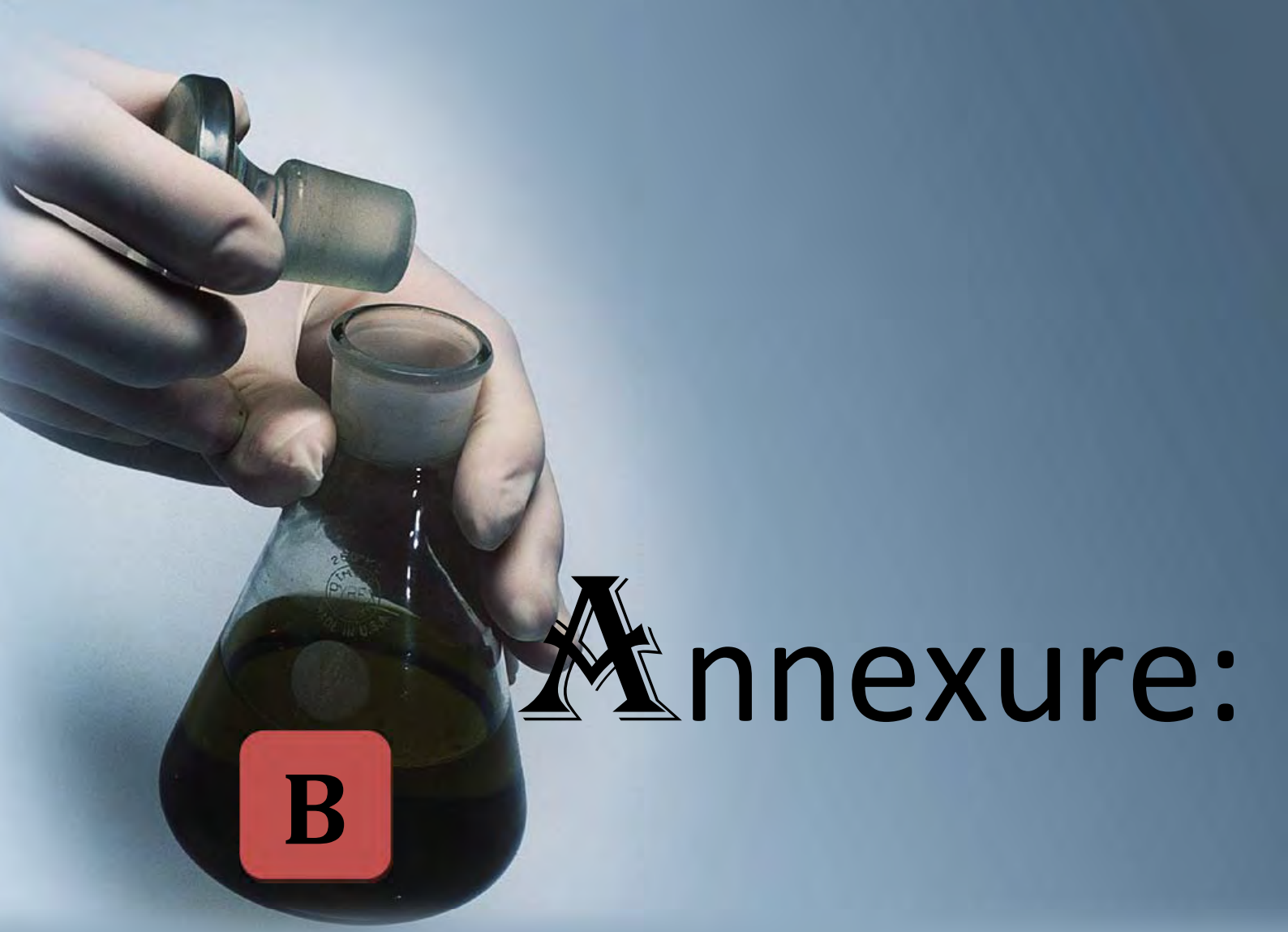
Stock solutions with different known concentrations.

Solution name	Description	Quantity to be dissolved	Unit	50% MeOH (ml)	Concentration (g/ml)
Stock solution	200 mg Proguanil in 50% MeOH	0.20	g	100	0.002
Dillution 1	50 µl of "stock solution" in 5ml 50% MeOH	50.00	µl	5	0.00002
Dillution 2	47.2 µl of "stock solution" in 5ml 50% MeOH	47.20	µl	5	0.00001888
Dillution 3	44.4 µl of "stock solution" in 5ml 50% MeOH	44.40	µl	5	0.00001776
Dillution 4	41.7 µl of "stock solution" in 5ml 50% MeOH	41.70	µl	5	0.00001668
Dillution 5	38.9 µl of "stock solution" in 5ml 50% MeOH	38.90	µl	5	0.00001556
Dillution 6	36.1 µl of "stock solution" in 5ml 50% MeOH	36.10	µl	5	0.00001444
Dillution 7	33.3 µl of "stock solution" in 5ml 50% MeOH	33.30	µl	5	0.00001332
Dillution 8	31.0 µl of "stock solution" in 5ml 50% MeOH	31.00	µl	5	0.0000124
Dillution 9	27.8 µl of "stock solution" in 5ml 50% MeOH	27.80	µl	5	0.00001112
Dillution 10	25.0 µl of "stock solution" in 5ml 50% MeOH	25.00	µl	5	0.00001

UV absorbance of the stock solutions at wavelengths of 259 nm.

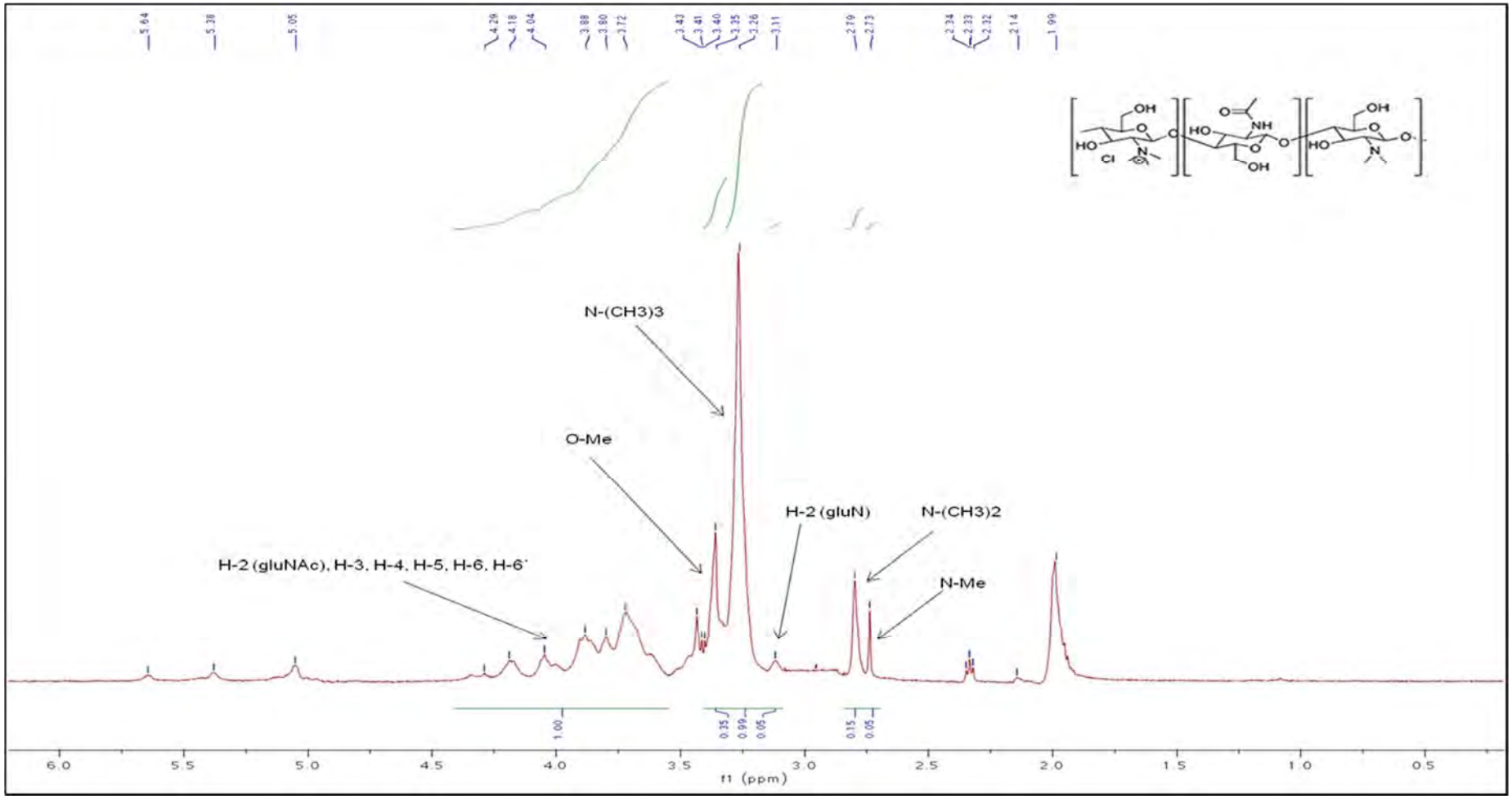
Solution name	Absorption at 259 nm			Average Absorption (259 nm)	Concentration (g/ml)
	1	2	3		
Dillution 1	1.351	1.267	1.310	1.309	0.00002
Dillution 2	1.281	1.280	1.234	1.265	0.00001888
Dillution 3	1.237	1.199	1.270	1.235	0.00001776
Dillution 4	1.133	1.216	1.088	1.146	0.00001668
Dillution 5	1.041	1.040	1.062	1.048	0.00001556
Dillution 6	1.040	0.989	0.996	1.008	0.00001444
Dillution 7	0.934	0.950	0.903	0.929	0.00001332
Dillution 8	0.838	0.884	0.834	0.852	0.0000124
Dillution 9	0.734	0.803	0.797	0.778	0.00001112
Dillution 10	0.697	0.707	0.682	0.695	0.00001
Zero Concentration				0	0


Proguanil UV Standard Curve
y = 66689x + 0.0229
R² = 0.9962
Absorbance (nm)
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
0.000005
0.00001
0.000015
0.00002
0.000025
Concentration (g/ml)
Absorption at 259 nm

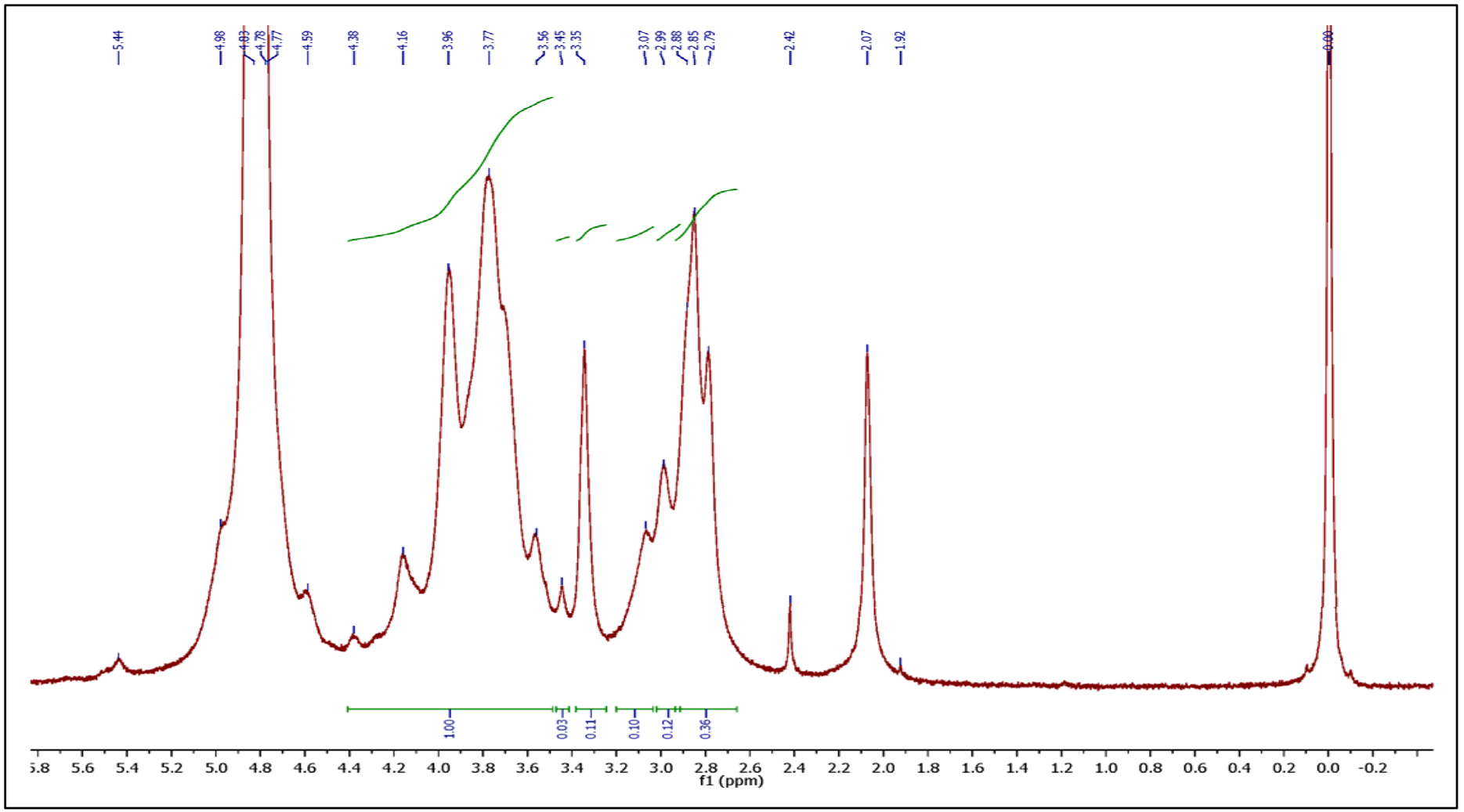


Annexure:

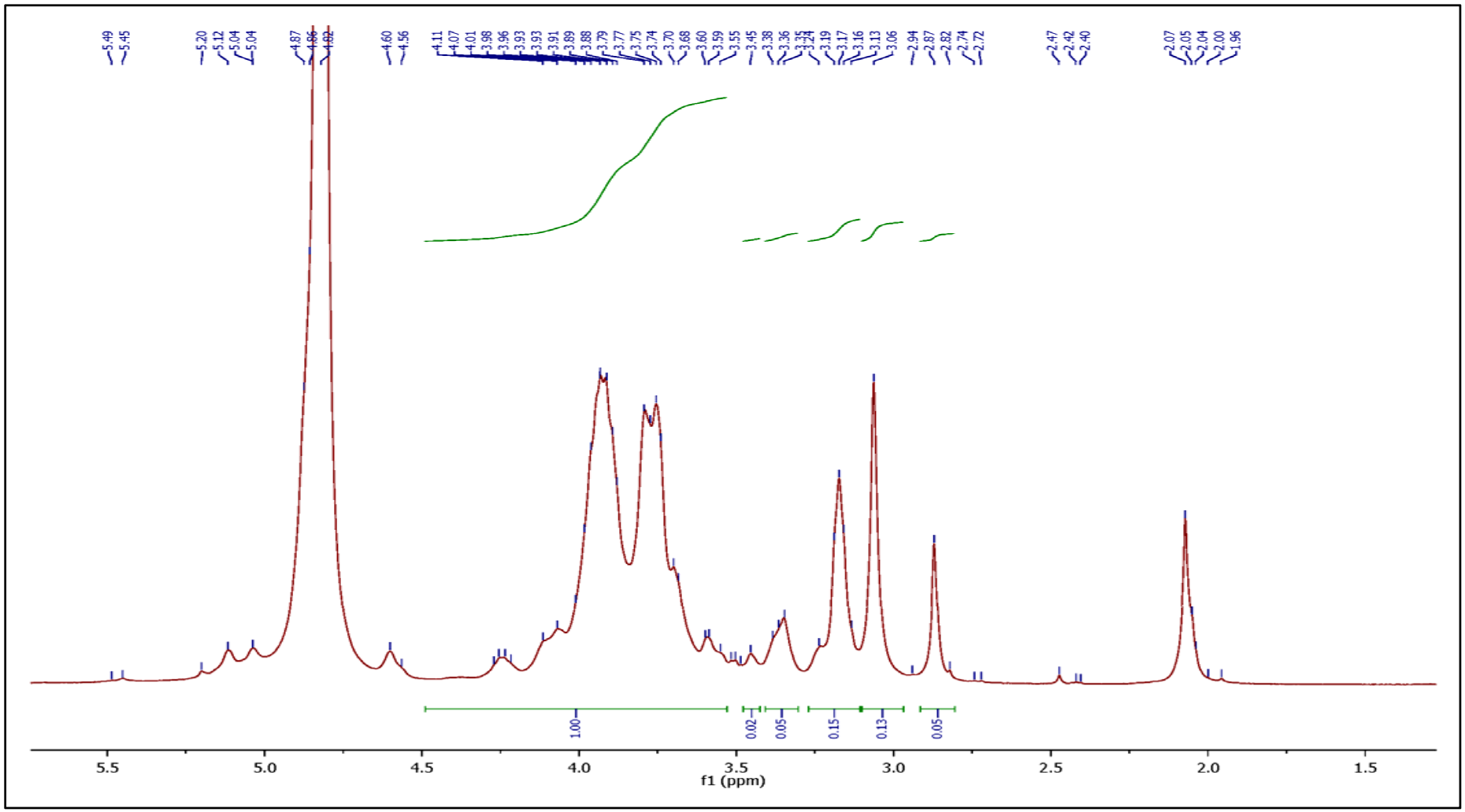
This annexure contains the ^{1}H-NMR spectra of the TMC which was prepared with the conventional and microwave irradiation synthesis method.



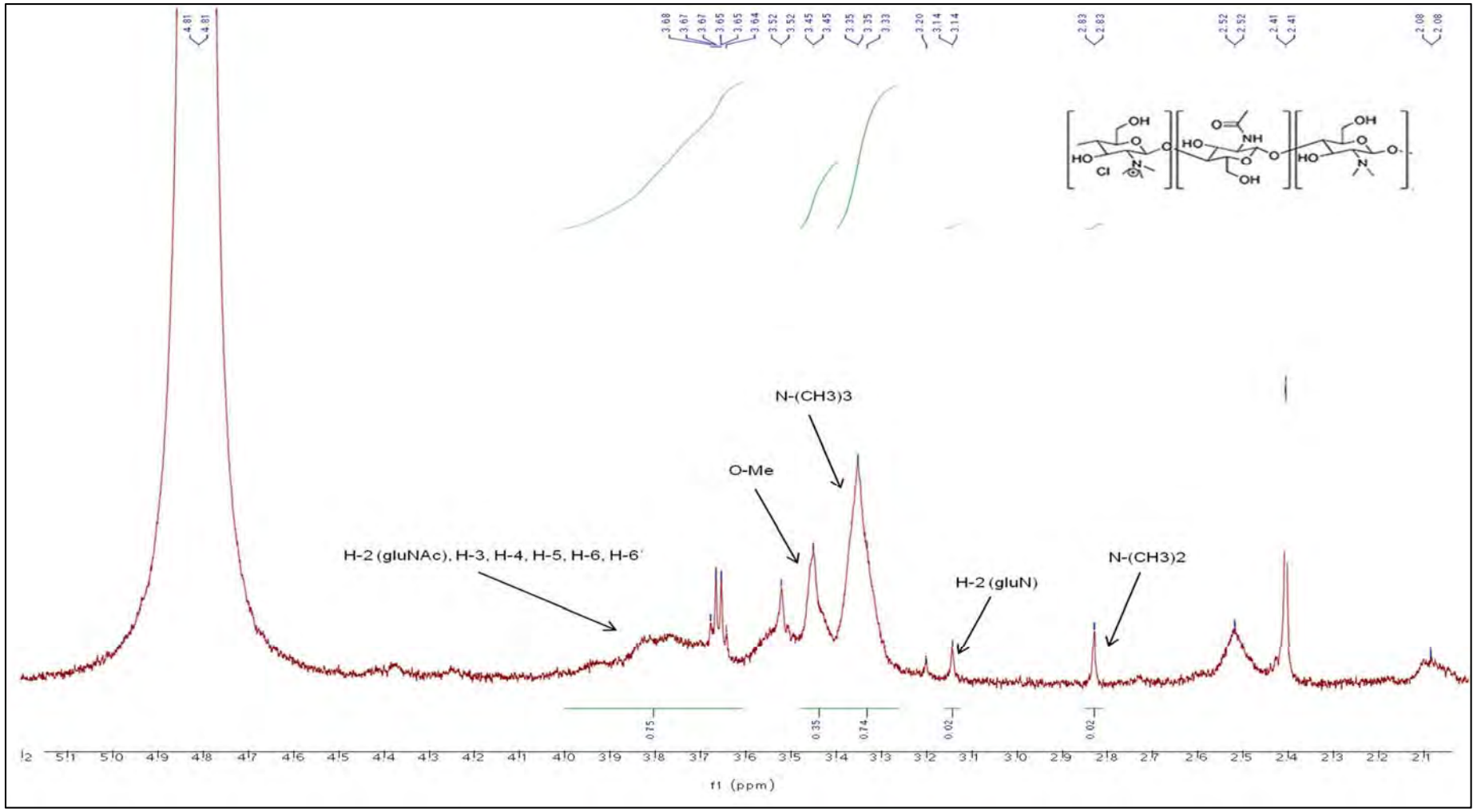
Conventionally synthesised TMC (TMCCM) with a 62.86 % degree of quaternisation.



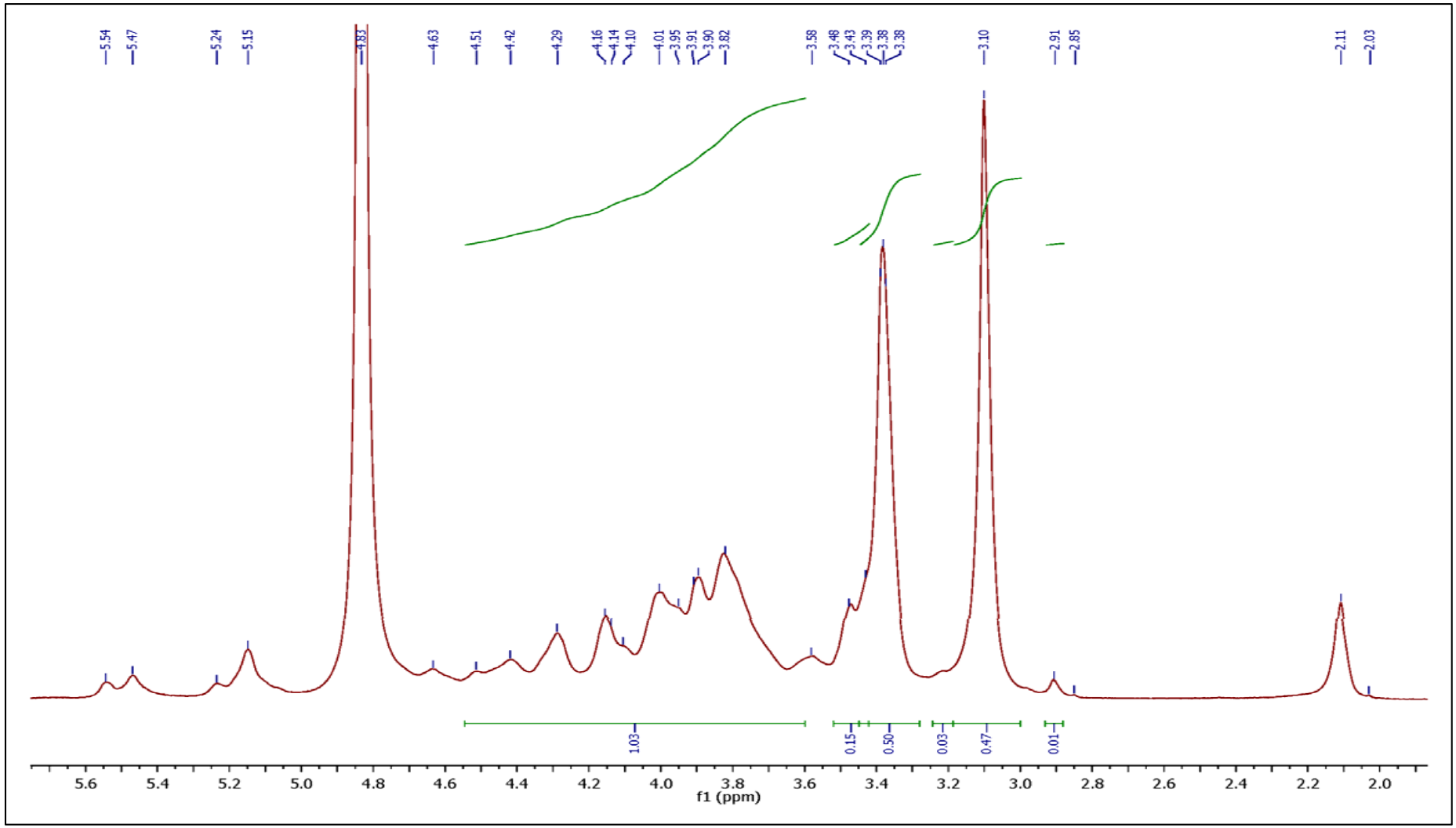
Microwave irradiation assisted synthesised TMC (TMCMWR1) with a 6.67 % degree of quaternisation.



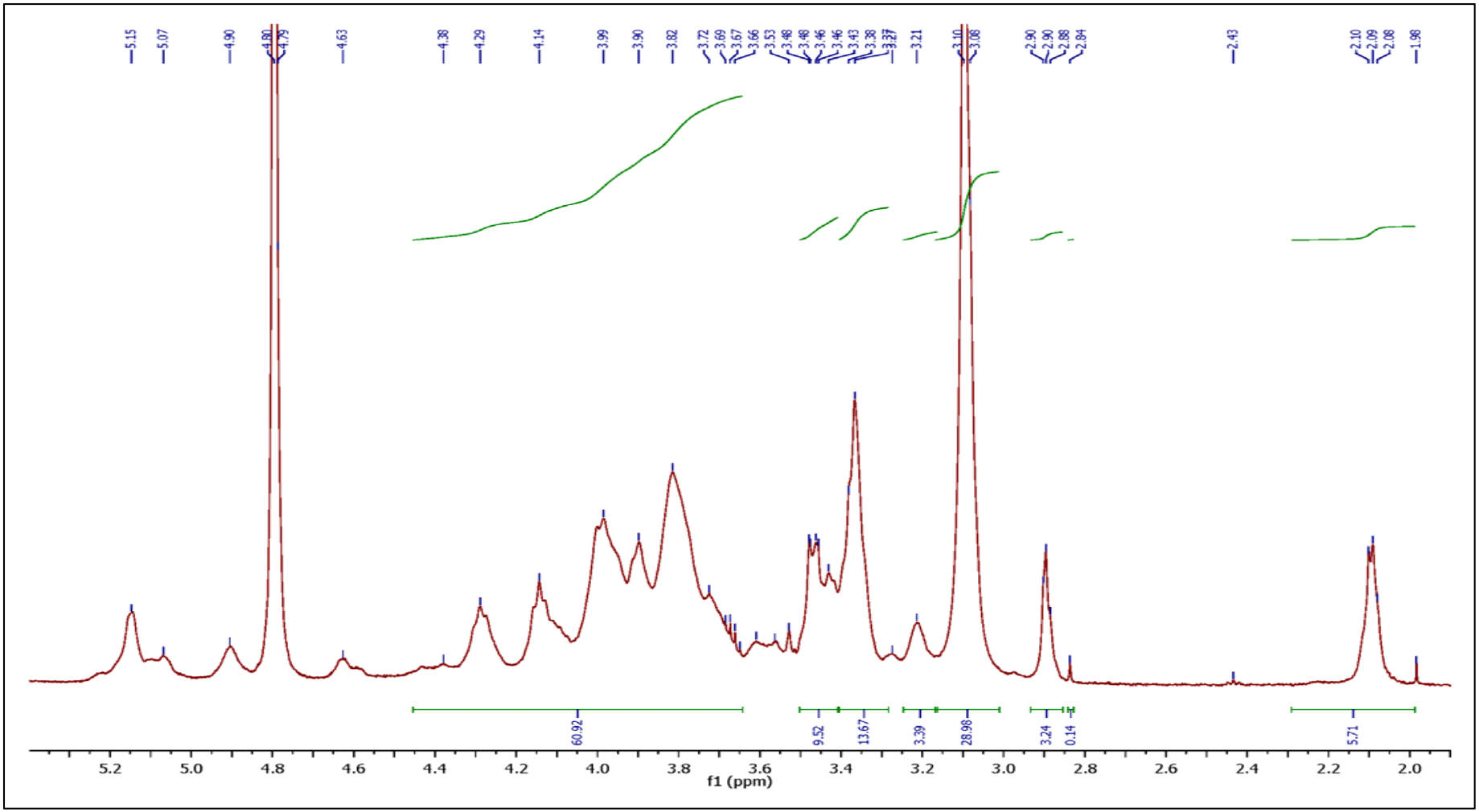
Microwave irradiation assisted synthesised TMC (TMCMWR2) with a 2.90 % degree of quaternisation.



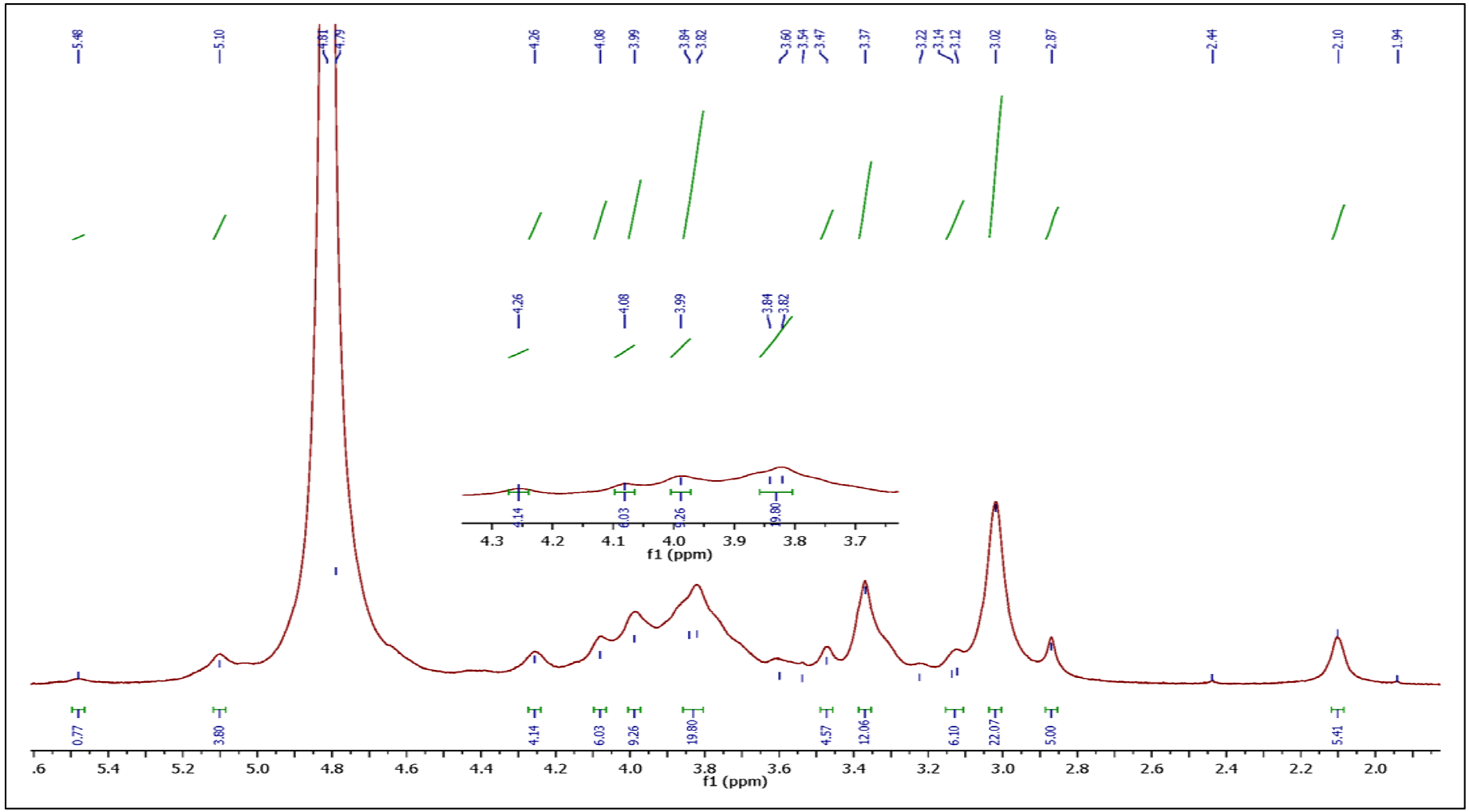
Microwave irradiation assisted synthesised TMC (TMCMWR3) with a 64.07 % degree of quaternisation.



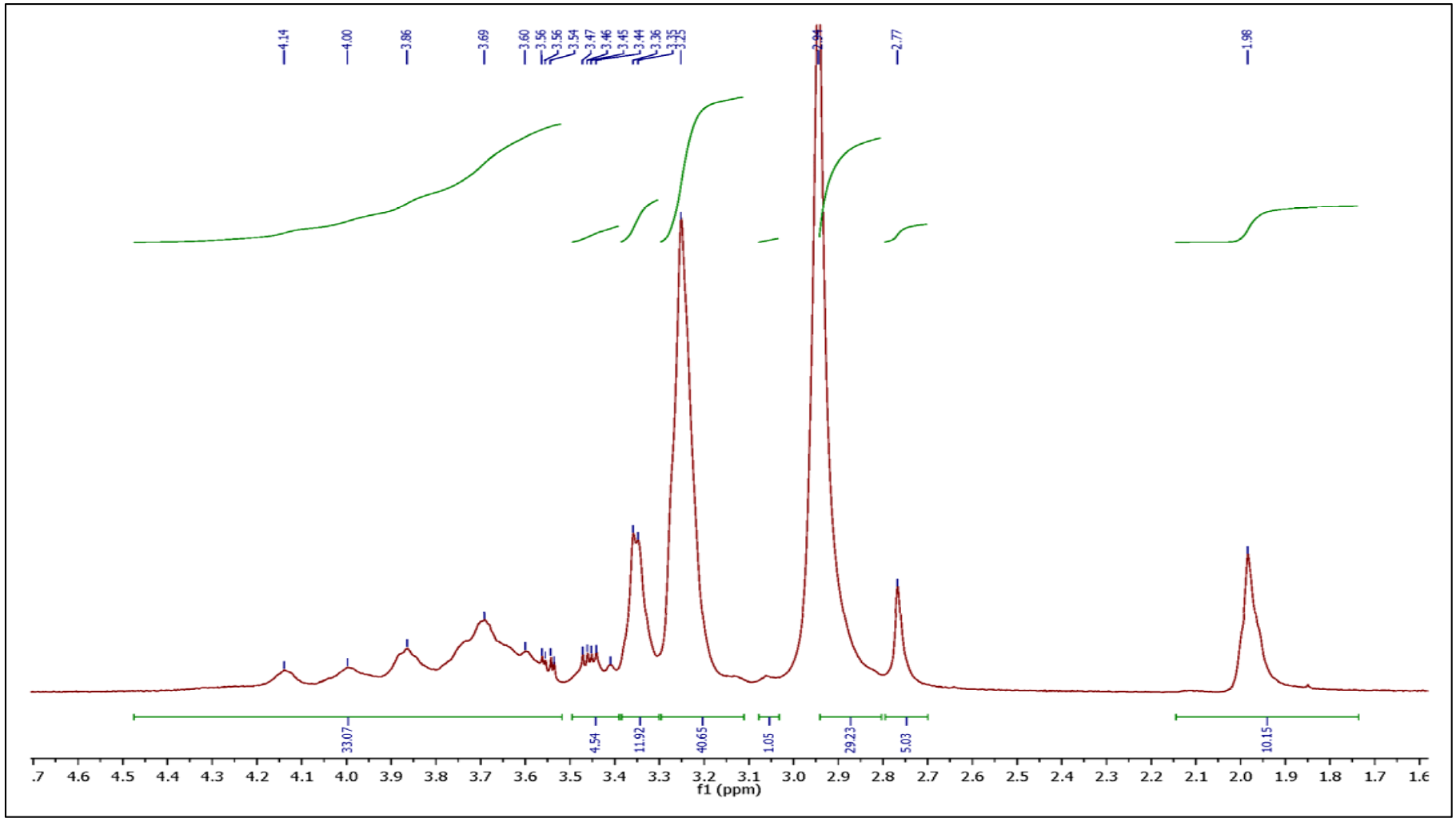
Microwave irradiation assisted synthesised TMC (TMCMWR4) with a 31.44 % degree of quaternisation.



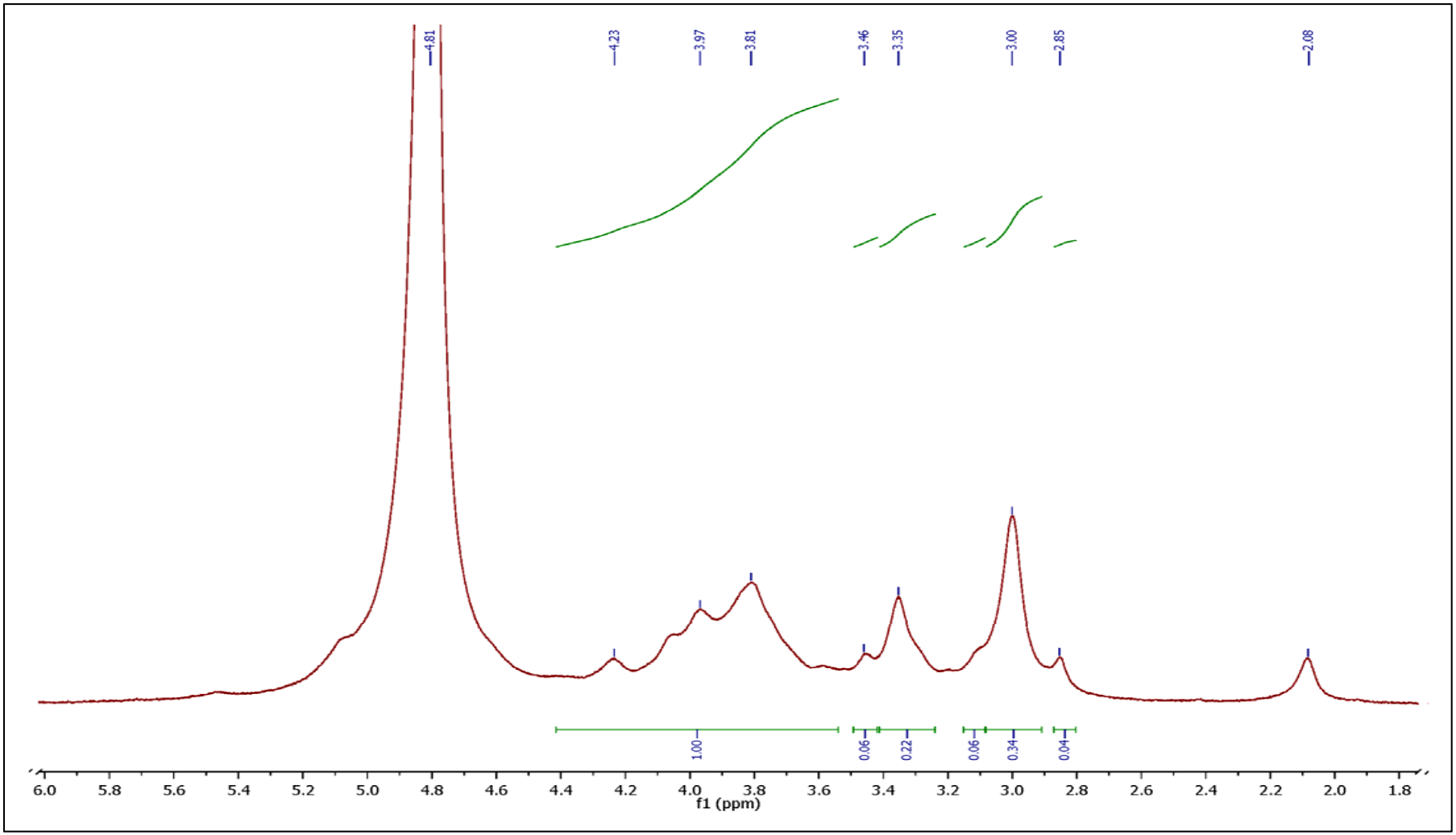
Microwave irradiation assisted synthesised TMC (TMCMWR5) with a 14.17 % degree of quaternisation.



Microwave irradiation assisted synthesised TMC (TMCMWR6) with a 17.70 % degree of quaternisation.



Microwave irradiation assisted synthesised TMC (TMCMWR7) with a 23.30 % degree of quaternisation.



Microwave irradiation assisted synthesised TMC (TMCMWR8) with a 13.84 % degree of quaternisation.

Annexure:

This annexure contains the validation of the HPLC method.

MATERIALS AND METHODS

Chemicals

All chemicals (Merck®, Johannesburg, South Africa) described herein were of analytical grade or HPLC grade (where applicable).

Instrumentation

All instruments used in this work are maintained, serviced and qualified and are in accordance with the quality standards described by the United States Pharmacopeia, British Pharmacopeia or has been certified to be calibrated by an independent accredited body.

HPLC

A HPLC method was used to quantitatively determine proguanil and dapsone from dissolution samples. The parameters were as follows:

System:	Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA)
Software:	Rev. B. 02.01-SR2 [260] ChemStation for LC 3D (Agilent Technologies, Santa Clara, CA)
Column:	C18, 3 µm, 4.6 mm x 10 cm (Phenomenex, Torrance, CA)
Mobile phase:	Mixture of 44 volumes acetonitrile and 56 volumes of buffer ph 3.0 (see below)
Buffer pH 3.0:	Dissolve 1.36 g of potassium dihydrogen phosphate in 900 ml of water, adjust the pH to 3.0 with phosphoric acid (~1440 g/l) TS and dilute to 1000 ml with water R.
Flow:	1ml/min
Temperature:	30 °C
Wavelength:	216 nm
Injection volume:	10 µl
Solvent:	Dissolution medium

The evaluation of *specificity, linearity, range, accuracy, repeatability, limit of detection and limit of quantitation* was based on the ICH guideline on validation of analytical procedures (ICH:Q2 (R1). 1997. Validation of analytical procedures: Text and methodology. Fed Regist 62 FR 27464).

Specificity

3 standard solutions were prepared. Solution 1 contained proguanil, solution 2 contained dapsone (solution 2) and a 3d solution contained a combination of both (solution 3). These standard solutions together with mobile phase, solvent, dissolution medium and placebo were injected and analysed separately using the HPLC conditions specified above. The analyses of

solutions 1 and 2 identified the retention time of each active ingredient. The analyses of the solvent, mobile phase dissolution medium and placebo identified the retention time of any of these contributors. Figure 1 depicts a schematic of a typical HPLC chromatogram of the sample/standard matrix. The solvent was identified at 0.7 and 0.9 minutes (C). Dapsone (D) was identified at 1.3 minutes and proguanil (P) at 2.8 minutes. The resolution factor between D-C were greater than 2.0, indicating sufficient separation between the contributor and the active. The method was regarded as specific, as 1) all peaks were identified 2) adequate resolution was obtained between the contributors and actives and 3) the peak positions did not vary between the sample and standard.

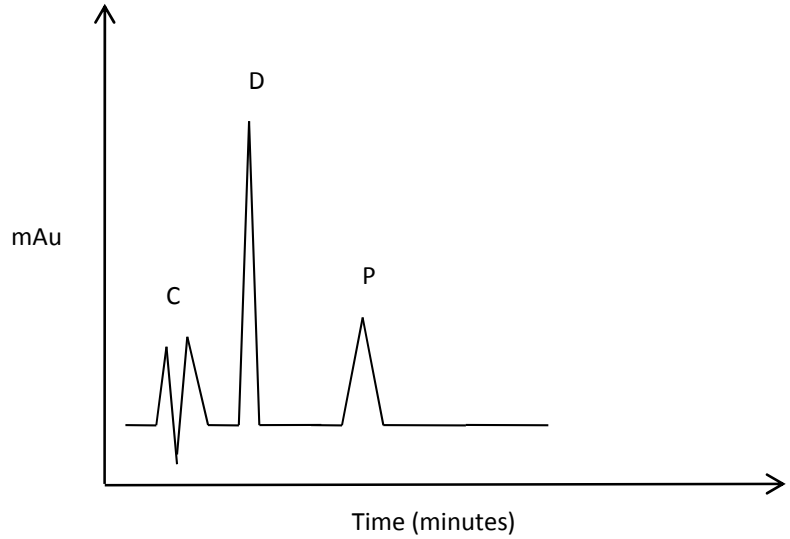


Figure 1. A schematic representation of a typical HPLC chromatogram of the analytical matrix.

Linearity, range, repeatability and accuracy

Linearity was established for each active ingredient, using five concentrations across the intended analytical concentration range (the range in which the sample is to be analysed). Dapsone and proguanil standard solutions were prepared with concentrations ranging between approximately 15.2 – 365.2 µg/ml. The correlation coefficient (r^2) for each calibration curve proved *linearity* across the intended analytical concentration range (Table 1) as the R^2 exceeded or equalled 0.99.

Table 1. Linearity across the analytical range.

Analysis	Regression line obtained*	Recovery (%RSD)
Dapsone dissolution	y = 28.05x + 684.08 (R^2 = 0.99)	101.5 % (0.9 %)
Proguanil dissolution	y = 29.75x – 24.99 (R^2 = 0.99)	99.8 % (0.2 %)

*Where y = HPLC response (mAu) and x = concentration (µg/ml)

The *accuracy* and *repeatability* were established across the specified range for each respective active ingredient. *Accuracy* was reported as percent (%) recovery. % recovery was determined by dividing the theoretical known concentration with the concentration derived from the linear function, multiplied by 100 (percentage). The *repeatability* of the method was assessed by calculating the relative standard deviation (% RSD) for the respective recovery values determined across the range. The recovery values obtained were found to be within 98 - 102 %, and the relative standard deviations of these determinations were less than 2.0 % in each case, proving the capability of the method to produce results that are accurate and repeatable in the specified range.

Limit of detection (LOD) and limit of quantification (LOQ)

The response of all the sample solutions were within the established analytical range for each respective active ingredient. No sample was analysed outside the minimum response of the standard calibration curve. The LOD and LOQ values fall below the minimum of the established concentration range, and with all sample responses above this threshold there is no need to elaborate on the LOD and LOQ as it do not pose a risk to the integrity of the results obtained.

Dissolution

The dissolution procedure was based on the dissolution methods specified by the 2011 USP monographs for dapsone tablets (www.uspnf.com) and the 2011 BP monograph for proguanil tablets (www.pharmacopeia.co.uk). An Erweka D700 dissolution system (Erweka, Heustenstamm, Germany) fitted with a paddle assembly set to rotate at 75 rpm, was used for powder dissolution experiment. 500 ml of 0.1 N HCl containing 0.2 % w/v NaCl, was used as the dissolution medium. The dissolution bath was maintained at 37.0±0.5°C using a calibrated thermostat. The execution of the dissolution was based on the technique described by Lötter and co-workers (Lötter *et al.*, 1983). The powdered samples (binary mixture of powdered sample equivalent to ±60 mg of dapsone and ±60 mg of proguanil per sample) were accurately transferred into test tubes. ±50 mg glass beads (Sigma Aldrich, Johannesburg, South Africa) with a diameter of 0.1 mm was added to each test tube in order to minimize possible agglomeration of particles. Thereafter 10 ml of dissolution medium was withdrawn from each vessel and transferred into the respective allocated test tube. The suspended samples were agitated using a Vortex Genie shaker (Scientific Industries Inc., Bohemia, New York) for 30 seconds, before being rinsed into the respective dissolution vessels. Samples were withdrawn after 7.5, 15, 22.5, 30, 45, 60, 90, 120 and 150 minutes. The samples were filtered using in-line Millipore 0.45 µm filters (Microsep, Sandton, South Africa) and analysed by HPLC using the conditions specified above.

RESULTS

A summary of the dissolution results is given in Table 2. The results depict the average across vessels 1 – 6 (6 individual determinations) per withdrawal timepoint.

Table 2. Dissolution results of proguanil and dapsone loaded TMC microparticles.

Active	**Dissolution (%) at the different withdrawal times (min)**								
	7.5 min	15 min	22.5 min	30 min	45 min	60 min	90 min	120 min	150 min
Dapsone	58% (6.3%)	61% (5.8%)	63% (6.1%)	65% (6.5%)	67% (4.7%)	69% (3.5%)	72% (3.3%)	73% (2.1%)	73% (1.7%)
Proguanil	14% (4.9%)	16% (5.3%)	19% (4.7%)	24% (5.2%)	30% (4.5%)	37% (3.1%)	45% (3.3%)	49% (2.7%)	55% (1.9%)

REFERENCES

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Annexure:

This annexure contains the Additional information regarding the *in vivo* pharmacodynamic evaluation.

1. Ethical committee approval:

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOMKAMPUS

Privaatsak X6001, Potchefstroom
Suid-Afrika, 2520

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

EENHEID VIR GENEESMIDDELNAVORSING EN -ONTWIKKELING
Tel: (018) 018 299-2274
Faks: (018) 018 293-5219
E-pos: jeanetta.duplessis@nwu.ac.za

Navorsings Etiekkomittee
Noordwes-Universiteit
Bussie116

20 Julie 2010

Geagte Mev. Halgryn

SAMEVATTENDE VERSLAG: NWU-00042-10-S5

In vivo antimalarial efficacy evaluation of novel antimalarial lead compounds in C57 BL6 mice

Die punte soos uiteengesit in in my skrywe gedateer 13 Julie 2010 is voldoende deur die aansoeker aangespreek, derhalwe kan goedkeuring nou verleen word.

Vriendelike groete

PROF. J. DU PLESSIS
DIREKTEUR

2. Animal handling and breeding conditions

Animal experiments were executed in compliance with the guidelines of Ethics Committee on the premises of North-West University Animal Research Centre. The experimental protocol was approved by the North-West University's Ethics Committee (protocol number: NWU-00042-10-S5). Healthy male C57 BL8 mice weighing 30 ± 5 g were used for the study. The mice were kept in a closed, controlled environment which ensured ideal growth and exposure to the minimal pathogens as depicted in table 1. The mice's diet consisted out of Epol® mice cubes (Epol (Pty) Ltd., Pretoria, South Africa).

Table 1. Controlled environmental conditions where under mice were kept.

Condition parameter	Value in the Animal Research Centre
Temperature (°C)	21 ± 2 °C
Relative Humidity (% RH)	55 ± 10 %
Air movement	18 changes per minute
Light intensity (Lux)	350 – 400 lux, one meter above the floor level
Period of exposure to light source (Hours)	12 hours exposure to light, 12 hours darkness

3. Parasites

In vivo antimalarial efficacy studies were performed on the rodent malaria parasite, *Plasmodium berghei* ANKA strain which is infective in mice. This strain's life cycle is in the essence similar to the human malaria parasite's one. It is lethal in mice, causing high mortality rates and its sensitivity to all currently used antimalarial drugs, provides a good model to estimate the efficacy and survival. The parasite was revived from frozen stocks and infection was initiated in the donor mice with intraperitoneal injection of 10^6 parasitized erythrocytes (pRBCs). Parasites were maintained in two host mice until the parasitemia reached a level of 25 % (Peters & Robinson, 1999).

4. Study protocol and drug treatment: Peters' four-day suppressive test

Blood taken from infected donor mice with approximately 25 % parasitemia was diluted suitably in phosphate buffered saline to contain approximately 10^6 parasitized erythrocytes. Experimental animals were infected intraperitoneal with this blood (200 µl) on day 0 to introduce

2 x 10^6 pRBS to each. Mice were randomly divided into fourteen groups (n = 8). Two hours post infection; mice were treated orally with single dose of the various formulations as depicted in table 2, 3 & 4. Further, on days 1, 2, and 3, mice were treated again similarly as on day 0. Then, throughout the study, blood was withdrawn from tail vein from day 1 at regular time intervals for the assessment of parasitemia (Borhade *et al.*, 2012; Fidock *et al.*, 2004).

Table 2. Treatment regimen for the dapsone experimental groups.

Groups	**A**	**B**	**C**	**D**	**I**	**J**
Drug	Vehicle	Vehicle	Dapsone	Dapsone	Dapsone	Dapsone
Formulation	DMSO/PBS (7.5:2.5, *v/v*)	TMC-TPP particles	DMSO/PBS (7.5:2.5, *v/v*)	DMSO/PBS (7.5:2.5, *v/v*)	TMC-TPP particles	TMC-TPP particles
Volume (µl)	200	200	200	200	200	200
Dose (mg/kg)	-	-	0.03*	0.15*	0.03*	0.15*

*EC50 and EC90 values according to Peters and co-workers (Peters *et al.*, 2005).

Table 3. Treatment regimen for the proguanil experimental groups.

Groups	**A**	**B**	**E**	**F**	**K**	**L**
Drug	Vehicle	Vehicle	Proguanil	Proguanil	Proguanil	Proguanil
Formulation	DMSO/PBS (7.5:2.5, *v/v*)	TMC-TPP particles	DMSO/PBS (7.5:2.5, *v/v*)	DMSO/PBS (7.5:2.5, *v/v*)	TMC-TPP particles	TMC-TPP particles
Volume (µl)	200	200	200	200	200	200
Dose (mg/kg)	-	-	16*	30*	16*	30*

*EC50 and EC90 values according to Steward and co-workers (Steward *et al.*, 2004).

Table 4. Treatment regimen for the dapsone/proguanil combination experimental groups.

Groups	A	B	G	H	M	N
Drug	Vehicle	Vehicle	Dapsone/ Proguanil	Dapsone/ Proguanil	Dapsone/ Proguanil	Dapsone/ Proguanil
Formulation	DMSO/ PBS (7.5:2.5, *v/v*)	TMC-TPP particles	DMSO/PBS (7.5:2.5, *v/v*)	DMSO/PBS (7.5:2.5, *v/v*)	TMC-TPP particles	TMC-TPP particles
Volume (µl)	200	200	200	200	200	200
Dose (mg/kg)	-	-	0.03* (Dapsone) 16@ (Proguanil)	0.15* (Dapsone) 30@ (Proguanil)	0.03* (Dapsone) 16@ (Proguanil)	0.15* (Dapsone) 30@ (Proguanil)

*EC50 and EC90 values according to Peters and co-workers (Peters *et al.*, 2005).

@EC50 and EC90 values according to Steward and co-workers (Steward *et al.*, 2004).

5. References

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Annexure:

This annexure contains the complete data set of the *in vivo* pharmacodynamic evaluation.

Table 1 and 2. The least square means of the parasitaemia for the dapsone formulations for each day.

Dapsone formulations compared to reference formulation A																
Formulation	**Least Square Means**															
	Day 0	**SEM**	**Day 1**	**SEM**	**Day 3**	**SEM**	**Day 5**	**SEM**	**Day 8**	**SEM**	**Day 11**	**SEM**	**Day 15**	**SEM**	**Day 18**	**SEM**
A (DMSO/PBS)	0	0	5.05	1.71	16.25	1.71	16.84	1.71	19.20	1.71	19.36	1.71	40.29	1.71	51.47	1.81
C (0.03 mg/kg)	0	0	3.06	1.71	11.80	1.71	6.08	1.71	9.36	1.71	11.58	1.71	33.34	1.71	45.48	1.71
D (0.15 mg/kg)	0	0	2.88	1.71	11.50	1.71	8.56	1.71	8.56	1.94	7.72	2.13	24.71	2.16	42.08	2.16
I (0.03 mg/kg)	0	0	4.03	1.71	3.96	1.80	4.72	1.82	7.23	2.12	8.08	2.16	27.10	2.16	39.46	2.16
J(0.15 mg/kg)	0	0	3.15	1.71	4.01	1.71	6.48	1.71	13.48	1.71	23.59	1.71	41.01	1.71	39.47	2.11

Dapsone formulations compared to reference formulation B																
Formulation	**Least Square Means**															
	Day 0	**SEM**	**Day 1**	**SEM**	**Day 3**	**SEM**	**Day 5**	**SEM**	**Day 8**	**SEM**	**Day 11**	**SEM**	**Day 15**	**SEM**	**Day 18**	**SEM**
B (TMC-TPP)	0	0	4.29	1.65	4.29	1.65	17.09	1.65	18.67	1.65	19.38	1.74	46.74	1.76	41.34	1.88
C (0.03 mg/kg)	0	0	3.06	1.65	11.80	1.65	6.08	1.65	9.36	1.65	11.58	1.65	33.34	1.65	45.48	1.65
D (0.15 mg/kg)	0	0	2.88	1.65	11.50	1.65	8.56	1.65	8.54	1.86	7.71	2.05	24.71	2.08	42.08	2.09
I (0.03 mg/kg)	0	0	4.03	1.65	3.98	1.73	4.75	1.75	7.19	2.03	8.04	2.08	27.08	2.09	39.45	2.09
J(0.15 mg/kg)	0	0	3.15	1.65	4.01	1.65	6.48	1.65	13.48	1.65	23.58	1.65	41.01	1.65	39.46	2.01

*SEM = Standard error of the mean

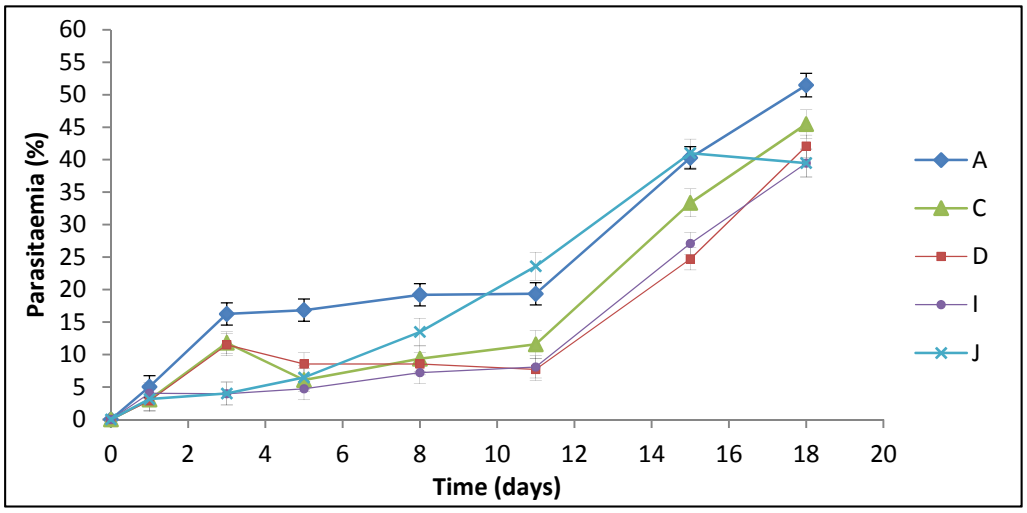


Fig. 1 Parasitaemia of dapsone formulations compared to formulation A.

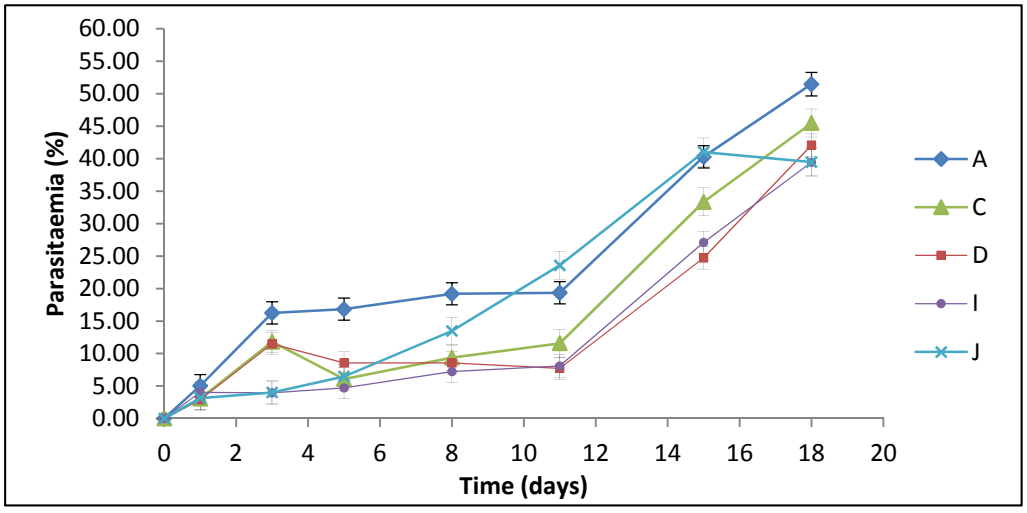


Fig. 2 Parasitaemia of dapsone formulations compared to formulation B.

Table 3 and 4. The least square means of the parasitaemia for the proguanil formulations for each day.

Proguanil formulations compared to reference formulation A																
Formulation	**Least Square Means**															
	Day 0	**SEM**	**Day 1**	**SEM**	**Day 3**	**SEM**	**Day 5**	**SEM**	**Day 8**	**SEM**	**Day 11**	**SEM**	**Day 15**	**SEM**	**Day 18**	**SEM**
A (DMSO/PBS)	0	0	5.05	1.71	16.25	1.71	16.84	1.71	19.20	1.71	19.36	1.71	40.29	1.71	51.47	1.81
E (16.00 mg/kg)	0	0	2.80	1.83	8.03	1.94	7.53	2.59	9.03	2.77	18.51	2.79	38.89	2.79	50.30	2.79
F (30.00 mg/kg)	0	0	3.40	2.42	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
K (16.00 mg/kg)	0	0	4.43	1.71	4.28	1.71	5.68	1.71	11.11	1.71	23.36	1.81	40.01	1.83	37.91	1.83
L (30.00 mg/kg)	0	0	3.89	1.71	6.21	1.71	3.84	1.71	5.51	1.81	11.86	1.83	26.39	1.83	39.74	1.83

Proguanil formulations compared to reference formulation B																
Formulation	**Least Square Means**															
	Day 0	**SEM**	**Day 1**	**SEM**	**Day 3**	**SEM**	**Day 5**	**SEM**	**Day 8**	**SEM**	**Day 11**	**SEM**	**Day 15**	**SEM**	**Day 18**	**SEM**
B	0	0	4.29	1.65	4.29	1.65	17.09	1.65	18.67	1.65	19.38	1.74	46.74	1.76	41.34	1.88
E (16.00 mg/kg)	0	0	2.80	1.76	8.02	1.86	7.53	2.45	9.03	2.65	18.51	2.69	38.89	2.69	50.30	2.69
F (30.00 mg/kg)	0	0	3.40	2.33	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
K (16.00 mg/kg)	0	0	4.43	1.65	4.28	1.65	5.68	1.65	11.11	1.65	23.40	1.74	40.04	1.76	37.93	1.76
L (30.00 mg/kg)	0	0	3.89	1.65	6.21	1.65	3.84	1.65	5.53	1.74	11.88	1.76	26.40	1.76	39.74	1.76

*SEM = Standard error of the mean, N/D = Not determined due to all the mice dying

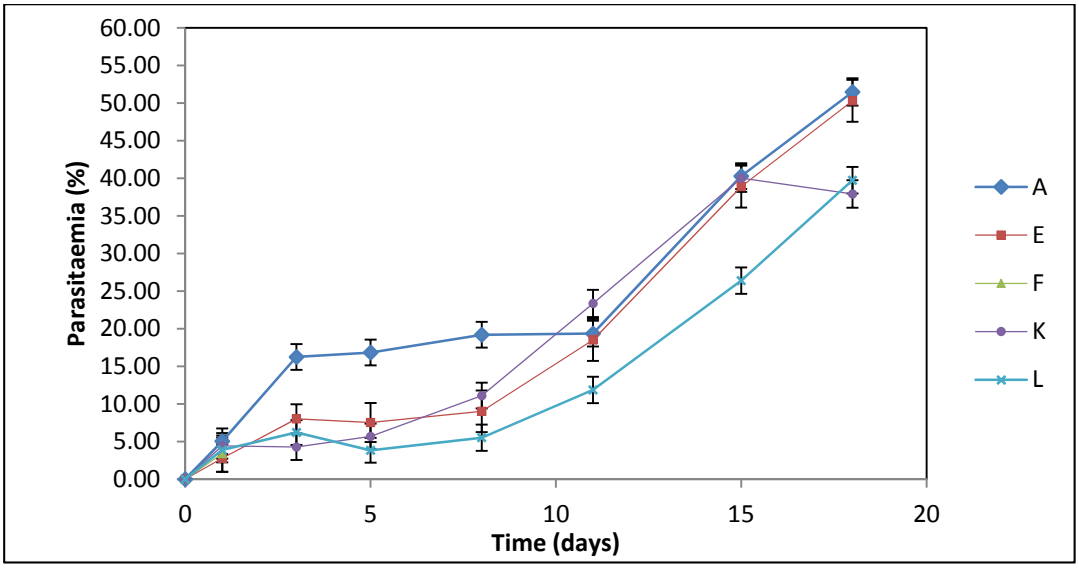


Fig. 3 Parasitaemia of proguanil formulations compared to formulation A.

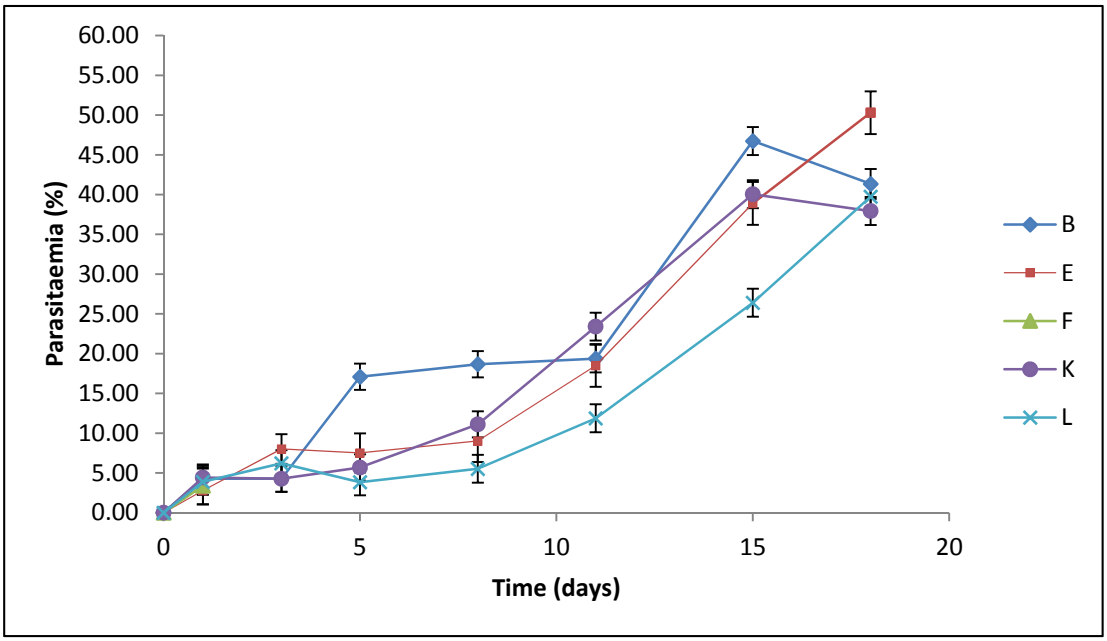


Fig. 4 Parasitaemia of proguanil formulations compared to formulation B.

Table 5 and 6. The least square means of the parasitaemia for the combination formulations for each day.

Combination formulations compared to reference formulation A																
Formulation	**Least Square Means**															
	Day 0	**SEM**	**Day 1**	**SEM**	**Day 3**	**SEM**	**Day 5**	**SEM**	**Day 8**	**SEM**	**Day 11**	**SEM**	**Day 15**	**SEM**	**Day 18**	**SEM**
A	0	0	5.05	1.71	16.25	1.71	16.84	1.71	19.20	1.71	19.36	1.71	40.29	1.71	51.47	1.81
G	0	0	2.80	1.71	5.41	1.71	4.26	1.80	3.55	1.82	1.60	1.95	N/D	N/D	N/D	N/D
H	0	0	0.56	3.42	3.07	4.50	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
M	0	0	1.30	1.71	5.35	1.71	5.93	1.71	6.93	1.71	16.08	1.81	40.31	1.83	42.10	1.83
N	0	0	2.81	1.71	5.93	1.71	5.91	1.71	6.25	1.71	13.60	1.81	30.15	1.83	44.93	1.83

Combination formulations compared to reference formulation B																
Formulation	**Least Square Means**															
B	0	0	4.29	1.65	4.29	1.65	17.09	1.65	18.67	1.65	19.38	1.74	46.74	1.76	41.34	1.88
G	0	0	2.80	1.65	5.41	1.65	4.27	1.73	3.56	1.76	1.61	1.88	N/D	N/D	N/D	N/D
H	0	0	0.56	3.30	3.10	4.27	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
M	0	0	1.30	1.65	5.35	1.65	5.93	1.65	6.93	1.65	16.06	1.74	40.30	1.76	42.10	1.76
N	0	0	2.81	1.65	5.93	1.65	5.91	1.65	6.25	1.65	13.63	1.74	30.17	1.76	44.95	1.76

*SEM = Standard error of the mean, N/D = Not determined due to all the mice dying

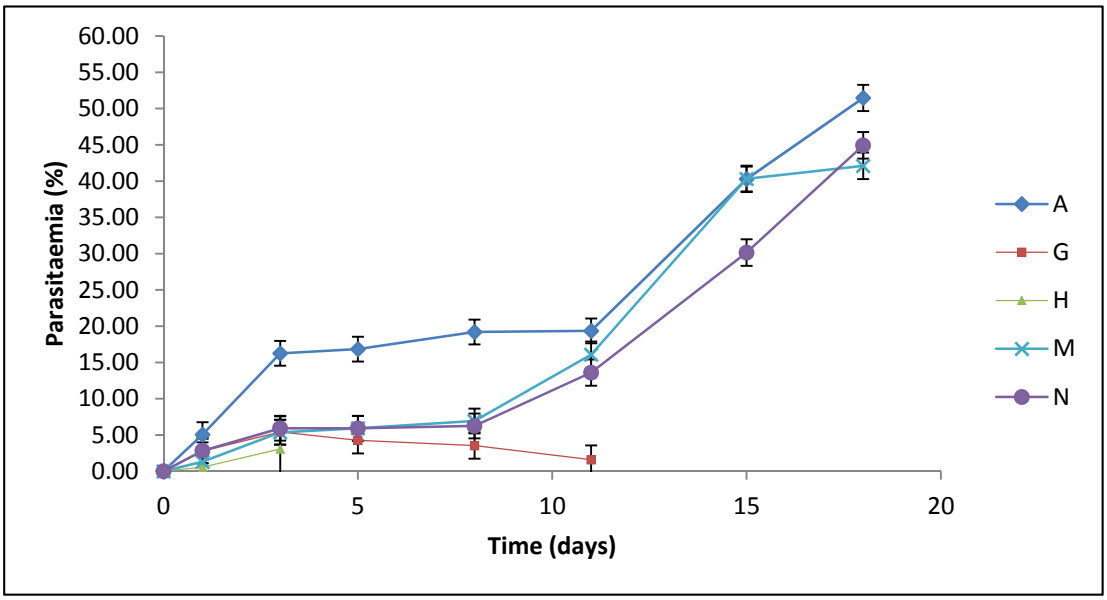


Fig. 5 Parasitaemia of combination formulations compared to formulation A.

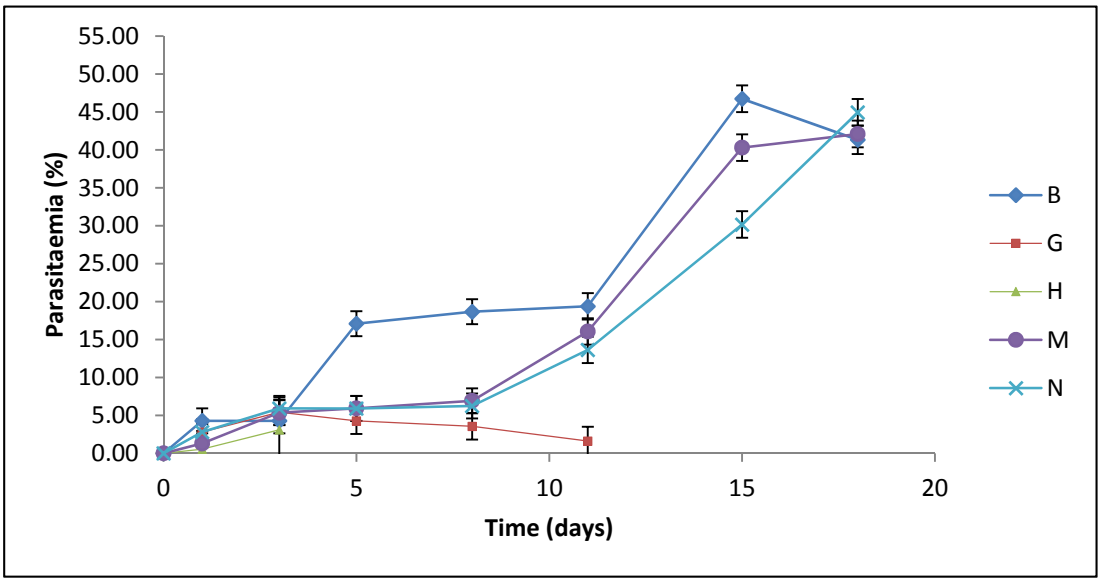


Fig. 6 Parasitaemia of combination formulations compared to formulation B.

Annexure:

This annexure contains the Malvern Mastersizer and Zetasizer data.

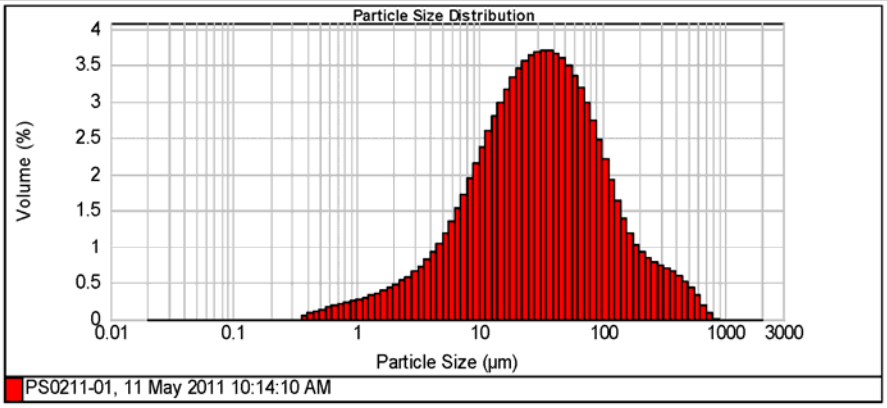
1. Mastersizer: TMC-TPP particles (TMC:TPP ratio – 2:1)

MASTERSIZER 2000

Result Analysis Report

Sample Name: PS0211-01	**SOP Name:** Jaco (TMC)	**Measured:** 11 May 2011 10:14:10 AM	
Sample Source & type:	**Measured by:** Jaco van Heerden	**Analysed:** 11 May 2011 10:14:11 AM	
Sample bulk lot ref:	**Result Source:** Measurement		
Particle Name: Titanium Dioxide	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.741	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 18.12 %
Dispersant Name: Alcohol	**Dispersant RI:** 1.320	**Weighted Residual:** 0.409 %	**Result Emulation:** Off
Concentration: 0.0262 %Vol	**Span :** 4.590	**Uniformity:** 1.61	**Result units:** Volume
Specific Surface Area: 0.56 m²/g	**Surface Weighted Mean D[3,2]:** 10.710 um	**Vol. Weighted Mean D[4,3]:** 63.454 um	

d(0.1): 5.358 um **d(0.5):** 30.869 um **d(0.9):** 147.044 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 %
0.020	0.00	0.142	0.00	1.002	0.28	7.096	1.73	50.238	3.49	355.656	0.66
0.022	0.00	0.159	0.00	1.125	0.31	7.962	1.94	56.368	3.36	399.052	0.60
0.025	0.00	0.178	0.00	1.262	0.33	8.934	2.15	63.246	3.19	447.744	0.53
0.028	0.00	0.200	0.00	1.416	0.36	10.024	2.38	70.963	2.99	502.377	0.43
0.032	0.00	0.224	0.00	1.589	0.40	11.247	2.59	79.621	2.75	563.677	0.33
0.036	0.00	0.252	0.00	1.783	0.44	12.619	2.80	89.337	2.49	632.456	0.19
0.040	0.00	0.283	0.00	2.000	0.48	14.159	3.00	100.237	2.21	709.627	0.10
0.045	0.00	0.317	0.00	2.244	0.53	15.887	3.17	112.468	1.92	796.214	0.01
0.050	0.00	0.356	0.05	2.518	0.59	17.825	3.33	126.191	1.65	893.367	0.00
0.056	0.00	0.399	0.08	2.825	0.66	20.000	3.46	141.589	1.40	1002.374	0.00
0.063	0.00	0.448	0.11	3.170	0.73	22.440	3.56	158.866	1.20	1124.683	0.00
0.071	0.00	0.502	0.14	3.557	0.82	25.179	3.64	178.250	1.04	1261.915	0.00
0.080	0.00	0.564	0.16	3.991	0.93	28.251	3.69	200.000	0.92	1415.892	0.00
0.089	0.00	0.632	0.19	4.477	1.05	31.698	3.71	224.404	0.84	1588.656	0.00
0.100	0.00	0.710	0.21	5.024	1.19	35.566	3.70	251.785	0.78	1782.502	0.00
0.112	0.00	0.796	0.24	5.637	1.35	39.905	3.66	282.508	0.74	2000.000	
0.126	0.00	0.893	0.26	6.325	1.53	44.774	3.59	316.979	0.70		
0.142		1.002		7.096		50.238		355.656			

Operator notes:

Malvern Instruments Ltd.
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007548

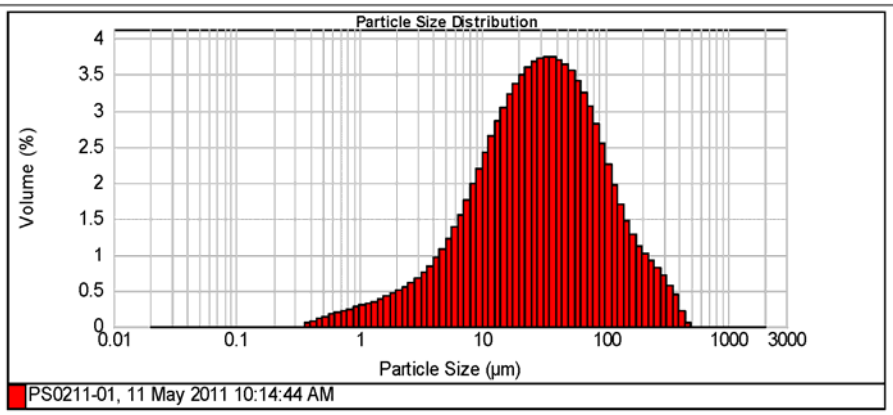
File name: 11 May 2011
Record Number: 1

MASTERSIZER 2000

Result Analysis Report

Sample Name: PS0211-01	**SOP Name:** Jaco (TMC)	**Measured:** 11 May 2011 10:14:44 AM	
Sample Source & type:	**Measured by:** Jaco van Heerden	**Analysed:** 11 May 2011 10:14:45 AM	
Sample bulk lot ref:	**Result Source:** Measurement		
Particle Name: Titanium Dioxide	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.741	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 18.31 %
Dispersant Name: Alcohol	**Dispersant RI:** 1.320	**Weighted Residual:** 0.761 %	**Result Emulation:** Off
Concentration: 0.0257 %Vol	**Span :** 4.143	**Uniformity:** 1.34	**Result units:** Volume
Specific Surface Area: 0.577 m²/g	**Surface Weighted Mean D[3,2]:** 10.400 um	**Vol. Weighted Mean D[4,3]:** 53.498 um	

d(0.1): 5.196 um **d(0.5):** 29.898 um **d(0.9):** 129.076 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 %
0.020	0.00	0.142	0.00	1.002	0.29	7.096	1.77	50.238	3.55	355.656	0.44
0.022	0.00	0.159	0.00	1.125	0.32	7.962	1.98	56.368	3.42	399.052	0.22
0.025	0.00	0.178	0.00	1.262	0.35	8.934	2.20	63.246	3.26	447.744	0.06
0.028	0.00	0.200	0.00	1.416	0.38	10.024	2.43	70.963	3.06	502.377	0.00
0.032	0.00	0.224	0.00	1.589	0.41	11.247	2.65	79.621	2.82	563.677	0.00
0.036	0.00	0.252	0.00	1.783	0.45	12.619	2.86	89.337	2.55	632.456	0.00
0.040	0.00	0.283	0.00	2.000	0.50	14.159	3.05	100.237	2.26	709.627	0.00
0.045	0.00	0.317	0.00	2.244	0.55	15.887	3.23	112.468	1.97	796.214	0.00
0.050	0.00	0.356	0.05	2.518	0.61	17.825	3.38	126.191	1.71	893.367	0.00
0.056	0.00	0.399	0.08	2.825	0.68	20.000	3.51	141.589	1.47	1002.374	0.00
0.063	0.00	0.448	0.12	3.170	0.75	22.440	3.61	158.866	1.28	1124.683	0.00
0.071	0.00	0.502	0.14	3.557	0.84	25.179	3.68	178.250	1.13	1261.915	0.00
0.080	0.00	0.564	0.17	3.991	0.95	28.251	3.73	200.000	1.02	1415.892	0.00
0.089	0.00	0.632	0.20	4.477	1.08	31.698	3.75	224.404	0.92	1588.656	0.00
0.100	0.00	0.710	0.22	5.024	1.22	35.566	3.75	251.785	0.82	1782.502	0.00
0.112	0.00	0.796	0.25	5.637	1.38	39.905	3.71	282.508	0.71	2000.000	0.00
0.126	0.00	0.893	0.27	6.325	1.57	44.774	3.65	316.979	0.57		
0.142	0.00	1.002		7.096		50.238		355.656			

Operator notes:

Malvern Instruments Ltd.
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007548

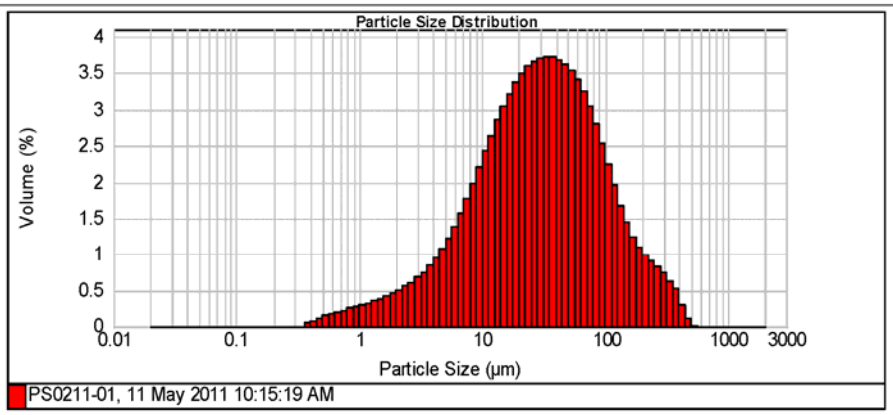
File name: 11 May 2011
Record Number: 2

MASTERSIZER 2000

Result Analysis Report

Sample Name: PS0211-01	**SOP Name:** Jaco (TMC)	**Measured:** 11 May 2011 10:15:19 AM	
Sample Source & type:	**Measured by:** Jaco van Heerden	**Analysed:** 11 May 2011 10:15:20 AM	
Sample bulk lot ref:	**Result Source:** Measurement		
Particle Name: Titanium Dioxide	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.741	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 18.51 %
Dispersant Name: Alcohol	**Dispersant RI:** 1.320	**Weighted Residual:** 0.810 %	**Result Emulation:** Off
Concentration: 0.0259 %Vol	**Span :** 4.205	**Uniformity:** 1.37	**Result units:** Volume
Specific Surface Area: 0.579 m²/g	**Surface Weighted Mean D[3,2]:** 10.354 um	**Vol. Weighted Mean D[4,3]:** 54.373 um	

d(0.1): 5.165 um **d(0.5):** 29.867 um **d(0.9):** 130.744 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 %
0.020	0.00	0.142	0.00	1.002	0.30	7.096	1.77	50.238	3.53	355.656	0.51
0.022	0.00	0.159	0.00	1.125	0.32	7.962	1.99	56.368	3.41	399.052	0.29
0.025	0.00	0.178	0.00	1.262	0.35	8.934	2.21	63.246	3.24	447.744	0.12
0.028	0.00	0.200	0.00	1.416	0.38	10.024	2.43	70.963	3.04	502.377	0.01
0.032	0.00	0.224	0.00	1.589	0.42	11.247	2.65	79.621	2.80	563.677	0.00
0.036	0.00	0.252	0.00	1.783	0.46	12.619	2.86	89.337	2.53	632.456	0.00
0.040	0.00	0.283	0.00	2.000	0.50	14.159	3.05	100.237	2.24	709.627	0.00
0.045	0.00	0.317	0.00	2.244	0.55	15.887	3.22	112.468	1.95	796.214	0.00
0.050	0.00	0.356	0.05	2.518	0.61	17.825	3.37	126.191	1.68	893.367	0.00
0.056	0.00	0.399	0.08	2.825	0.68	20.000	3.50	141.589	1.44	1002.374	0.00
0.063	0.00	0.448	0.12	3.170	0.76	22.440	3.60	158.866	1.25	1124.683	0.00
0.071	0.00	0.502	0.14	3.557	0.85	25.179	3.67	178.250	1.10	1261.915	0.00
0.080	0.00	0.564	0.17	3.991	0.95	28.251	3.71	200.000	1.00	1415.892	0.00
0.089	0.00	0.632	0.20	4.477	1.08	31.698	3.73	224.404	0.91	1588.656	0.00
0.100	0.00	0.710	0.23	5.024	1.22	35.566	3.72	251.785	0.84	1782.502	0.00
0.112	0.00	0.796	0.25	5.637	1.39	39.905	3.69	282.508	0.75	2000.000	0.00
0.126	0.00	0.893	0.27	6.325	1.57	44.774	3.63	316.979	0.63		
0.142	0.00	1.002		7.096		50.238		355.656			

Operator notes:

Malvern Instruments Ltd.
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007548

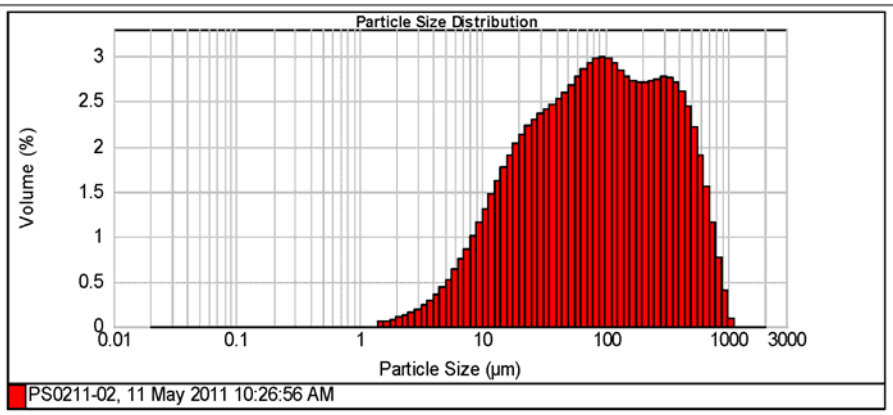
File name: 11 May 2011
Record Number: 3

MASTERSIZER 2000

Result Analysis Report

Sample Name: PS0211-02	**SOP Name:** Jaco (TMC)	**Measured:** 11 May 2011 10:26:56 AM	
Sample Source & type:	**Measured by:** Jaco van Heerden	**Analysed:** 11 May 2011 10:26:57 AM	
Sample bulk lot ref:	**Result Source:** Measurement		
Particle Name: Titanium Dioxide	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.741	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 17.44 %
Dispersant Name: Alcohol	**Dispersant RI:** 1.320	**Weighted Residual:** 0.750 %	**Result Emulation:** Off
Concentration: 0.0825 %Vol	**Span :** 5.079	**Uniformity:** 1.52	**Result units:** Volume
Specific Surface Area: 0.186 m²/g	**Surface Weighted Mean D[3,2]:** 32.199 um	**Vol. Weighted Mean D[4,3]:** 168.989 um	

d(0.1): 12.793 um **d(0.5):** 87.929 um **d(0.9):** 459.350 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 %
0.020	0.00	0.142	0.00	1.002	0.00	7.096	0.87	50.238	2.68	355.656	2.72
0.022	0.00	0.159	0.00	1.125	0.00	7.962	1.01	56.368	2.77	399.052	2.62
0.025	0.00	0.178	0.00	1.262	0.00	8.934	1.16	63.246	2.86	447.744	2.45
0.028	0.00	0.200	0.00	1.416	0.05	10.024	1.31	70.963	2.93	502.377	2.21
0.032	0.00	0.224	0.00	1.589	0.06	11.247	1.47	79.621	2.98	563.677	1.91
0.036	0.00	0.252	0.00	1.783	0.08	12.619	1.62	89.337	3.00	632.456	1.55
0.040	0.00	0.283	0.00	2.000	0.10	14.159	1.77	100.237	2.98	709.627	1.16
0.045	0.00	0.317	0.00	2.244	0.12	15.887	1.91	112.468	2.92	796.214	0.77
0.050	0.00	0.356	0.00	2.518	0.16	17.825	2.03	126.191	2.85	893.367	0.41
0.056	0.00	0.399	0.00	2.825	0.19	20.000	2.14	141.589	2.78	1002.374	0.10
0.063	0.00	0.448	0.00	3.170	0.24	22.440	2.23	158.866	2.73	1124.683	0.00
0.071	0.00	0.502	0.00	3.557	0.29	25.179	2.30	178.250	2.71	1261.915	0.00
0.080	0.00	0.564	0.00	3.991	0.36	28.251	2.36	200.000	2.71	1415.892	0.00
0.089	0.00	0.632	0.00	4.477	0.44	31.698	2.41	224.404	2.73	1588.656	0.00
0.100	0.00	0.710	0.00	5.024	0.53	35.566	2.47	251.785	2.75	1782.502	0.00
0.112	0.00	0.796	0.00	5.637	0.63	39.905	2.53	282.508	2.77	2000.000	
0.126	0.00	0.893	0.00	6.325	0.74	44.774	2.60	316.979	2.76		
0.142		1.002		7.096		50.238		355.656			

Operator notes:

Malvern Instruments Ltd.
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007548

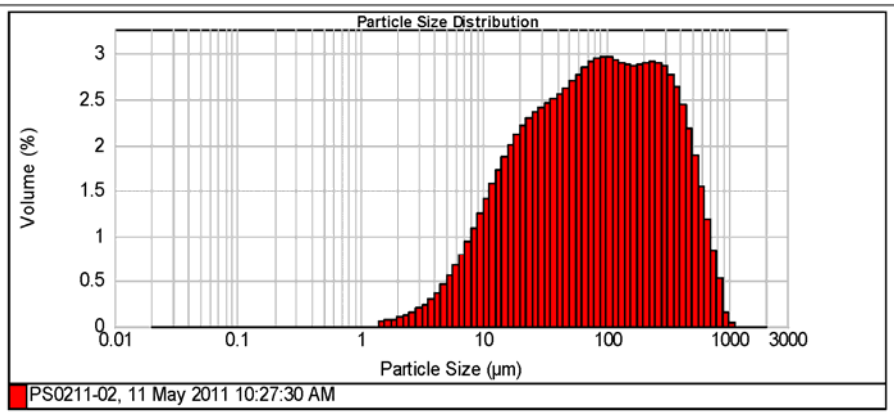
File name: 11 May 2011
Record Number: 4

MASTERSIZER 2000

Result Analysis Report

Sample Name: PS0211-02	**SOP Name:** Jaco (TMC)	**Measured:** 11 May 2011 10:27:30 AM	
Sample Source & type:	**Measured by:** Jaco van Heerden	**Analysed:** 11 May 2011 10:27:31 AM	
Sample bulk lot ref:	**Result Source:** Measurement		
Particle Name: Titanium Dioxide	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.741	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 18.26 %
Dispersant Name: Alcohol	**Dispersant RI:** 1.320	**Weighted Residual:** 0.708 %	**Result Emulation:** Off
Concentration: 0.0833 %Vol	**Span :** 4.825	**Uniformity:** 1.47	**Result units:** Volume
Specific Surface Area: 0.194 m²/g	**Surface Weighted Mean D[3,2]:** 30.919 um	**Vol. Weighted Mean D[4,3]:** 155.294 um	

d(0.1): 12.221 um **d(0.5):** 83.263 um **d(0.9):** 413.944 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 %
0.020	0.00	0.142	0.00	1.002	0.00	7.096	0.94	50.238	2.70	355.656	2.64
0.022	0.00	0.159	0.00	1.125	0.00	7.962	1.09	56.368	2.77	399.052	2.44
0.025	0.00	0.178	0.00	1.262	0.00	8.934	1.25	63.246	2.85	447.744	2.19
0.028	0.00	0.200	0.00	1.416	0.05	10.024	1.41	70.963	2.91	502.377	1.88
0.032	0.00	0.224	0.00	1.589	0.07	11.247	1.57	79.621	2.95	563.677	1.55
0.036	0.00	0.252	0.00	1.783	0.08	12.619	1.73	89.337	2.97	632.456	1.19
0.040	0.00	0.283	0.00	2.000	0.10	14.159	1.88	100.237	2.96	709.627	0.83
0.045	0.00	0.317	0.00	2.244	0.13	15.887	2.01	112.468	2.93	796.214	0.52
0.050	0.00	0.356	0.00	2.518	0.16	17.825	2.12	126.191	2.90	893.367	0.15
0.056	0.00	0.399	0.00	2.825	0.20	20.000	2.22	141.589	2.88	1002.374	0.04
0.063	0.00	0.448	0.00	3.170	0.24	22.440	2.30	158.866	2.87	1124.683	0.00
0.071	0.00	0.502	0.00	3.557	0.30	25.179	2.36	178.250	2.88	1261.915	0.00
0.080	0.00	0.564	0.00	3.991	0.37	28.251	2.41	200.000	2.90	1415.892	0.00
0.089	0.00	0.632	0.00	4.477	0.46	31.698	2.46	224.404	2.91	1588.656	0.00
0.100	0.00	0.710	0.00	5.024	0.56	35.566	2.51	251.785	2.90	1782.502	0.00
0.112	0.00	0.796	0.00	5.637	0.67	39.905	2.56	282.508	2.86	2000.000	
0.126	0.00	0.893	0.00	6.325	0.80	44.774	2.63	316.979	2.78		
0.142		1.002		7.096		50.238		355.656			

Operator notes:

Malvern Instruments Ltd.
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007548

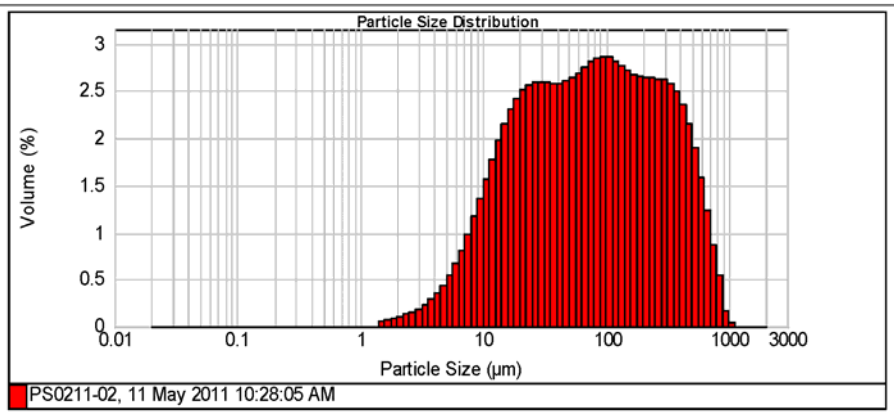
File name: 11 May 2011
Record Number: 5

MASTERSIZER 2000

Result Analysis Report

Sample Name: PS0211-02	**SOP Name:** Jaco (TMC)	**Measured:** 11 May 2011 10:28:05 AM	
Sample Source & type:	**Measured by:** Jaco van Heerden	**Analysed:** 11 May 2011 10:28:06 AM	
Sample bulk lot ref:	**Result Source:** Measurement		
Particle Name: Titanium Dioxide	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.741	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 19.41 %
Dispersant Name: Alcohol	**Dispersant RI:** 1.320	**Weighted Residual:** 0.640 %	**Result Emulation:** Off
Concentration: 0.0854 %Vol	**Span :** 5.356	**Uniformity:** 1.6	**Result units:** Volume
Specific Surface Area: 0.203 m²/g	**Surface Weighted Mean D[3,2]:** 29.618 um	**Vol. Weighted Mean D[4,3]:** 150.796 um	

d(0.1): 11.871 um **d(0.5): 75.383 um** **d(0.9): 415.640 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 %
0.020	0.00	0.142	0.00	1.002	0.00	7.096	0.98	50.238	2.64	355.656	2.50
0.022	0.00	0.159	0.00	1.125	0.00	7.962	1.17	56.368	2.69	399.052	2.36
0.025	0.00	0.178	0.00	1.262	0.00	8.934	1.37	63.246	2.75	447.744	2.16
0.028	0.00	0.200	0.00	1.416	0.06	10.024	1.57	70.963	2.81	502.377	1.90
0.032	0.00	0.224	0.00	1.589	0.07	11.247	1.78	79.621	2.85	563.677	1.59
0.036	0.00	0.252	0.00	1.783	0.09	12.619	1.98	89.337	2.87	632.456	1.23
0.040	0.00	0.283	0.00	2.000	0.10	14.159	2.15	100.237	2.86	709.627	0.87
0.045	0.00	0.317	0.00	2.244	0.13	15.887	2.30	112.468	2.82	796.214	0.55
0.050	0.00	0.356	0.00	2.518	0.15	17.825	2.42	126.191	2.77	893.367	0.16
0.056	0.00	0.399	0.00	2.825	0.19	20.000	2.51	141.589	2.72	1002.374	0.04
0.063	0.00	0.448	0.00	3.170	0.23	22.440	2.56	158.866	2.68	1124.683	0.00
0.071	0.00	0.502	0.00	3.557	0.28	25.179	2.59	178.250	2.65	1261.915	0.00
0.080	0.00	0.564	0.00	3.991	0.35	28.251	2.59	200.000	2.64	1415.892	0.00
0.089	0.00	0.632	0.00	4.477	0.44	31.698	2.59	224.404	2.64	1588.656	0.00
0.100	0.00	0.710	0.00	5.024	0.54	35.566	2.58	251.785	2.63	1782.502	0.00
0.112	0.00	0.796	0.00	5.637	0.66	39.905	2.59	282.508	2.62	2000.000	
0.126	0.00	0.893	0.00	6.325	0.81	44.774	2.60	316.979	2.58		
0.142		1.002		7.096		50.238		355.656			

Operator notes:

Malvern Instruments Ltd.
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007548

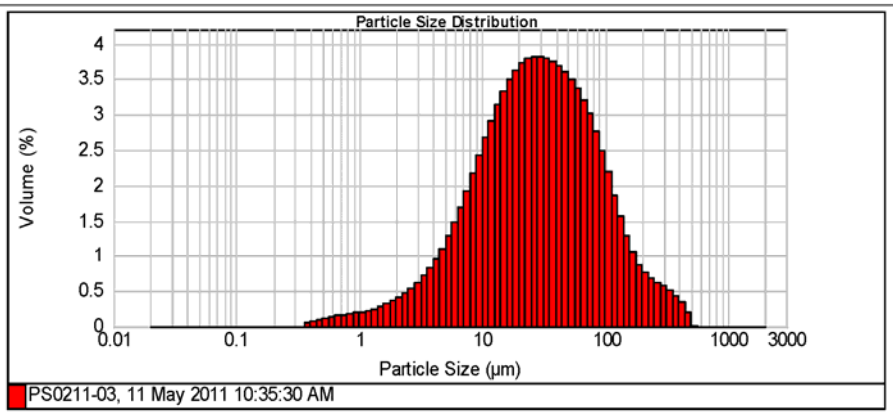
File name: 11 May 2011
Record Number: 6

MASTERSIZER 2000

Result Analysis Report

Sample Name: PS0211-03	**SOP Name:** Jaco (TMC)	**Measured:** 11 May 2011 10:35:30 AM	
Sample Source & type:	**Measured by:** Jaco van Heerden	**Analysed:** 11 May 2011 10:35:31 AM	
Sample bulk lot ref:	**Result Source:** Measurement		
Particle Name: Titanium Dioxide	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.741	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 18.24 %
Dispersant Name: Alcohol	**Dispersant RI:** 1.320	**Weighted Residual:** 0.845 %	**Result Emulation:** Off
Concentration: 0.0281 %Vol	**Span :** 3.973	**Uniformity:** 1.35	**Result units:** Volume
Specific Surface Area: 0.532 m²/g	**Surface Weighted Mean D[3,2]:** 11.280 um	**Vol. Weighted Mean D[4,3]:** 51.143 um	

d(0.1): 5.738 um **d(0.5):** 28.210 um **d(0.9):** 117.817 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 %
0.020	0.00	0.142	0.00	1.002	0.21	7.096	1.93	50.238	3.49	355.656	0.44
0.022	0.00	0.159	0.00	1.125	0.23	7.962	2.18	56.368	3.36	399.052	0.35
0.025	0.00	0.178	0.00	1.262	0.25	8.934	2.43	63.246	3.20	447.744	0.21
0.028	0.00	0.200	0.00	1.416	0.28	10.024	2.68	70.963	3.01	502.377	0.01
0.032	0.00	0.224	0.00	1.589	0.32	11.247	2.92	79.621	2.77	563.677	0.00
0.036	0.00	0.252	0.00	1.783	0.36	12.619	3.14	89.337	2.49	632.456	0.00
0.040	0.00	0.283	0.00	2.000	0.41	14.159	3.34	100.237	2.19	709.627	0.00
0.045	0.00	0.317	0.00	2.244	0.48	15.887	3.50	112.468	1.87	796.214	0.00
0.050	0.00	0.356	0.05	2.518	0.55	17.825	3.63	126.191	1.56	893.367	0.00
0.056	0.00	0.399	0.08	2.825	0.63	20.000	3.73	141.589	1.28	1002.374	0.00
0.063	0.00	0.448	0.09	3.170	0.72	22.440	3.79	158.866	1.06	1124.683	0.00
0.071	0.00	0.502	0.12	3.557	0.83	25.179	3.82	178.250	0.88	1261.915	0.00
0.080	0.00	0.564	0.13	3.991	0.96	28.251	3.82	200.000	0.76	1415.892	0.00
0.089	0.00	0.632	0.15	4.477	1.11	31.698	3.79	224.404	0.68	1588.656	0.00
0.100	0.00	0.710	0.17	5.024	1.29	35.566	3.75	251.785	0.62	1782.502	0.00
0.112	0.00	0.796	0.18	5.637	1.48	39.905	3.68	282.508	0.57	2000.000	
0.126	0.00	0.893	0.19	6.325	1.70	44.774	3.60	316.979	0.52		
0.142	0.00	1.002		7.096		50.238		355.656			

Operator notes:

Malvern Instruments Ltd.
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007548

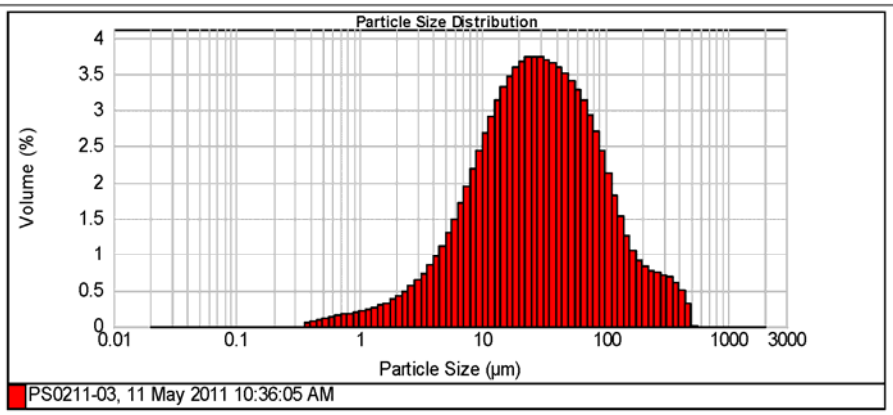
File name: 11 May 2011
Record Number: 7

MASTERSIZER 2000

Result Analysis Report

Sample Name: PS0211-03	**SOP Name:** Jaco (TMC)	**Measured:** 11 May 2011 10:36:05 AM	
Sample Source & type:	**Measured by:** Jaco van Heerden	**Analysed:** 11 May 2011 10:36:06 AM	
Sample bulk lot ref:	**Result Source:** Measurement		
Particle Name: Titanium Dioxide	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.741	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 18.63 %
Dispersant Name: Alcohol	**Dispersant RI:** 1.320	**Weighted Residual:** 1.144 %	**Result Emulation:** Off
Concentration: 0.0285 %Vol	**Span :** 4.258	**Uniformity:** 1.45	**Result units:** Volume
Specific Surface Area: 0.536 m²/g	**Surface Weighted Mean D[3,2]:** 11.190 um	**Vol. Weighted Mean D[4,3]:** 53.987 um	

d(0.1): 5.647 um **d(0.5):** 28.225 um **d(0.9):** 125.816 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 %
0.020	0.00	0.142	0.00	1.002	0.21	7.096	1.94	50.238	3.41	355.656	0.60
0.022	0.00	0.159	0.00	1.125	0.23	7.962	2.19	56.368	3.29	399.052	0.50
0.025	0.00	0.178	0.00	1.262	0.26	8.934	2.44	63.246	3.13	447.744	0.32
0.028	0.00	0.200	0.00	1.416	0.29	10.024	2.69	70.963	2.94	502.377	0.01
0.032	0.00	0.224	0.00	1.589	0.33	11.247	2.92	79.621	2.70	563.677	0.00
0.036	0.00	0.252	0.00	1.783	0.37	12.619	3.13	89.337	2.43	632.456	0.00
0.040	0.00	0.283	0.00	2.000	0.43	14.159	3.32	100.237	2.13	709.627	0.00
0.045	0.00	0.317	0.00	2.244	0.49	15.887	3.47	112.468	1.82	796.214	0.00
0.050	0.00	0.356	0.05	2.518	0.56	17.825	3.59	126.191	1.53	893.367	0.00
0.056	0.00	0.399	0.08	2.825	0.64	20.000	3.67	141.589	1.27	1002.374	0.00
0.063	0.00	0.448	0.10	3.170	0.74	22.440	3.72	158.866	1.07	1124.683	0.00
0.071	0.00	0.502	0.12	3.557	0.85	25.179	3.74	178.250	0.92	1261.915	0.00
0.080	0.00	0.564	0.14	3.991	0.98	28.251	3.73	200.000	0.83	1415.892	0.00
0.089	0.00	0.632	0.15	4.477	1.13	31.698	3.70	224.404	0.77	1588.656	0.00
0.100	0.00	0.710	0.17	5.024	1.30	35.566	3.65	251.785	0.74	1782.502	0.00
0.112	0.00	0.796	0.18	5.637	1.50	39.905	3.59	282.508	0.72	2000.000	0.00
0.126	0.00	0.893	0.20	6.325	1.71	44.774	3.51	316.979	0.68		
0.142	0.00	1.002		7.096		50.238		355.656			

Operator notes:

Malvern Instruments Ltd.
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007548

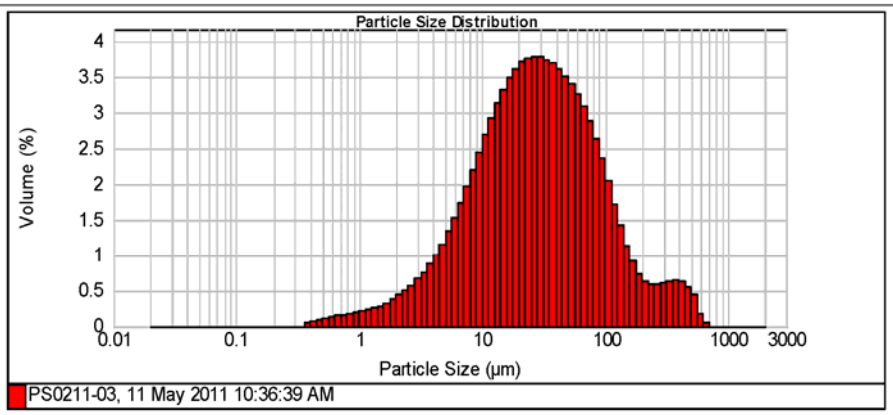
File name: 11 May 2011
Record Number: 8

MASTERSIZER 2000

Result Analysis Report

Sample Name: PS0211-03	**SOP Name:** Jaco (TMC)	**Measured:** 11 May 2011 10:36:39 AM	
Sample Source & type:	**Measured by:** Jaco van Heerden	**Analysed:** 11 May 2011 10:36:40 AM	
Sample bulk lot ref:	**Result Source:** Measurement		
Particle Name: Titanium Dioxide	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.741	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 19.04 %
Dispersant Name: Alcohol	**Dispersant RI:** 1.320	**Weighted Residual:** 0.756 %	**Result Emulation:** Off
Concentration: 0.0289 %Vol	**Span :** 4.271	**Uniformity:** 1.57	**Result units:** Volume
Specific Surface Area: 0.541 m²/g	**Surface Weighted Mean D[3,2]:** 11.090 um	**Vol. Weighted Mean D[4,3]:** 56.642 um	

d(0.1): 5.539 um **d(0.5):** 27.825 um **d(0.9):** 124.384 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 %
0.020	0.00	0.142	0.00	1.002	0.21	7.096	1.96	50.238	3.40	355.656	0.65
0.022	0.00	0.159	0.00	1.125	0.23	7.962	2.20	56.368	3.26	399.052	0.63
0.025	0.00	0.178	0.00	1.262	0.26	8.934	2.44	63.246	3.09	447.744	0.56
0.028	0.00	0.200	0.00	1.416	0.29	10.024	2.69	70.963	2.88	502.377	0.45
0.032	0.00	0.224	0.00	1.589	0.33	11.247	2.92	79.621	2.64	563.677	0.18
0.036	0.00	0.252	0.00	1.783	0.38	12.619	3.14	89.337	2.35	632.456	0.06
0.040	0.00	0.283	0.00	2.000	0.44	14.159	3.33	100.237	2.05	709.627	0.00
0.045	0.00	0.317	0.00	2.244	0.51	15.887	3.49	112.468	1.73	796.214	0.00
0.050	0.00	0.356	0.05	2.518	0.58	17.825	3.62	126.191	1.42	893.367	0.00
0.056	0.00	0.399	0.08	2.825	0.67	20.000	3.71	141.589	1.14	1002.374	0.00
0.063	0.00	0.448	0.10	3.170	0.77	22.440	3.76	158.866	0.92	1124.683	0.00
0.071	0.00	0.502	0.12	3.557	0.88	25.179	3.78	178.250	0.75	1261.915	0.00
0.080	0.00	0.564	0.14	3.991	1.01	28.251	3.77	200.000	0.64	1415.892	0.00
0.089	0.00	0.632	0.15	4.477	1.16	31.698	3.74	224.404	0.59	1588.656	0.00
0.100	0.00	0.710	0.17	5.024	1.33	35.566	3.68	251.785	0.58	1782.502	0.00
0.112	0.00	0.796	0.18	5.637	1.52	39.905	3.61	282.508	0.61	2000.000	
0.126	0.00	0.893	0.19	6.325	1.73	44.774	3.51	316.979	0.63		
0.142		1.002		7.096		50.238		355.656			

Operator notes:

2. Zetasizer: TMC-TPP particles (TMC:TPP ratio – 2:1)

MALVERN ZETASIZER

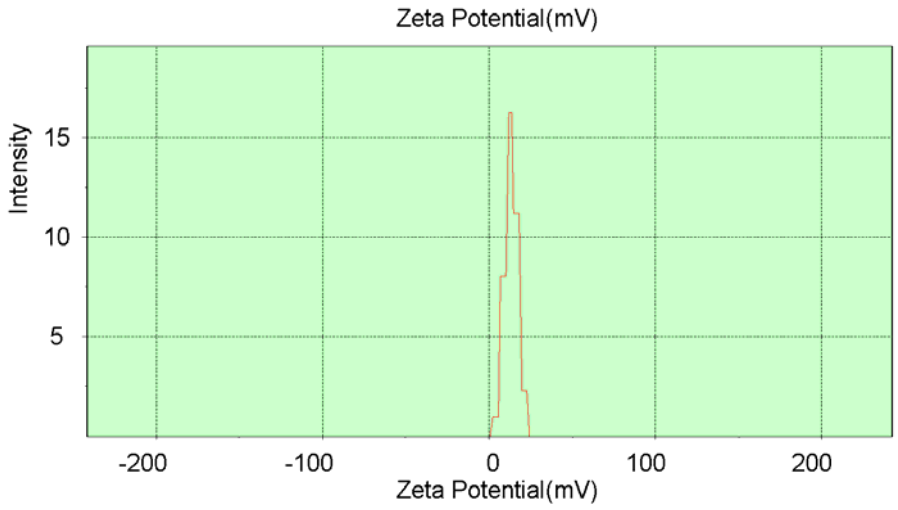
Zeta Potential Report

PS0211-01
TMC partikels 2:1 (TMC:TPP)
Jaco van Heerden

Sample		System	
Record Number:	1	Instrument Type:	Zetasizer 2000
Filename:	ZSIZER.zet	Temperature (°C):	25.0
File Path:	G:\MALVERN\ZETASI~1\JACOV	Count rate (kCps):	4492.5
Dielectric Constant:	79.7	Cell Type:	Capillary cell
pH:	N/A	Cell Position (%):	50.00
Viscosity (cP):	0.890	Cell field (V/cm):	29.0
Date (DMY):	11/05/11	Current (mA):	7.7
Time:	14:15:01		

Result

Zeta Potential (mV):	13.2	Mobility (umcm/V.s):	0.989
StDev (mV):	1.6	StDev (umcm/V.s):	0.126
Conductivity (mS/cm):	2.67	F(ka):	1.50



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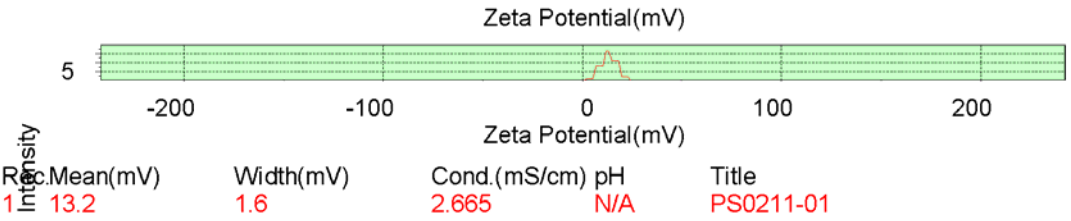
Int.	Zeta(mV)	Mob(µmcm/Vs)	Freq.(Hz)	Int.	Zeta(mV)	Mob(µmcm/Vs)
0.0	-200.0	-15.859	32.3	4.3	3.2	0.252
0.0	-193.7	-15.356	39.2	35.8	9.5	0.755
0.0	-187.3	-14.852	46.1	49.7	15.9	1.259
0.0	-181.0	-14.349	53.0	10.1	22.2	1.762
0.0	-174.6	-13.846	59.9	0.0	28.6	2.266
0.0	-168.3	-13.342	66.8	0.0	34.9	2.769
0.0	-161.9	-12.839	73.8	0.0	41.3	3.273
0.0	-155.6	-12.335	80.7	0.0	47.6	3.776
0.0	-149.2	-11.832	87.6	0.0	54.0	4.280
0.0	-142.9	-11.328	94.5	0.0	60.3	4.783
0.0	-136.5	-10.825	101.4	0.0	66.7	5.286
0.0	-130.2	-10.321	108.3	0.0	73.0	5.790
0.0	-123.8	-9.818	115.2	0.0	79.4	6.293
0.0	-117.5	-9.314	122.1	0.0	85.7	6.797
0.0	-111.1	-8.811	129.1	0.0	92.1	7.300
0.0	-104.8	-8.307	136.0	0.0	98.4	7.804
0.0	-98.4	-7.804	142.9	0.0	104.8	8.307
0.0	-92.1	-7.300	149.8	0.0	111.1	8.811
0.0	-85.7	-6.797	156.7	0.0	117.5	9.314
0.0	-79.4	-6.293	163.6	0.0	123.8	9.818
0.0	-73.0	-5.790	170.5	0.0	130.2	10.321
0.0	-66.7	-5.286	177.4	0.0	136.5	10.825
0.0	-60.3	-4.783	184.3	0.0	142.9	11.328
0.0	-54.0	-4.280	191.3	0.0	149.2	11.832
0.0	-47.6	-3.776	198.2	0.0	155.6	12.335
0.0	-41.3	-3.273	205.1	0.0	161.9	12.839
0.0	-34.9	-2.769	212.0	0.0	168.3	13.342
0.0	-28.6	-2.266	218.9	0.0	174.6	13.846
0.0	-22.2	-1.762	225.8	0.0	181.0	14.349
0.0	-15.9	-1.259	232.7	0.0	187.3	14.852
0.0	-9.5	-0.755	239.6	0.0	193.7	15.356
0.0	-3.2	-0.252	246.5	0.0	200.0	15.859



Rec.	Mean(mV)	Width(mV)	Cond.(mS/cm)	pH	Title
1	13.2	1.6	2.665	N/A	PS0211-01

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MALVERN ZETASIZER

Zeta Potential Report

PS0211-01
TMC partikels 2:1 (TMC:TPP)
Jaco van Heerden

Sample

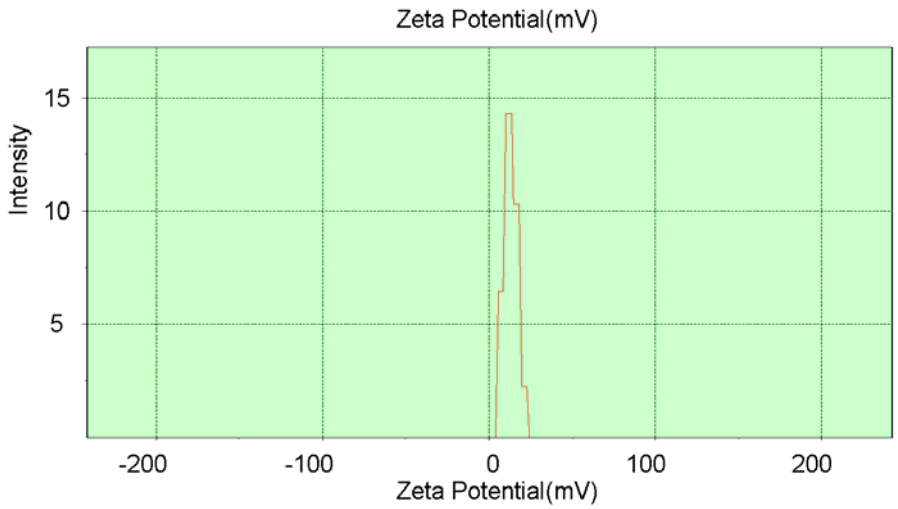
Record Number:	2
Filename:	ZSIZER.zet
File Path:	G:\MALVERN\ZETASI~1\JACOV
Dielectric Constant:	79.7
pH:	N/A
Viscosity (cP):	0.890
Date (DMY):	11/05/11
Time:	14:16:00

System

Instrument Type:	Zetasizer 2000
Temperature (°C):	25.0
Count rate (kCps):	4807.8
Cell Type:	Capillary cell
Cell Position (%):	50.00
Cell field (V/cm):	29.0
Current (mA):	7.8

Result

Zeta Potential (mV):	12.7	Mobility (umcm/V.s):	0.994
StDev (mV):	1.6	StDev (umcm/V.s):	0.126
Conductivity (mS/cm):	2.68	F(ka):	1.50



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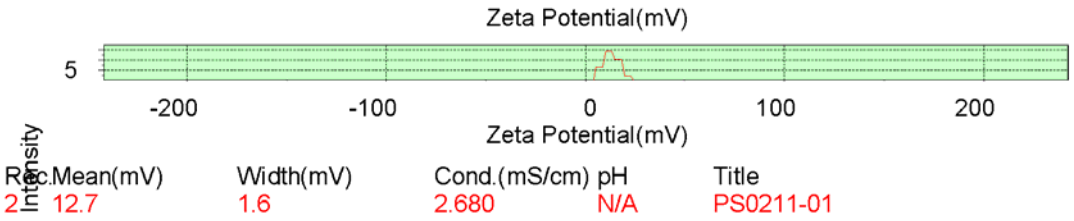
Int.	Zeta(mV)	Mob(µmcm/Vs)	Freq.(Hz)	Int.	Zeta(mV)	Mob(µmcm/Vs)
0.0	-200.0	-15.856	33.2	0.0	3.2	0.252
0.0	-193.7	-15.353	40.1	53.2	9.5	0.755
0.0	-187.3	-14.849	47.0	38.4	15.9	1.258
0.0	-181.0	-14.346	53.9	8.4	22.2	1.762
0.0	-174.6	-13.843	60.8	0.0	28.6	2.265
0.0	-168.3	-13.339	67.8	0.0	34.9	2.769
0.0	-161.9	-12.836	74.7	0.0	41.3	3.272
0.0	-155.6	-12.333	81.6	0.0	47.6	3.775
0.0	-149.2	-11.829	88.5	0.0	54.0	4.279
0.0	-142.9	-11.326	95.4	0.0	60.3	4.782
0.0	-136.5	-10.822	102.3	0.0	66.7	5.285
0.0	-130.2	-10.319	109.3	0.0	73.0	5.789
0.0	-123.8	-9.816	116.2	0.0	79.4	6.292
0.0	-117.5	-9.312	123.1	0.0	85.7	6.795
0.0	-111.1	-8.809	130.0	0.0	92.1	7.299
0.0	-104.8	-8.306	136.9	0.0	98.4	7.802
0.0	-98.4	-7.802	143.8	0.0	104.8	8.306
0.0	-92.1	-7.299	150.7	0.0	111.1	8.809
0.0	-85.7	-6.795	157.7	0.0	117.5	9.312
0.0	-79.4	-6.292	164.6	0.0	123.8	9.816
0.0	-73.0	-5.789	171.5	0.0	130.2	10.319
0.0	-66.7	-5.285	178.4	0.0	136.5	10.822
0.0	-60.3	-4.782	185.3	0.0	142.9	11.326
0.0	-54.0	-4.279	192.2	0.0	149.2	11.829
0.0	-47.6	-3.775	199.1	0.0	155.6	12.333
0.0	-41.3	-3.272	206.1	0.0	161.9	12.836
0.0	-34.9	-2.769	213.0	0.0	168.3	13.339
0.0	-28.6	-2.265	219.9	0.0	174.6	13.843
0.0	-22.2	-1.762	226.8	0.0	181.0	14.346
0.0	-15.9	-1.258	233.7	0.0	187.3	14.849
0.0	-9.5	-0.755	240.6	0.0	193.7	15.353
0.0	-3.2	-0.252	247.5	0.0	200.0	15.856



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ZETASIZER

Zeta Potential Report

PS0211-01
TMC partikels 2:1 (TMC:TPP)
Jaco van Heerden

Sample

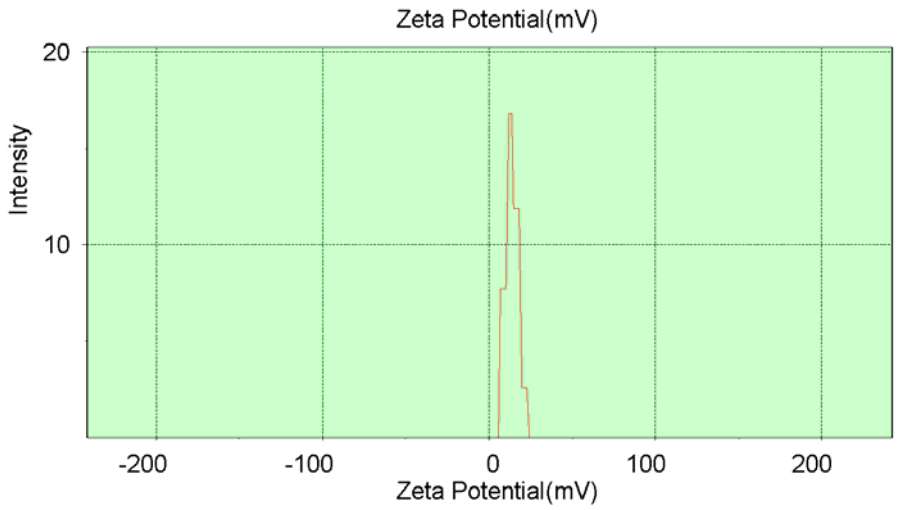
Record Number:	3
Filename:	ZSIZER.zet
File Path:	G:\MALVERN\ZETASI~1\JACOV
Dielectric Constant:	79.7
pH:	N/A
Viscosity (cP):	0.891
Date (DMY):	11/05/11
Time:	14:16:59

System

Instrument Type:	Zetasizer 2000
Temperature (°C):	25.0
Count rate (kCps):	5070.4
Cell Type:	Capillary cell
Cell Position (%):	50.00
Cell field (V/cm):	29.0
Current (mA):	7.8

Result

Zeta Potential (mV):	13.6	Mobility (umcm/V.s):	1.020
StDev (mV):	1.6	StDev (umcm/V.s):	0.126
Conductivity (mS/cm):	2.68	F(ka):	1.50



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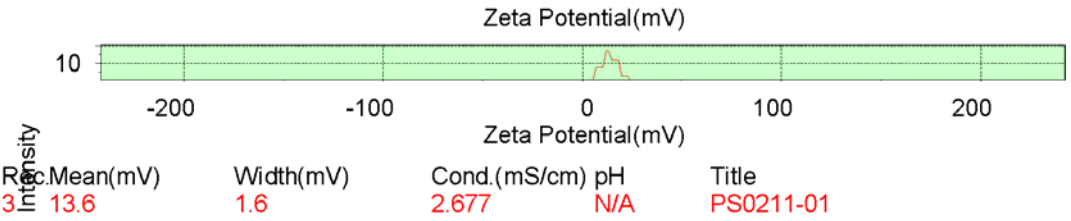
Int.	Zeta(mV)	Mob(µmcm/Vs)	Freq.(Hz)	Int.	Zeta(mV)	Mob(µmcm/Vs)
0.0	-200.0	-15.852	32.1	1.9	3.2	0.252
0.0	-193.7	-15.349	39.0	34.0	9.5	0.755
0.0	-187.3	-14.846	45.9	52.7	15.9	1.258
0.0	-181.0	-14.342	52.9	11.4	22.2	1.761
0.0	-174.6	-13.839	59.8	0.0	28.6	2.265
0.0	-168.3	-13.336	66.7	0.0	34.9	2.768
0.0	-161.9	-12.833	73.6	0.0	41.3	3.271
0.0	-155.6	-12.329	80.5	0.0	47.6	3.774
0.0	-149.2	-11.826	87.4	0.0	54.0	4.278
0.0	-142.9	-11.323	94.4	0.0	60.3	4.781
0.0	-136.5	-10.820	101.3	0.0	66.7	5.284
0.0	-130.2	-10.316	108.2	0.0	73.0	5.787
0.0	-123.8	-9.813	115.1	0.0	79.4	6.291
0.0	-117.5	-9.310	122.0	0.0	85.7	6.794
0.0	-111.1	-8.807	128.9	0.0	92.1	7.297
0.0	-104.8	-8.303	135.9	0.0	98.4	7.800
0.0	-98.4	-7.800	142.8	0.0	104.8	8.303
0.0	-92.1	-7.297	149.7	0.0	111.1	8.807
0.0	-85.7	-6.794	156.6	0.0	117.5	9.310
0.0	-79.4	-6.291	163.5	0.0	123.8	9.813
0.0	-73.0	-5.787	170.4	0.0	130.2	10.316
0.0	-66.7	-5.284	177.4	0.0	136.5	10.820
0.0	-60.3	-4.781	184.3	0.0	142.9	11.323
0.0	-54.0	-4.278	191.2	0.0	149.2	11.826
0.0	-47.6	-3.774	198.1	0.0	155.6	12.329
0.0	-41.3	-3.271	205.0	0.0	161.9	12.833
0.0	-34.9	-2.768	212.0	0.0	168.3	13.336
0.0	-28.6	-2.265	218.9	0.0	174.6	13.839
0.0	-22.2	-1.761	225.8	0.0	181.0	14.342
0.0	-15.9	-1.258	232.7	0.0	187.3	14.846
0.0	-9.5	-0.755	239.6	0.0	193.7	15.349
0.0	-3.2	-0.252	246.5	0.0	200.0	15.852



Rec.Mean(mV)	Width(mV)	Cond.(mS/cm)	pH	Title
3 13.6	1.6	2.677	N/A	PS0211-01

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ZETASIZER

Zeta Potential Report

PS0211-02
TMC partikels 2:1 (TMC:TPP) Tween 80
Jaco van Heerden

Sample

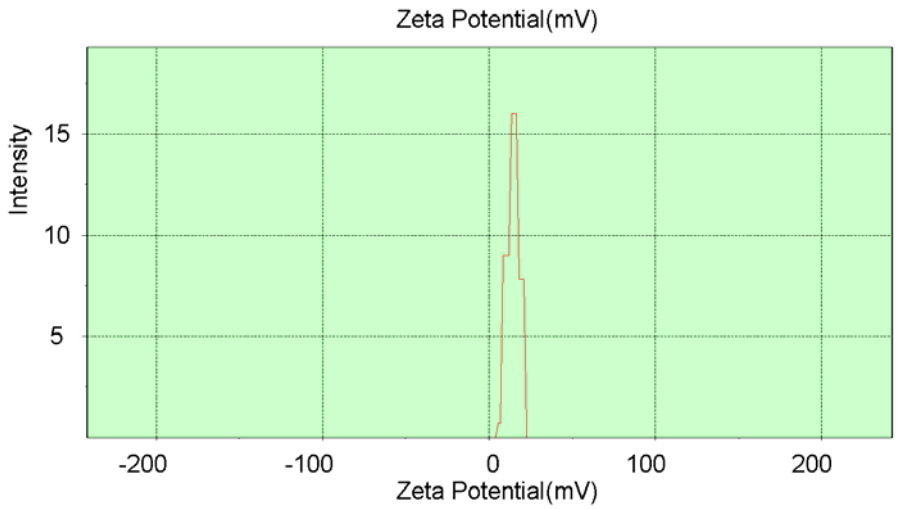
Record Number:	4
Filename:	ZSIZER.zet
File Path:	G:\MALVERN\ZETASI~1\JACOV
Dielectric Constant:	79.7
pH:	N/A
Viscosity (cP):	0.891
Date (DMY):	11/05/11
Time:	14:28:01

System

Instrument Type:	Zetasizer 2000
Temperature (°C):	25.0
Count rate (kCps):	3769.3
Cell Type:	Capillary cell
Cell Position (%):	50.00
Cell field (V/cm):	29.1
Current (mA):	4.5

Result

Zeta Potential (mV):	14.7	Mobility (umcm/V.s):	1.143
StDev (mV):	1.6	StDev (umcm/V.s):	0.125
Conductivity (mS/cm):	1.54	F(ka):	1.50



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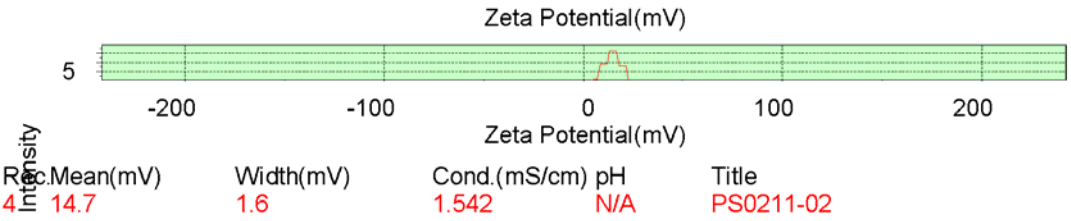
Int.	Zeta(mV)	Mob(µmcm/Vs)	Freq.(Hz)	Int.	Zeta(mV)	Mob(µmcm/Vs)
0.0	-200.0	-15.846	33.4	0.0	3.2	0.252
0.0	-193.7	-15.343	40.4	36.0	9.5	0.755
0.0	-187.3	-14.840	47.3	64.0	15.9	1.258
0.0	-181.0	-14.337	54.3	0.0	22.2	1.761
0.0	-174.6	-13.834	61.2	0.0	28.6	2.264
0.0	-168.3	-13.331	68.1	0.0	34.9	2.767
0.0	-161.9	-12.828	75.1	0.0	41.3	3.270
0.0	-155.6	-12.325	82.0	0.0	47.6	3.773
0.0	-149.2	-11.822	88.9	0.0	54.0	4.276
0.0	-142.9	-11.319	95.9	0.0	60.3	4.779
0.0	-136.5	-10.816	102.8	0.0	66.7	5.282
0.0	-130.2	-10.313	109.8	0.0	73.0	5.785
0.0	-123.8	-9.810	116.7	0.0	79.4	6.288
0.0	-117.5	-9.307	123.6	0.0	85.7	6.791
0.0	-111.1	-8.804	130.6	0.0	92.1	7.294
0.0	-104.8	-8.301	137.5	0.0	98.4	7.797
0.0	-98.4	-7.797	144.5	0.0	104.8	8.301
0.0	-92.1	-7.294	151.4	0.0	111.1	8.804
0.0	-85.7	-6.791	158.3	0.0	117.5	9.307
0.0	-79.4	-6.288	165.3	0.0	123.8	9.810
0.0	-73.0	-5.785	172.2	0.0	130.2	10.313
0.0	-66.7	-5.282	179.1	0.0	136.5	10.816
0.0	-60.3	-4.779	186.1	0.0	142.9	11.319
0.0	-54.0	-4.276	193.0	0.0	149.2	11.822
0.0	-47.6	-3.773	200.0	0.0	155.6	12.325
0.0	-41.3	-3.270	206.9	0.0	161.9	12.828
0.0	-34.9	-2.767	213.8	0.0	168.3	13.331
0.0	-28.6	-2.264	220.8	0.0	174.6	13.834
0.0	-22.2	-1.761	227.7	0.0	181.0	14.337
0.0	-15.9	-1.258	234.7	0.0	187.3	14.840
0.0	-9.5	-0.755	241.6	0.0	193.7	15.343
0.0	-3.2	-0.252	248.5	0.0	200.0	15.846



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ZETASIZER

Zeta Potential Report

PS0211-02
TMC partikels 2:1 (TMC:TPP) Tween 80
Jaco van Heerden

Sample

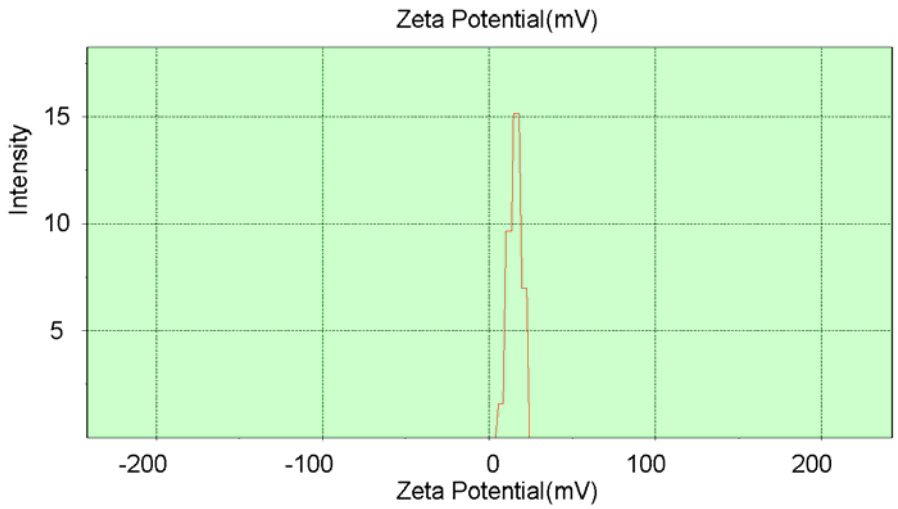
Record Number:	5
Filename:	ZSIZER.zet
File Path:	G:\MALVERN\ZETASI~1\JACOV
Dielectric Constant:	79.7
pH:	N/A
Viscosity (cP):	0.891
Date (DMY):	11/05/11
Time:	14:29:00

System

Instrument Type:	Zetasizer 2000
Temperature (°C):	25.0
Count rate (kCps):	3770.0
Cell Type:	Capillary cell
Cell Position (%):	50.00
Cell field (V/cm):	29.1
Current (mA):	4.5

Result

Zeta Potential (mV):	15.1	Mobility (umcm/V.s):	1.194
StDev (mV):	1.6	StDev (umcm/V.s):	0.125
Conductivity (mS/cm):	1.55	F(ka):	1.50



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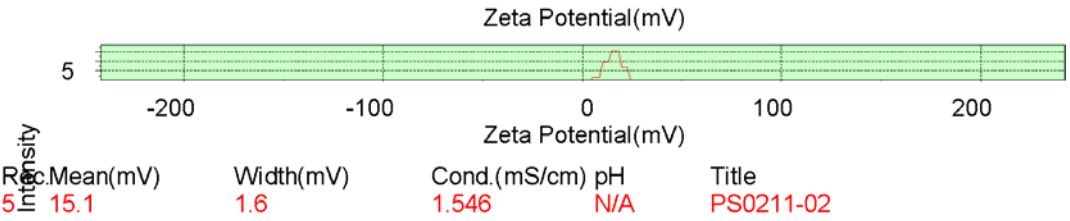
Int.	Zeta(mV)	Mob(µmcm/Vs)	Freq.(Hz)	Int.	Zeta(mV)	Mob(µmcm/Vs)
0.0	-200.0	-15.850	32.5	0.0	3.2	0.252
0.0	-193.7	-15.347	39.5	30.4	9.5	0.755
0.0	-187.3	-14.843	46.4	47.7	15.9	1.258
0.0	-181.0	-14.340	53.3	21.9	22.2	1.761
0.0	-174.6	-13.837	60.3	0.0	28.6	2.264
0.0	-168.3	-13.334	67.2	0.0	34.9	2.767
0.0	-161.9	-12.831	74.2	0.0	41.3	3.271
0.0	-155.6	-12.328	81.1	0.0	47.6	3.774
0.0	-149.2	-11.824	88.0	0.0	54.0	4.277
0.0	-142.9	-11.321	95.0	0.0	60.3	4.780
0.0	-136.5	-10.818	101.9	0.0	66.7	5.283
0.0	-130.2	-10.315	108.8	0.0	73.0	5.786
0.0	-123.8	-9.812	115.8	0.0	79.4	6.290
0.0	-117.5	-9.309	122.7	0.0	85.7	6.793
0.0	-111.1	-8.805	129.6	0.0	92.1	7.296
0.0	-104.8	-8.302	136.6	0.0	98.4	7.799
0.0	-98.4	-7.799	143.5	0.0	104.8	8.302
0.0	-92.1	-7.296	150.4	0.0	111.1	8.805
0.0	-85.7	-6.793	157.4	0.0	117.5	9.309
0.0	-79.4	-6.290	164.3	0.0	123.8	9.812
0.0	-73.0	-5.786	171.2	0.0	130.2	10.315
0.0	-66.7	-5.283	178.2	0.0	136.5	10.818
0.0	-60.3	-4.780	185.1	0.0	142.9	11.321
0.0	-54.0	-4.277	192.1	0.0	149.2	11.824
0.0	-47.6	-3.774	199.0	0.0	155.6	12.328
0.0	-41.3	-3.271	205.9	0.0	161.9	12.831
0.0	-34.9	-2.767	212.9	0.0	168.3	13.334
0.0	-28.6	-2.264	219.8	0.0	174.6	13.837
0.0	-22.2	-1.761	226.7	0.0	181.0	14.340
0.0	-15.9	-1.258	233.7	0.0	187.3	14.843
0.0	-9.5	-0.755	240.6	0.0	193.7	15.347
0.0	-3.2	-0.252	247.5	0.0	200.0	15.850



Rec.Mean(mV)	Width(mV)	Cond.(mS/cm)	pH	Title
5 15.1	1.6	1.546	N/A	PS0211-02

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ZETASIZER

Zeta Potential Report

PS0211-02
TMC partikels 2:1 (TMC:TPP) Tween 80
Jaco van Heerden

Sample

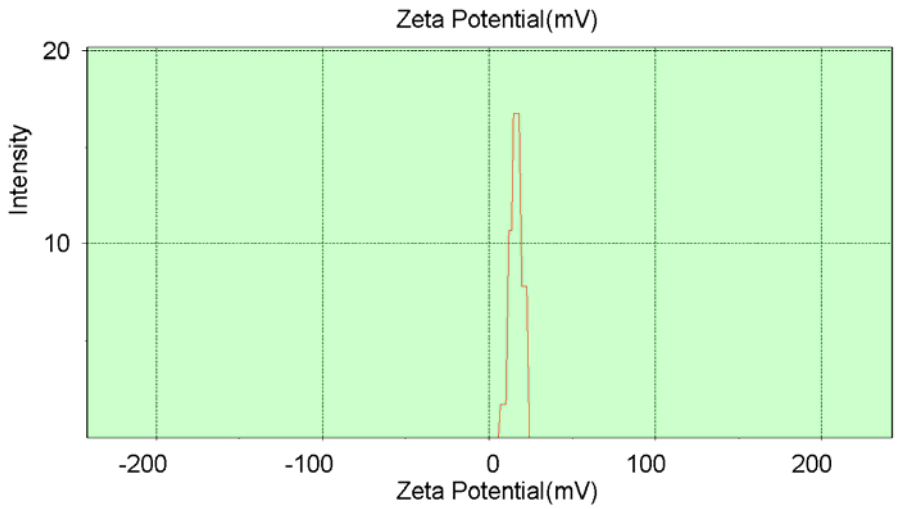
Record Number:	6
Filename:	ZSIZER.zet
File Path:	G:\MALVERN\ZETASI~1\JACOV
Dielectric Constant:	79.7
pH:	N/A
Viscosity (cP):	0.891
Date (DMY):	11/05/11
Time:	14:29:58

System

Instrument Type:	Zetasizer 2000
Temperature (°C):	25.0
Count rate (kCps):	3608.7
Cell Type:	Capillary cell
Cell Position (%):	50.00
Cell field (V/cm):	29.1
Current (mA):	4.5

Result

Zeta Potential (mV):	16.1	Mobility (umcm/V.s):	1.248
StDev (mV):	1.6	StDev (umcm/V.s):	0.125
Conductivity (mS/cm):	1.55	F(ka):	1.50



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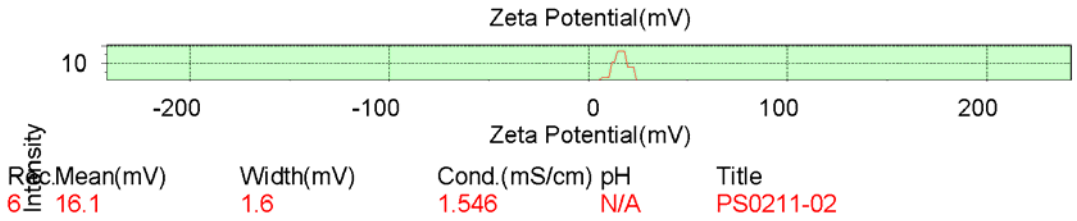
Int.	Zeta(mV)	Mob(µmcm/Vs)	Freq.(Hz)	Int.	Zeta(mV)	Mob(µmcm/Vs)
0.0	-200.0	-15.852	31.7	0.0	3.2	0.252
0.0	-193.7	-15.349	38.6	6.4	9.5	0.755
0.0	-187.3	-14.846	45.5	63.9	15.9	1.258
0.0	-181.0	-14.342	52.5	29.7	22.2	1.761
0.0	-174.6	-13.839	59.4	0.0	28.6	2.265
0.0	-168.3	-13.336	66.3	0.0	34.9	2.768
0.0	-161.9	-12.833	73.2	0.0	41.3	3.271
0.0	-155.6	-12.329	80.2	0.0	47.6	3.774
0.0	-149.2	-11.826	87.1	0.0	54.0	4.278
0.0	-142.9	-11.323	94.0	0.0	60.3	4.781
0.0	-136.5	-10.820	101.0	0.0	66.7	5.284
0.0	-130.2	-10.316	107.9	0.0	73.0	5.787
0.0	-123.8	-9.813	114.8	0.0	79.4	6.291
0.0	-117.5	-9.310	121.8	0.0	85.7	6.794
0.0	-111.1	-8.807	128.7	0.0	92.1	7.297
0.0	-104.8	-8.303	135.6	0.0	98.4	7.800
0.0	-98.4	-7.800	142.6	0.0	104.8	8.303
0.0	-92.1	-7.297	149.5	0.0	111.1	8.807
0.0	-85.7	-6.794	156.4	0.0	117.5	9.310
0.0	-79.4	-6.291	163.4	0.0	123.8	9.813
0.0	-73.0	-5.787	170.3	0.0	130.2	10.316
0.0	-66.7	-5.284	177.2	0.0	136.5	10.820
0.0	-60.3	-4.781	184.2	0.0	142.9	11.323
0.0	-54.0	-4.278	191.1	0.0	149.2	11.826
0.0	-47.6	-3.774	198.0	0.0	155.6	12.329
0.0	-41.3	-3.271	204.9	0.0	161.9	12.833
0.0	-34.9	-2.768	211.9	0.0	168.3	13.336
0.0	-28.6	-2.265	218.8	0.0	174.6	13.839
0.0	-22.2	-1.761	225.7	0.0	181.0	14.342
0.0	-15.9	-1.258	232.7	0.0	187.3	14.846
0.0	-9.5	-0.755	239.6	0.0	193.7	15.349
0.0	-3.2	-0.252	246.5	0.0	200.0	15.852



Rec.Mean(mV)	Width(mV)	Cond.(mS/cm)	pH	Title
6 16.1	1.6	1.546	N/A	PS0211-02

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ZETASIZER

Zeta Potential Report

PS0211-03
TMC partikels 2:1 (TMC:TPP) sonder Tween 80 en 10 min Ultra sound
Jaco van Heerden

Sample

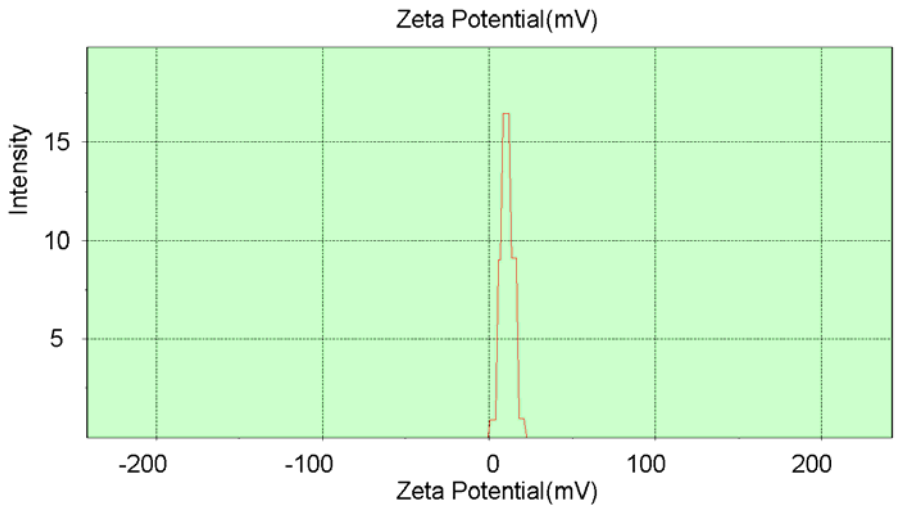
Record Number:	7
Filename:	ZSIZER.zet
File Path:	G:\MALVERN\ZETASI~1\JACOV
Dielectric Constant:	79.7
pH:	N/A
Viscosity (cP):	0.891
Date (DMY):	11/05/11
Time:	14:42:32

System

Instrument Type:	Zetasizer 2000
Temperature (°C):	25.0
Count rate (kCps):	5027.0
Cell Type:	Capillary cell
Cell Position (%):	50.00
Cell field (V/cm):	29.0
Current (mA):	6.8

Result

Zeta Potential (mV):	10.7	Mobility (umcm/V.s):	0.798
StDev (mV):	1.6	StDev (umcm/V.s):	0.125
Conductivity (mS/cm):	2.35	F(ka):	1.50



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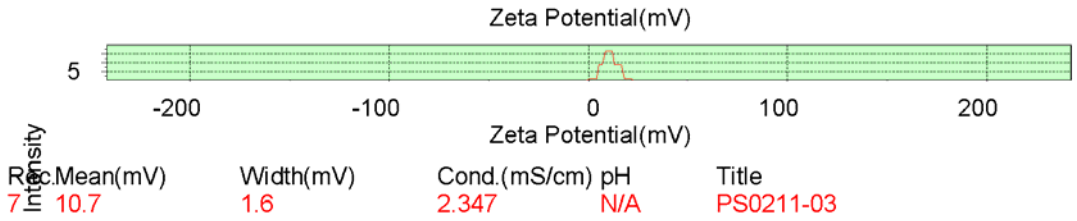
Int.	Zeta(mV)	Mob(µmcm/Vs)	Freq.(Hz)	Int.	Zeta(mV)	Mob(µmcm/Vs)
0.0	-200.0	-15.848	33.9	3.3	3.2	0.252
0.0	-193.7	-15.345	40.8	62.3	9.5	0.755
0.0	-187.3	-14.842	47.7	34.5	15.9	1.258
0.0	-181.0	-14.339	54.6	0.0	22.2	1.761
0.0	-174.6	-13.836	61.6	0.0	28.6	2.264
0.0	-168.3	-13.332	68.5	0.0	34.9	2.767
0.0	-161.9	-12.829	75.4	0.0	41.3	3.270
0.0	-155.6	-12.326	82.3	0.0	47.6	3.773
0.0	-149.2	-11.823	89.3	0.0	54.0	4.276
0.0	-142.9	-11.320	96.2	0.0	60.3	4.780
0.0	-136.5	-10.817	103.1	0.0	66.7	5.283
0.0	-130.2	-10.314	110.0	0.0	73.0	5.786
0.0	-123.8	-9.811	117.0	0.0	79.4	6.289
0.0	-117.5	-9.308	123.9	0.0	85.7	6.792
0.0	-111.1	-8.804	130.8	0.0	92.1	7.295
0.0	-104.8	-8.301	137.7	0.0	98.4	7.798
0.0	-98.4	-7.798	144.7	0.0	104.8	8.301
0.0	-92.1	-7.295	151.6	0.0	111.1	8.804
0.0	-85.7	-6.792	158.5	0.0	117.5	9.308
0.0	-79.4	-6.289	165.4	0.0	123.8	9.811
0.0	-73.0	-5.786	172.4	0.0	130.2	10.314
0.0	-66.7	-5.283	179.3	0.0	136.5	10.817
0.0	-60.3	-4.780	186.2	0.0	142.9	11.320
0.0	-54.0	-4.276	193.1	0.0	149.2	11.823
0.0	-47.6	-3.773	200.1	0.0	155.6	12.326
0.0	-41.3	-3.270	207.0	0.0	161.9	12.829
0.0	-34.9	-2.767	213.9	0.0	168.3	13.332
0.0	-28.6	-2.264	220.8	0.0	174.6	13.836
0.0	-22.2	-1.761	227.8	0.0	181.0	14.339
0.0	-15.9	-1.258	234.7	0.0	187.3	14.842
0.0	-9.5	-0.755	241.6	0.0	193.7	15.345
0.0	-3.2	-0.252	248.5	0.0	200.0	15.848



Rec.Mean(mV)	Width(mV)	Cond.(mS/cm)	pH	Title
7 10.7	1.6	2.347	N/A	PS0211-03

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ZETASIZER

Zeta Potential Report

PS0211-03
TMC partikels 2:1 (TMC:TPP) sonder Tween 80 en 10 min Ultra sound
Jaco van Heerden

Sample

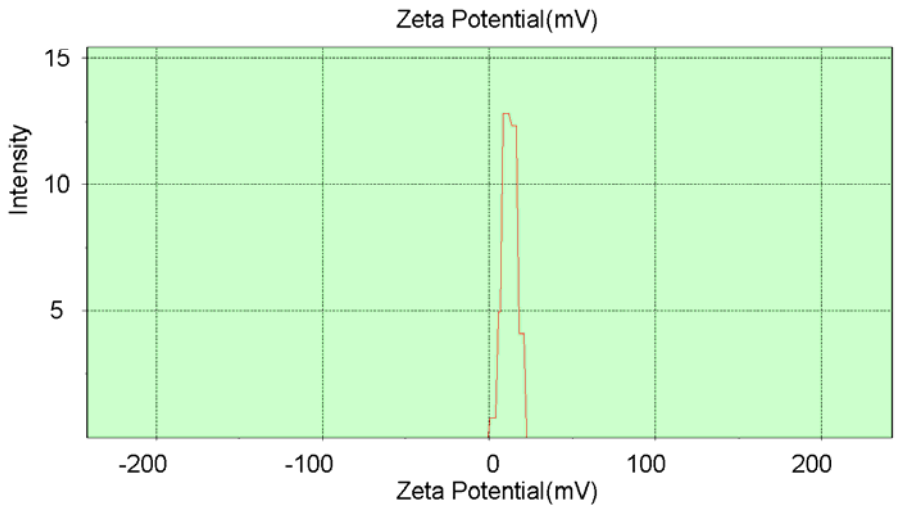
Record Number:	8
Filename:	ZSIZER.zet
File Path:	G:\MALVERN\ZETASI~1\JACOV
Dielectric Constant:	79.7
pH:	N/A
Viscosity (cP):	0.891
Date (DMY):	11/05/11
Time:	14:43:31

System

Instrument Type:	Zetasizer 2000
Temperature (°C):	25.0
Count rate (kCps):	5526.0
Cell Type:	Capillary cell
Cell Position (%):	50.00
Cell field (V/cm):	29.0
Current (mA):	6.9

Result

Zeta Potential (mV):	12.4	Mobility (umcm/V.s):	0.938
StDev (mV):	1.6	StDev (umcm/V.s):	0.126
Conductivity (mS/cm):	2.36	F(ka):	1.50



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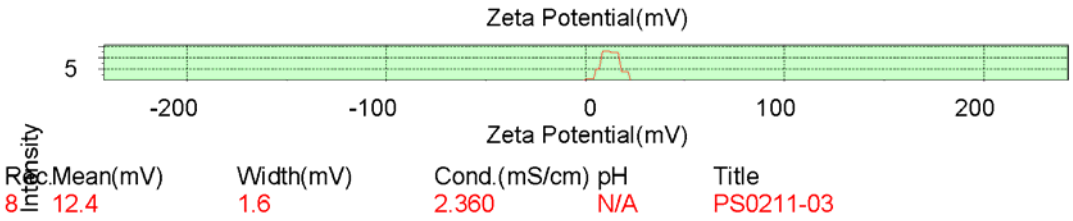
Int.	Zeta(mV)	Mob(µmcm/Vs)	Freq.(Hz)	Int.	Zeta(mV)	Mob(µmcm/Vs)
0.0	-200.0	-15.850	34.0	2.9	3.2	0.252
0.0	-193.7	-15.347	40.9	49.5	9.5	0.755
0.0	-187.3	-14.843	47.8	47.6	15.9	1.258
0.0	-181.0	-14.340	54.7	0.0	22.2	1.761
0.0	-174.6	-13.837	61.7	0.0	28.6	2.264
0.0	-168.3	-13.334	68.6	0.0	34.9	2.767
0.0	-161.9	-12.831	75.5	0.0	41.3	3.271
0.0	-155.6	-12.328	82.4	0.0	47.6	3.774
0.0	-149.2	-11.824	89.4	0.0	54.0	4.277
0.0	-142.9	-11.321	96.3	0.0	60.3	4.780
0.0	-136.5	-10.818	103.2	0.0	66.7	5.283
0.0	-130.2	-10.315	110.1	0.0	73.0	5.786
0.0	-123.8	-9.812	117.0	0.0	79.4	6.290
0.0	-117.5	-9.309	124.0	0.0	85.7	6.793
0.0	-111.1	-8.805	130.9	0.0	92.1	7.296
0.0	-104.8	-8.302	137.8	0.0	98.4	7.799
0.0	-98.4	-7.799	144.7	0.0	104.8	8.302
0.0	-92.1	-7.296	151.6	0.0	111.1	8.805
0.0	-85.7	-6.793	158.6	0.0	117.5	9.309
0.0	-79.4	-6.290	165.5	0.0	123.8	9.812
0.0	-73.0	-5.786	172.4	0.0	130.2	10.315
0.0	-66.7	-5.283	179.3	0.0	136.5	10.818
0.0	-60.3	-4.780	186.2	0.0	142.9	11.321
0.0	-54.0	-4.277	193.2	0.0	149.2	11.824
0.0	-47.6	-3.774	200.1	0.0	155.6	12.328
0.0	-41.3	-3.271	207.0	0.0	161.9	12.831
0.0	-34.9	-2.767	213.9	0.0	168.3	13.334
0.0	-28.6	-2.264	220.9	0.0	174.6	13.837
0.0	-22.2	-1.761	227.8	0.0	181.0	14.340
0.0	-15.9	-1.258	234.7	0.0	187.3	14.843
0.0	-9.5	-0.755	241.6	0.0	193.7	15.347
0.0	-3.2	-0.252	248.5	0.0	200.0	15.850



Rec.Mean(mV)	Width(mV)	Cond.(mS/cm)	pH	Title
8 12.4	1.6	2.360	N/A	PS0211-03

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ZETASIZER

Zeta Potential Report

PS0211-03
TMC partikels 2:1 (TMC:TPP) sonder Tween 80 en 10 min Ultra sound
Jaco van Heerden

Sample

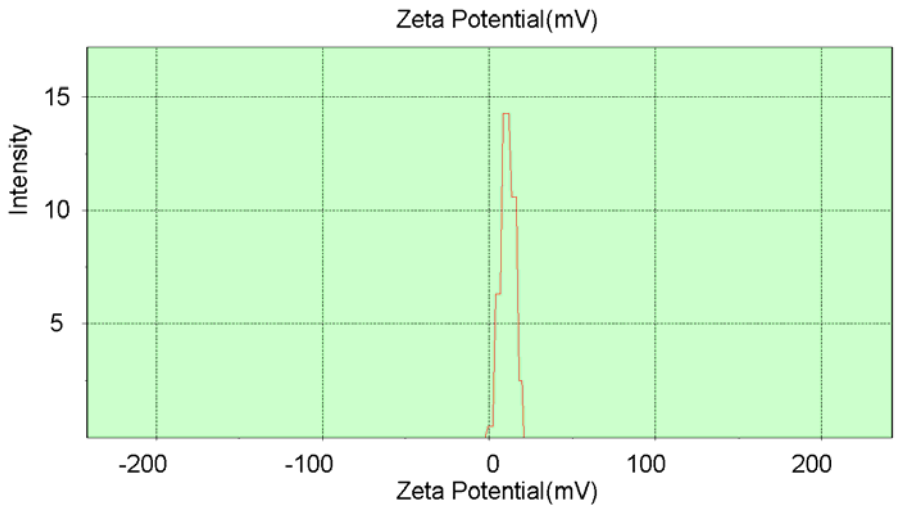
Record Number:	9
Filename:	ZSIZER.zet
File Path:	G:\MALVERN\ZETASI~1\JACOV
Dielectric Constant:	79.7
pH:	N/A
Viscosity (cP):	0.890
Date (DMY):	11/05/11
Time:	14:44:30

System

Instrument Type:	Zetasizer 2000
Temperature (°C):	25.0
Count rate (kCps):	4993.0
Cell Type:	Capillary cell
Cell Position (%):	50.00
Cell field (V/cm):	29.0
Current (mA):	6.8

Result

Zeta Potential (mV):	10.9	Mobility (umcm/V.s):	0.854
StDev (mV):	1.6	StDev (umcm/V.s):	0.126
Conductivity (mS/cm):	2.36	F(ka):	1.50



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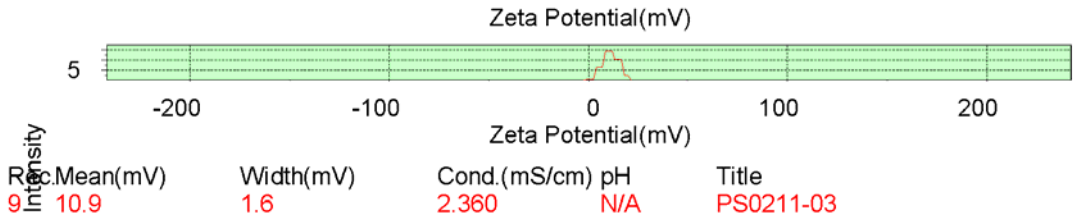
Int.	Zeta(mV)	Mob(µmcm/Vs)	Freq.(Hz)	Int.	Zeta(mV)	Mob(µmcm/Vs)
0.0	-200.0	-15.857	35.3	20.3	3.2	0.252
0.0	-193.7	-15.354	42.2	45.7	9.5	0.755
0.0	-187.3	-14.850	49.1	33.9	15.9	1.258
0.0	-181.0	-14.347	56.1	0.0	22.2	1.762
0.0	-174.6	-13.843	63.0	0.0	28.6	2.265
0.0	-168.3	-13.340	69.9	0.0	34.9	2.769
0.0	-161.9	-12.837	76.8	0.0	41.3	3.272
0.0	-155.6	-12.333	83.7	0.0	47.6	3.775
0.0	-149.2	-11.830	90.6	0.0	54.0	4.279
0.0	-142.9	-11.326	97.5	0.0	60.3	4.782
0.0	-136.5	-10.823	104.4	0.0	66.7	5.286
0.0	-130.2	-10.320	111.3	0.0	73.0	5.789
0.0	-123.8	-9.816	118.2	0.0	79.4	6.292
0.0	-117.5	-9.313	125.2	0.0	85.7	6.796
0.0	-111.1	-8.809	132.1	0.0	92.1	7.299
0.0	-104.8	-8.306	139.0	0.0	98.4	7.803
0.0	-98.4	-7.803	145.9	0.0	104.8	8.306
0.0	-92.1	-7.299	152.8	0.0	111.1	8.809
0.0	-85.7	-6.796	159.7	0.0	117.5	9.313
0.0	-79.4	-6.292	166.6	0.0	123.8	9.816
0.0	-73.0	-5.789	173.5	0.0	130.2	10.320
0.0	-66.7	-5.286	180.4	0.0	136.5	10.823
0.0	-60.3	-4.782	187.4	0.0	142.9	11.326
0.0	-54.0	-4.279	194.3	0.0	149.2	11.830
0.0	-47.6	-3.775	201.2	0.0	155.6	12.333
0.0	-41.3	-3.272	208.1	0.0	161.9	12.837
0.0	-34.9	-2.769	215.0	0.0	168.3	13.340
0.0	-28.6	-2.265	221.9	0.0	174.6	13.843
0.0	-22.2	-1.762	228.8	0.0	181.0	14.347
0.0	-15.9	-1.258	235.7	0.0	187.3	14.850
0.0	-9.5	-0.755	242.6	0.0	193.7	15.354
0.0	-3.2	-0.252	249.5	0.0	200.0	15.857



Malvern Instruments Ltd, Malvern UK +44 1684 892456

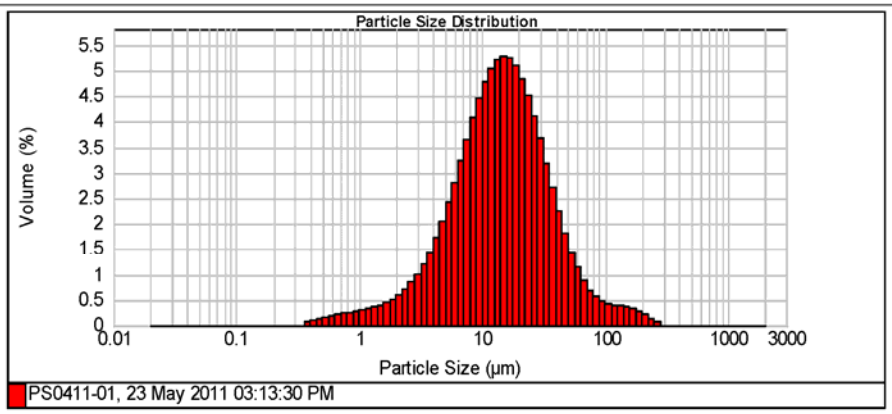
3. Mastersizer: TMC-TPP particles (TMC:TPP ratio – 3:1)

MASTERSIZER 2000

Result Analysis Report

Sample Name: PS0411-01	**SOP Name:** Jaco (TMC)	**Measured:** 23 May 2011 03:13:30 PM	
Sample Source & type:	**Measured by:** Jaco van Heerden	**Analysed:** 23 May 2011 03:13:31 PM	
Sample bulk lot ref:	**Result Source:** Measurement		
Particle Name: Titanium Dioxide	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.741	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 18.08 %
Dispersant Name: Alcohol	**Dispersant RI:** 1.320	**Weighted Residual:** 1.327 %	**Result Emulation:** Off
Concentration: 0.0187 %Vol	**Span :** 2.782	**Uniformity:** 1.01	**Result units:** Volume
Specific Surface Area: 0.791 m²/g	**Surface Weighted Mean D[3,2]:** 7.590 um	**Vol. Weighted Mean D[4,3]:** 22.209 um	

d(0.1): 4.033 um **d(0.5):** 14.353 um **d(0.9):** 43.955 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 %
0.020	0.00	0.142	0.00	1.002	0.30	7.096	3.66	50.238	1.45	355.656	0.00
0.022	0.00	0.159	0.00	1.125	0.32	7.962	4.08	56.368	1.14	399.052	0.00
0.025	0.00	0.178	0.00	1.262	0.35	8.934	4.46	63.246	0.88	447.744	0.00
0.028	0.00	0.200	0.00	1.416	0.40	10.024	4.80	70.963	0.69	502.377	0.00
0.032	0.00	0.224	0.00	1.589	0.45	11.247	5.05	79.621	0.56	563.677	0.00
0.036	0.00	0.252	0.00	1.783	0.52	12.619	5.22	89.337	0.48	632.456	0.00
0.040	0.00	0.283	0.00	2.000	0.61	14.159	5.29	100.237	0.43	709.627	0.00
0.045	0.00	0.317	0.00	2.244	0.71	15.887	5.25	112.468	0.40	796.214	0.00
0.050	0.00	0.356	0.00	2.518	0.85	17.825	5.10	126.191	0.38	893.367	0.00
0.056	0.00	0.399	0.07	2.825	1.01	20.000	4.85	141.589	0.36	1002.374	0.00
0.063	0.00	0.448	0.10	3.170	1.21	22.440	4.52	158.866	0.33	1124.683	0.00
0.071	0.00	0.502	0.14	3.557	1.45	25.179	4.12	178.250	0.29	1261.915	0.00
0.080	0.00	0.564	0.17	3.991	1.73	28.251	3.67	200.000	0.23	1415.892	0.00
0.089	0.00	0.632	0.20	4.477	2.05	31.698	3.19	224.404	0.14	1588.656	0.00
0.100	0.00	0.710	0.22	5.024	2.41	35.566	2.72	251.785	0.08	1782.502	0.00
0.112	0.00	0.796	0.24	5.637	2.81	39.905	2.25	282.508	0.00	2000.000	0.00
0.126	0.00	0.893	0.26	6.325	3.24	44.774	1.83	316.979	0.00		
0.142	0.00	1.002	0.28	7.096		50.238		355.656			

Operator notes:

Malvern Instruments Ltd.
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007548

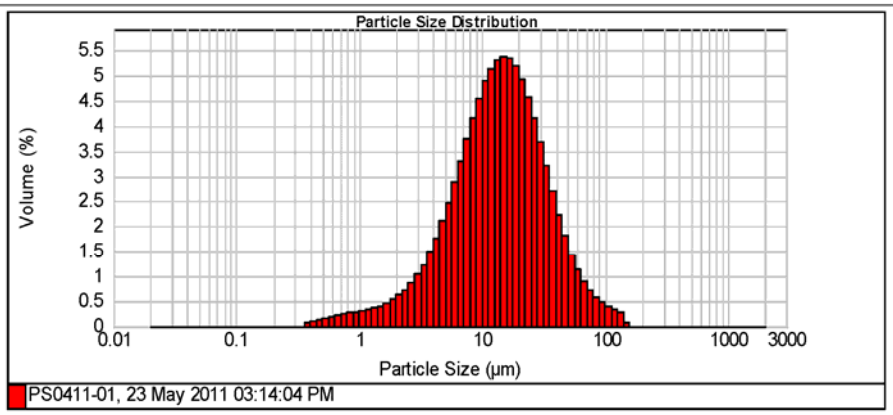
File name: 19 May 2011
Record Number: 1

MASTERSIZER 2000

Result Analysis Report

Sample Name: PS0411-01	**SOP Name:** Jaco (TMC)	**Measured:** 23 May 2011 03:14:04 PM	
Sample Source & type:	**Measured by:** Jaco van Heerden	**Analysed:** 23 May 2011 03:14:05 PM	
Sample bulk lot ref:	**Result Source:** Measurement		
Particle Name: Titanium Dioxide	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.741	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 18.19 %
Dispersant Name: Alcohol	**Dispersant RI:** 1.320	**Weighted Residual:** 1.311 %	**Result Emulation:** Off
Concentration: 0.0184 %Vol	**Span :** 2.622	**Uniformity:** 0.862	**Result units:** Volume
Specific Surface Area: 0.808 m²/g	**Surface Weighted Mean D[3,2]:** 7.428 um	**Vol. Weighted Mean D[4,3]:** 19.643 um	

d(0.1): 3.955 um **d(0.5):** 14.036 um **d(0.9):** 40.757 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 %
0.020	0.00	0.142	0.00	1.002	0.31	7.096	3.73	50.238	1.45	355.656	0.00
0.022	0.00	0.159	0.00	1.125	0.33	7.962	4.16	56.368	1.14	399.052	0.00
0.025	0.00	0.178	0.00	1.262	0.36	8.934	4.55	63.246	0.90	447.744	0.00
0.028	0.00	0.200	0.00	1.416	0.41	10.024	4.88	70.963	0.72	502.377	0.00
0.032	0.00	0.224	0.00	1.589	0.46	11.247	5.14	79.621	0.59	563.677	0.00
0.036	0.00	0.252	0.00	1.783	0.53	12.619	5.31	89.337	0.49	632.456	0.00
0.040	0.00	0.283	0.00	2.000	0.62	14.159	5.37	100.237	0.41	709.627	0.00
0.045	0.00	0.317	0.00	2.244	0.74	15.887	5.33	112.468	0.33	796.214	0.00
0.050	0.00	0.356	0.07	2.518	0.87	17.825	5.17	126.191	0.27	893.367	0.00
0.056	0.00	0.399	0.10	2.825	1.04	20.000	4.92	141.589	0.08	1002.374	0.00
0.063	0.00	0.448	0.15	3.170	1.24	22.440	4.57	158.866	0.00	1124.683	0.00
0.071	0.00	0.502	0.17	3.557	1.49	25.179	4.16	178.250	0.00	1261.915	0.00
0.080	0.00	0.564	0.20	3.991	1.77	28.251	3.69	200.000	0.00	1415.892	0.00
0.089	0.00	0.632	0.23	4.477	2.10	31.698	3.21	224.404	0.00	1588.656	0.00
0.100	0.00	0.710	0.25	5.024	2.47	35.566	2.72	251.785	0.00	1782.502	0.00
0.112	0.00	0.796	0.27	5.637	2.87	39.905	2.25	282.508	0.00	2000.000	
0.126	0.00	0.893	0.29	6.325	3.30	44.774	1.82	316.979	0.00		
0.142		1.002		7.096		50.238		355.656			

Operator notes:

Malvern Instruments Ltd.
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007548

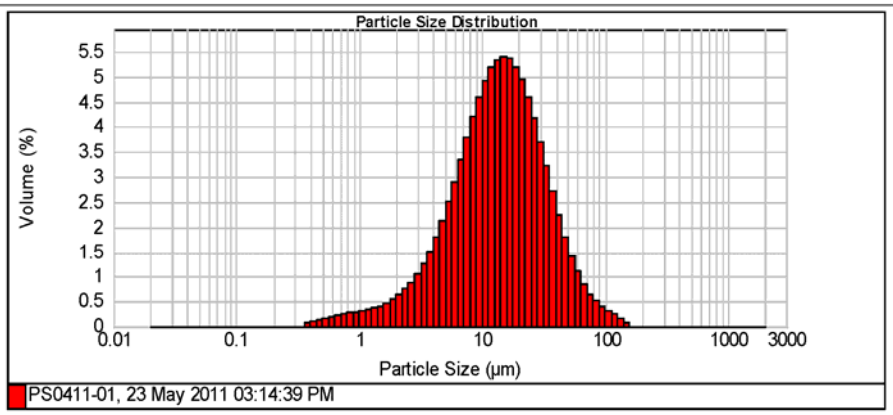
File name: 19 May 2011
Record Number: 2

MASTERSIZER 2000

Result Analysis Report

Sample Name: PS0411-01	**SOP Name:** Jaco (TMC)	**Measured:** 23 May 2011 03:14:39 PM	
Sample Source & type:	**Measured by:** Jaco van Heerden	**Analysed:** 23 May 2011 03:14:40 PM	
Sample bulk lot ref:	**Result Source:** Measurement		
Particle Name: Titanium Dioxide	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.741	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 18.26 %
Dispersant Name: Alcohol	**Dispersant RI:** 1.320	**Weighted Residual:** 1.295 %	**Result Emulation:** Off
Concentration: 0.0183 %Vol	**Span :** 2.564	**Uniformity:** 0.834	**Result units:** Volume
Specific Surface Area: 0.817 m²/g	**Surface Weighted Mean D[3,2]:** 7.341 um	**Vol. Weighted Mean D[4,3]:** 19.026 um	

d(0.1): 3.908 um **d(0.5):** 13.866 um **d(0.9):** 39.462 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 %
0.020	0.00	0.142	0.00	1.002	0.31	7.096	3.77	50.238	1.42	355.656	0.00
0.022	0.00	0.159	0.00	1.125	0.34	7.962	4.20	56.368	1.11	399.052	0.00
0.025	0.00	0.178	0.00	1.262	0.37	8.934	4.59	63.246	0.85	447.744	0.00
0.028	0.00	0.200	0.00	1.416	0.41	10.024	4.92	70.963	0.65	502.377	0.00
0.032	0.00	0.224	0.00	1.589	0.47	11.247	5.18	79.621	0.51	563.677	0.00
0.036	0.00	0.252	0.00	1.783	0.54	12.619	5.34	89.337	0.40	632.456	0.00
0.040	0.00	0.283	0.00	2.000	0.63	14.159	5.40	100.237	0.32	709.627	0.00
0.045	0.00	0.317	0.00	2.244	0.75	15.887	5.35	112.468	0.24	796.214	0.00
0.050	0.00	0.356	0.07	2.518	0.89	17.825	5.19	126.191	0.15	893.367	0.00
0.056	0.00	0.399	0.11	2.825	1.06	20.000	4.93	141.589	0.06	1002.374	0.00
0.063	0.00	0.448	0.15	3.170	1.26	22.440	4.58	158.866	0.00	1124.683	0.00
0.071	0.00	0.502	0.18	3.557	1.51	25.179	4.17	178.250	0.00	1261.915	0.00
0.080	0.00	0.564	0.21	3.991	1.80	28.251	3.70	200.000	0.00	1415.892	0.00
0.089	0.00	0.632	0.23	4.477	2.13	31.698	3.21	224.404	0.00	1588.656	0.00
0.100	0.00	0.710	0.25	5.024	2.50	35.566	2.72	251.785	0.00	1782.502	0.00
0.112	0.00	0.796	0.27	5.637	2.91	39.905	2.24	282.508	0.00	2000.000	
0.126	0.00	0.893	0.29	6.325	3.34	44.774	1.81	316.979	0.00		
0.142		1.002		7.096		50.238		355.656			

Operator notes:

Malvern Instruments Ltd.
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007548

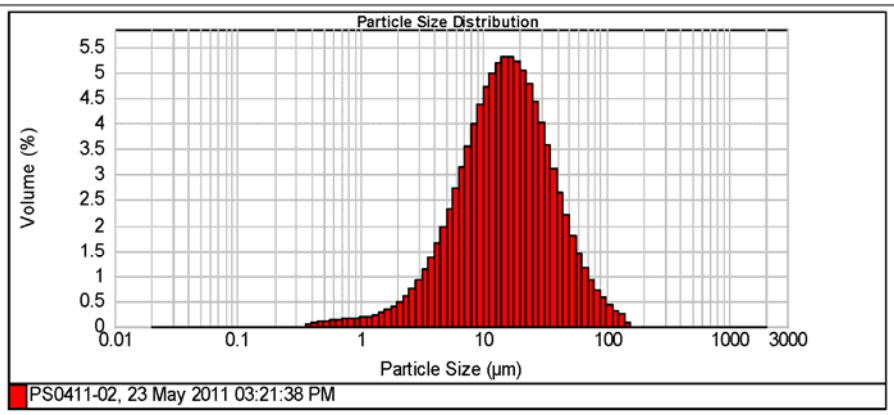
File name: 19 May 2011
Record Number: 3

MASTERSIZER 2000

Result Analysis Report

Sample Name: PS0411-02	**SOP Name:** Jaco (TMC)	**Measured:** 23 May 2011 03:21:38 PM	
Sample Source & type:	**Measured by:** Jaco van Heerden	**Analysed:** 23 May 2011 03:21:39 PM	
Sample bulk lot ref:	**Result Source:** Measurement		
Particle Name: Titanium Dioxide	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.741	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 17.60 %
Dispersant Name: Alcohol	**Dispersant RI:** 1.320	**Weighted Residual:** 1.362 %	**Result Emulation:** Off
Concentration: 0.0211 %Vol	**Span :** 2.623	**Uniformity:** 0.844	**Result units:** Volume
Specific Surface Area: 0.69 m²/g	**Surface Weighted Mean D[3,2]:** 8.690 um	**Vol. Weighted Mean D[4,3]:** 21.190 um	

d(0.1): 4.588 um **d(0.5): 15.243 um** **d(0.9): 44.564 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 %
0.020	0.00	0.142	0.00	1.002	0.18	7.096	3.56	50.238	1.81	355.656	0.00
0.022	0.00	0.159	0.00	1.125	0.20	7.962	3.98	56.368	1.46	399.052	0.00
0.025	0.00	0.178	0.00	1.262	0.23	8.934	4.37	63.246	1.16	447.744	0.00
0.028	0.00	0.200	0.00	1.416	0.27	10.024	4.71	70.963	0.91	502.377	0.00
0.032	0.00	0.224	0.00	1.589	0.33	11.247	4.99	79.621	0.72	563.677	0.00
0.036	0.00	0.252	0.00	1.783	0.40	12.619	5.19	89.337	0.56	632.456	0.00
0.040	0.00	0.283	0.00	2.000	0.49	14.159	5.30	100.237	0.43	709.627	0.00
0.045	0.00	0.317	0.00	2.244	0.61	15.887	5.32	112.468	0.32	796.214	0.00
0.050	0.00	0.356	0.05	2.518	0.75	17.825	5.23	126.191	0.24	893.367	0.00
0.056	0.00	0.399	0.08	2.825	0.92	20.000	5.05	141.589	0.07	1002.374	0.00
0.063	0.00	0.448	0.09	3.170	1.13	22.440	4.78	158.866	0.00	1124.683	0.00
0.071	0.00	0.502	0.11	3.557	1.37	25.179	4.43	178.250	0.00	1261.915	0.00
0.080	0.00	0.564	0.13	3.991	1.65	28.251	4.03	200.000	0.00	1415.892	0.00
0.089	0.00	0.632	0.14	4.477	1.98	31.698	3.58	224.404	0.00	1588.656	0.00
0.100	0.00	0.710	0.15	5.024	2.34	35.566	3.12	251.785	0.00	1782.502	0.00
0.112	0.00	0.796	0.16	5.637	2.73	39.905	2.66	282.508	0.00	2000.000	
0.126	0.00	0.893	0.17	6.325	3.14	44.774	2.22	316.979	0.00		
0.142		1.002		7.096		50.238		355.656			

Operator notes:

Malvern Instruments Ltd.
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007548

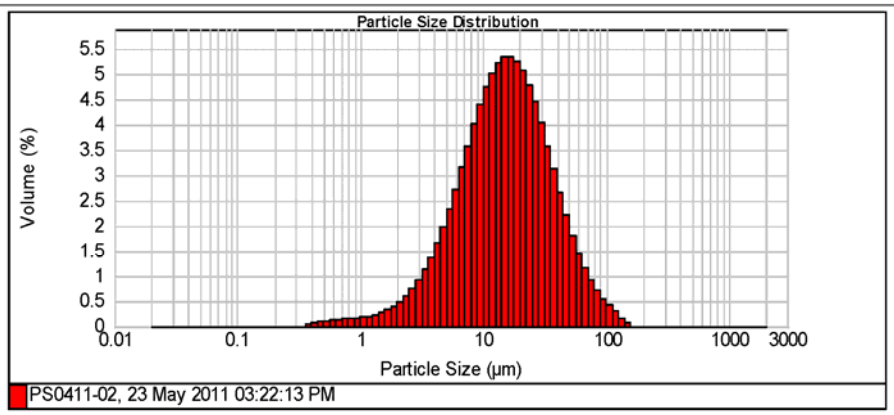
File name: 19 May 2011
Record Number: 4

MASTERSIZER 2000

Result Analysis Report

Sample Name: PS0411-02	**SOP Name:** Jaco (TMC)	**Measured:** 23 May 2011 03:22:13 PM	
Sample Source & type:	**Measured by:** Jaco van Heerden	**Analysed:** 23 May 2011 03:22:14 PM	
Sample bulk lot ref:	**Result Source:** Measurement		
Particle Name: Titanium Dioxide	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.741	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 17.65 %
Dispersant Name: Alcohol	**Dispersant RI:** 1.320	**Weighted Residual:** 1.385 %	**Result Emulation:** Off
Concentration: 0.0211 %Vol	**Span :** 2.613	**Uniformity:** 0.837	**Result units:** Volume
Specific Surface Area: 0.692 m²/g	**Surface Weighted Mean D[3,2]:** 8.672 um	**Vol. Weighted Mean D[4,3]:** 21.044 um	

d(0.1): 4.582 um **d(0.5): 15.210 um** **d(0.9): 44.319 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 %
0.020	0.00	0.142	0.00	1.002	0.18	7.096	3.57	50.238	1.81	355.656	0.00
0.022	0.00	0.159	0.00	1.125	0.20	7.962	3.99	56.368	1.46	399.052	0.00
0.025	0.00	0.178	0.00	1.262	0.23	8.934	4.38	63.246	1.16	447.744	0.00
0.028	0.00	0.200	0.00	1.416	0.27	10.024	4.73	70.963	0.92	502.377	0.00
0.032	0.00	0.224	0.00	1.589	0.33	11.247	5.01	79.621	0.72	563.677	0.00
0.036	0.00	0.252	0.00	1.783	0.40	12.619	5.21	89.337	0.56	632.456	0.00
0.040	0.00	0.283	0.00	2.000	0.50	14.159	5.32	100.237	0.42	709.627	0.00
0.045	0.00	0.317	0.00	2.244	0.61	15.887	5.33	112.468	0.30	796.214	0.00
0.050	0.00	0.356	0.05	2.518	0.75	17.825	5.24	126.191	0.16	893.367	0.00
0.056	0.00	0.399	0.08	2.825	0.92	20.000	5.06	141.589	0.06	1002.374	0.00
0.063	0.00	0.448	0.09	3.170	1.13	22.440	4.78	158.866	0.00	1124.683	0.00
0.071	0.00	0.502	0.11	3.557	1.37	25.179	4.43	178.250	0.00	1261.915	0.00
0.080	0.00	0.564	0.13	3.991	1.65	28.251	4.02	200.000	0.00	1415.892	0.00
0.089	0.00	0.632	0.14	4.477	1.97	31.698	3.57	224.404	0.00	1588.656	0.00
0.100	0.00	0.710	0.15	5.024	2.33	35.566	3.11	251.785	0.00	1782.502	0.00
0.112	0.00	0.796	0.16	5.637	2.73	39.905	2.65	282.508	0.00	2000.000	0.00
0.126	0.00	0.893	0.17	6.325	3.15	44.774	2.21	316.979	0.00		
0.142	0.00	1.002		7.096		50.238		355.656	0.00		

Operator notes:

Malvern Instruments Ltd.
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007548

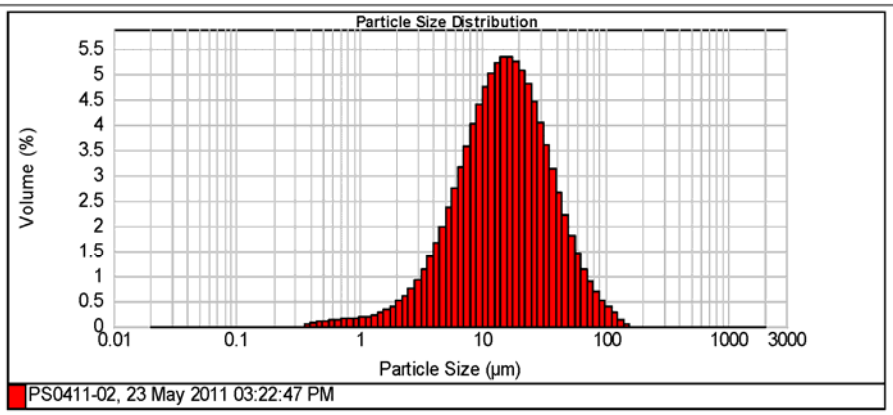
File name: 19 May 2011
Record Number: 5

MASTERSIZER 2000

Result Analysis Report

Sample Name: PS0411-02	**SOP Name:** Jaco (TMC)	**Measured:** 23 May 2011 03:22:47 PM	
Sample Source & type:	**Measured by:** Jaco van Heerden	**Analysed:** 23 May 2011 03:22:48 PM	
Sample bulk lot ref:	**Result Source:** Measurement		
Particle Name: Titanium Dioxide	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.741	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 17.71 %
Dispersant Name: Alcohol	**Dispersant RI:** 1.320	**Weighted Residual:** 1.372 %	**Result Emulation:** Off
Concentration: 0.0211 %Vol	**Span :** 2.590	**Uniformity:** 0.83	**Result units:** Volume
Specific Surface Area: 0.695 m²/g	**Surface Weighted Mean D[3,2]:** 8.635 um	**Vol. Weighted Mean D[4,3]:** 20.863 um	

d(0.1): 4.561 um **d(0.5): 15.167 um** **d(0.9): 43.838 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 %
0.020	0.00	0.142	0.00	1.002	0.18	7.096	3.58	50.238	1.80	355.656	0.00
0.022	0.00	0.159	0.00	1.125	0.20	7.962	4.00	56.368	1.44	399.052	0.00
0.025	0.00	0.178	0.00	1.262	0.23	8.934	4.38	63.246	1.14	447.744	0.00
0.028	0.00	0.200	0.00	1.416	0.28	10.024	4.73	70.963	0.89	502.377	0.00
0.032	0.00	0.224	0.00	1.589	0.33	11.247	5.01	79.621	0.68	563.677	0.00
0.036	0.00	0.252	0.00	1.783	0.41	12.619	5.21	89.337	0.52	632.456	0.00
0.040	0.00	0.283	0.00	2.000	0.50	14.159	5.32	100.237	0.39	709.627	0.00
0.045	0.00	0.317	0.00	2.244	0.62	15.887	5.33	112.468	0.27	796.214	0.00
0.050	0.00	0.356	0.05	2.518	0.76	17.825	5.25	126.191	0.14	893.367	0.00
0.056	0.00	0.399	0.08	2.825	0.93	20.000	5.07	141.589	0.06	1002.374	0.00
0.063	0.00	0.448	0.10	3.170	1.14	22.440	4.79	158.866	0.00	1124.683	0.00
0.071	0.00	0.502	0.11	3.557	1.38	25.179	4.45	178.250	0.00	1261.915	0.00
0.080	0.00	0.564	0.13	3.991	1.66	28.251	4.04	200.000	0.00	1415.892	0.00
0.089	0.00	0.632	0.14	4.477	1.99	31.698	3.59	224.404	0.00	1588.656	0.00
0.100	0.00	0.710	0.15	5.024	2.35	35.566	3.12	251.785	0.00	1782.502	0.00
0.112	0.00	0.796	0.16	5.637	2.74	39.905	2.66	282.508	0.00	2000.000	
0.126	0.00	0.893	0.17	6.325	3.16	44.774	2.21	316.979	0.00		
0.142		1.002		7.096		50.238		355.656			

Operator notes:

Malvern Instruments Ltd.
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007548

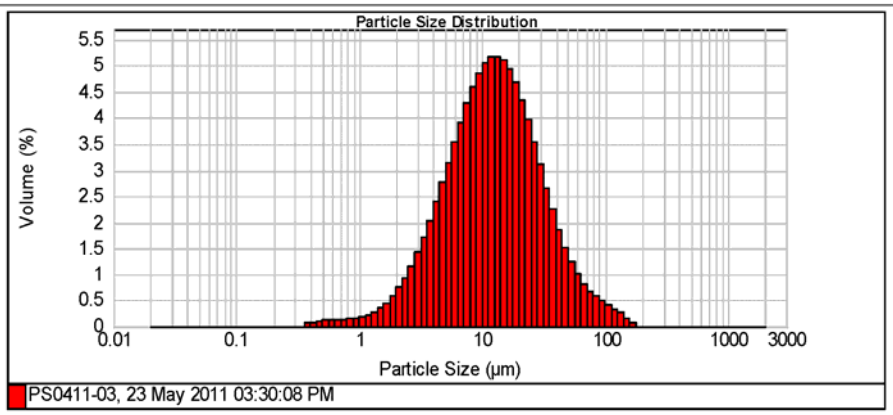
File name: 19 May 2011
Record Number: 6

MASTERSIZER 2000

Result Analysis Report

Sample Name: PS0411-03	**SOP Name:** Jaco (TMC)	**Measured:** 23 May 2011 03:30:08 PM	
Sample Source & type:	**Measured by:** Jaco van Heerden	**Analysed:** 23 May 2011 03:30:09 PM	
Sample bulk lot ref:	**Result Source:** Measurement		
Particle Name: Titanium Dioxide	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.741	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 17.15 %
Dispersant Name: Alcohol	**Dispersant RI:** 1.320	**Weighted Residual:** 1.181 %	**Result Emulation:** Off
Concentration: 0.0176 %Vol	**Span :** 2.818	**Uniformity:** 0.944	**Result units:** Volume
Specific Surface Area: 0.805 m²/g	**Surface Weighted Mean D[3,2]:** 7.451 um	**Vol. Weighted Mean D[4,3]:** 18.470 um	

d(0.1): 3.757 um **d(0.5):** 12.394 um **d(0.9):** 38.686 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 %
0.020	0.00	0.142	0.00	1.002	0.18	7.096	4.28	50.238	1.23	355.656	0.00
0.022	0.00	0.159	0.00	1.125	0.21	7.962	4.60	56.368	1.00	399.052	0.00
0.025	0.00	0.178	0.00	1.262	0.27	8.934	4.86	63.246	0.81	447.744	0.00
0.028	0.00	0.200	0.00	1.416	0.34	10.024	5.05	70.963	0.68	502.377	0.00
0.032	0.00	0.224	0.00	1.589	0.45	11.247	5.15	79.621	0.57	563.677	0.00
0.036	0.00	0.252	0.00	1.783	0.58	12.619	5.17	89.337	0.49	632.456	0.00
0.040	0.00	0.283	0.00	2.000	0.74	14.159	5.09	100.237	0.41	709.627	0.00
0.045	0.00	0.317	0.00	2.244	0.93	15.887	4.93	112.468	0.34	796.214	0.00
0.050	0.00	0.356	0.06	2.518	1.16	17.825	4.67	126.191	0.27	893.367	0.00
0.056	0.00	0.399	0.08	2.825	1.42	20.000	4.35	141.589	0.15	1002.374	0.00
0.063	0.00	0.448	0.11	3.170	1.71	22.440	3.97	158.866	0.08	1124.683	0.00
0.071	0.00	0.502	0.12	3.557	2.04	25.179	3.55	178.250	0.00	1261.915	0.00
0.080	0.00	0.564	0.13	3.991	2.39	28.251	3.11	200.000	0.00	1415.892	0.00
0.089	0.00	0.632	0.13	4.477	2.76	31.698	2.67	224.404	0.00	1588.656	0.00
0.100	0.00	0.710	0.14	5.024	3.15	35.566	2.25	251.785	0.00	1782.502	0.00
0.112	0.00	0.796	0.15	5.637	3.54	39.905	1.87	282.508	0.00	2000.000	
0.126	0.00	0.893	0.16	6.325	3.92	44.774	1.53	316.979	0.00		
0.142		1.002		7.096		50.238		355.656			

Operator notes:

Malvern Instruments Ltd.
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007548

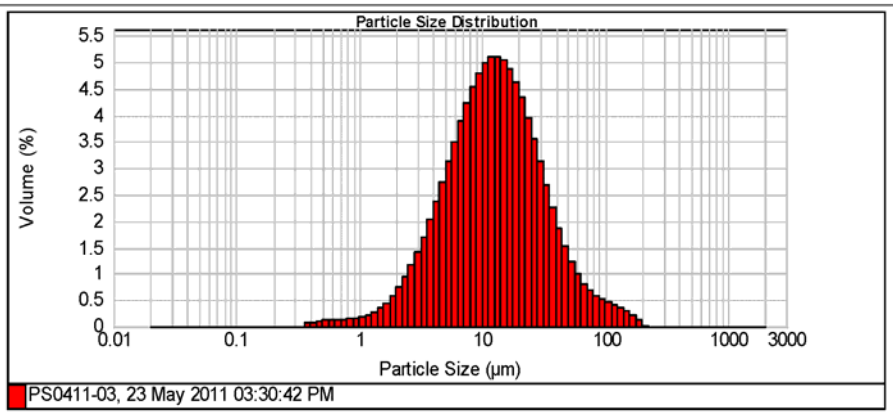
File name: 19 May 2011
Record Number: 7

MASTERSIZER 2000

Result Analysis Report

Sample Name: PS0411-03	**SOP Name:** Jaco (TMC)	**Measured:** 23 May 2011 03:30:42 PM	
Sample Source & type:	**Measured by:** Jaco van Heerden	**Analysed:** 23 May 2011 03:30:43 PM	
Sample bulk lot ref:	**Result Source:** Measurement		
Particle Name: Titanium Dioxide	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.741	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 17.27 %
Dispersant Name: Alcohol	**Dispersant RI:** 1.320	**Weighted Residual:** 1.178 %	**Result Emulation:** Off
Concentration: 0.0178 %Vol	**Span :** 2.896	**Uniformity:** 1	**Result units:** Volume
Specific Surface Area: 0.801 m²/g	**Surface Weighted Mean D[3,2]:** 7.490 um	**Vol. Weighted Mean D[4,3]:** 19.316 um	

d(0.1): 3.763 um **d(0.5):** 12.509 um **d(0.9):** 39.985 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 %
0.020	0.00	0.142	0.00	1.002	0.18	7.096	4.24	50.238	1.24	355.656	0.00
0.022	0.00	0.159	0.00	1.125	0.21	7.962	4.55	56.368	1.00	399.052	0.00
0.025	0.00	0.178	0.00	1.262	0.27	8.934	4.80	63.246	0.81	447.744	0.00
0.028	0.00	0.200	0.00	1.416	0.34	10.024	4.99	70.963	0.68	502.377	0.00
0.032	0.00	0.224	0.00	1.589	0.45	11.247	5.09	79.621	0.58	563.677	0.00
0.036	0.00	0.252	0.00	1.783	0.58	12.619	5.11	89.337	0.51	632.456	0.00
0.040	0.00	0.283	0.00	2.000	0.74	14.159	5.04	100.237	0.45	709.627	0.00
0.045	0.00	0.317	0.00	2.244	0.93	15.887	4.88	112.468	0.40	796.214	0.00
0.050	0.00	0.356	0.06	2.518	1.16	17.825	4.64	126.191	0.34	893.367	0.00
0.056	0.00	0.399	0.08	2.825	1.42	20.000	4.33	141.589	0.29	1002.374	0.00
0.063	0.00	0.448	0.10	3.170	1.71	22.440	3.96	158.866	0.20	1124.683	0.00
0.071	0.00	0.502	0.12	3.557	2.03	25.179	3.55	178.250	0.12	1261.915	0.00
0.080	0.00	0.564	0.13	3.991	2.38	28.251	3.12	200.000	0.02	1415.892	0.00
0.089	0.00	0.632	0.13	4.477	2.75	31.698	2.69	224.404	0.00	1588.656	0.00
0.100	0.00	0.710	0.14	5.024	3.13	35.566	2.27	251.785	0.00	1782.502	0.00
0.112	0.00	0.796	0.15	5.637	3.51	39.905	1.88	282.508	0.00	2000.000	
0.126	0.00	0.893	0.16	6.325	3.89	44.774	1.53	316.979	0.00		
0.142		1.002		7.096		50.238		355.656			

Operator notes:

Malvern Instruments Ltd.
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007548

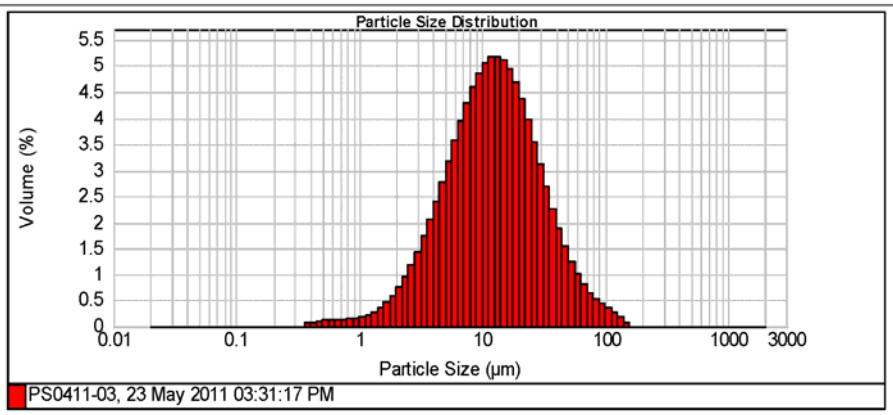
File name: 19 May 2011
Record Number: 8

MASTERSIZER 2000

Result Analysis Report

Sample Name: PS0411-03	**SOP Name:** Jaco (TMC)	**Measured:** 23 May 2011 03:31:17 PM	
Sample Source & type:	**Measured by:** Jaco van Heerden	**Analysed:** 23 May 2011 03:31:19 PM	
Sample bulk lot ref:	**Result Source:** Measurement		
Particle Name: Titanium Dioxide	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.741	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 17.31 %
Dispersant Name: Alcohol	**Dispersant RI:** 1.320	**Weighted Residual:** 1.133 %	**Result Emulation:** Off
Concentration: 0.0176 %Vol	**Span :** 2.770	**Uniformity:** 0.91	**Result units:** Volume
Specific Surface Area: 0.813 m²/g	**Surface Weighted Mean D[3,2]:** 7.377 um	**Vol. Weighted Mean D[4,3]:** 17.889 um	

d(0.1): 3.717 um **d(0.5):** 12.293 um **d(0.9):** 37.769 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 %
0.020	0.00	0.142	0.00	1.002	0.18	7.096	4.29	50.238	1.25	355.656	0.00
0.022	0.00	0.159	0.00	1.125	0.22	7.962	4.61	56.368	1.01	399.052	0.00
0.025	0.00	0.178	0.00	1.262	0.28	8.934	4.87	63.246	0.81	447.744	0.00
0.028	0.00	0.200	0.00	1.416	0.35	10.024	5.06	70.963	0.65	502.377	0.00
0.032	0.00	0.224	0.00	1.589	0.46	11.247	5.16	79.621	0.53	563.677	0.00
0.036	0.00	0.252	0.00	1.783	0.59	12.619	5.18	89.337	0.43	632.456	0.00
0.040	0.00	0.283	0.00	2.000	0.76	14.159	5.10	100.237	0.34	709.627	0.00
0.045	0.00	0.317	0.00	2.244	0.95	15.887	4.93	112.468	0.27	796.214	0.00
0.050	0.00	0.356	0.06	2.518	1.18	17.825	4.68	126.191	0.18	893.367	0.00
0.056	0.00	0.399	0.08	2.825	1.45	20.000	4.36	141.589	0.08	1002.374	0.00
0.063	0.00	0.448	0.11	3.170	1.74	22.440	3.97	158.866	0.00	1124.683	0.00
0.071	0.00	0.502	0.12	3.557	2.07	25.179	3.56	178.250	0.00	1261.915	0.00
0.080	0.00	0.564	0.13	3.991	2.42	28.251	3.12	200.000	0.00	1415.892	0.00
0.089	0.00	0.632	0.14	4.477	2.79	31.698	2.69	224.404	0.00	1588.656	0.00
0.100	0.00	0.710	0.14	5.024	3.17	35.566	2.27	251.785	0.00	1782.502	0.00
0.112	0.00	0.796	0.15	5.637	3.56	39.905	1.88	282.508	0.00	2000.000	
0.126	0.00	0.893	0.16	6.325	3.94	44.774	1.54	316.979	0.00		
0.142		1.002		7.096		50.238		355.656			

Operator notes:

Malvern Instruments Ltd.
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007548

File name: 19 May 2011
Record Number: 9

4. Zetasizer: TMC-TPP particles (TMC:TPP ratio – 3:1)

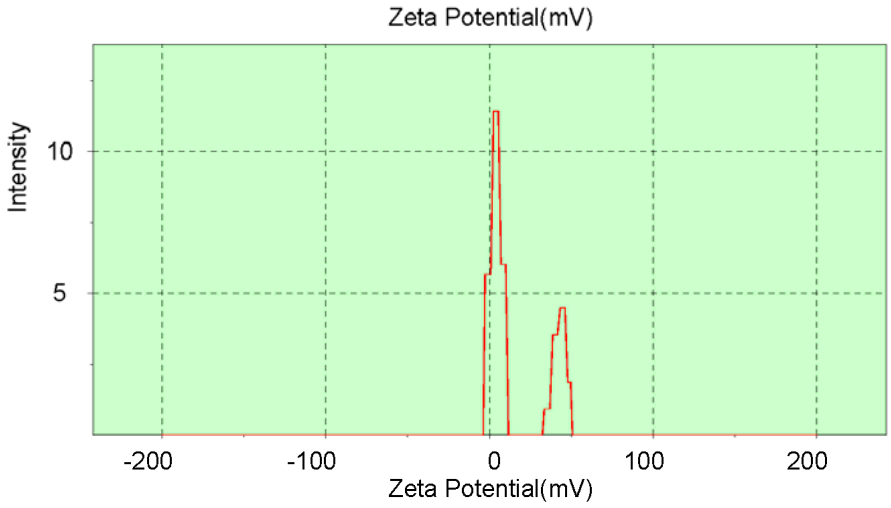
MALVERN ZETASIZER

Zeta Potential Report

PS0411-01
TMC particle 3:1 , without Tween 80
Jaco van Heerden

Sample		System	
Record Number:	4	Instrument Type:	Zetasizer 2000
Filename:	19MAY.zet	Temperature (°C):	25.0
File Path:	G:\MALVERN\ZETASI~1\19MAY	Count rate (kCps):	3932.0
Dielectric Constant:	79.7	Cell Type:	Capillary cell
pH:	N/A	Cell Position (%):	50.00
Viscosity (cP):	0.891	Cell field (V/cm):	29.0
Date (DMY):	23/05/11	Current (mA):	5.6
Time:	15:38:44		

Result			
Zeta Potential (mV):	14.8	Mobility (umcm/V.s):	1.135
StDev (mV):	17.5	StDev (umcm/V.s):	1.384
Conductivity (mS/cm):	1.92	F(ka):	1.50



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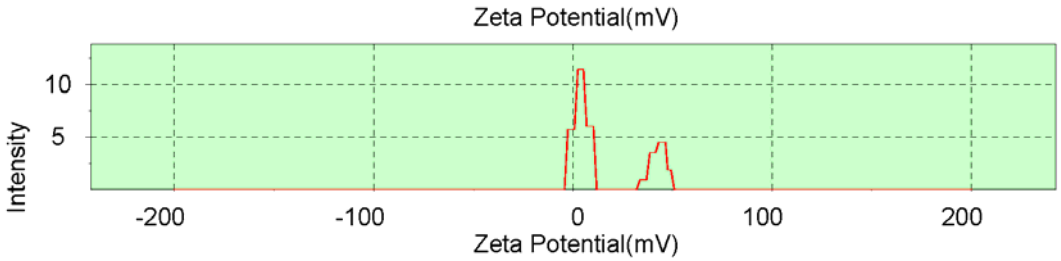
Int.	Zeta(mV)	Mob(µmcm/Vs)	Freq.(Hz)	Int.	Zeta(mV)	Mob(µmcm/V
0.0	-200.0	-15.846	21.9	48.0	3.2	0.252
0.0	-193.7	-15.343	28.8	25.4	9.5	0.755
0.0	-187.3	-14.840	35.7	0.0	15.9	1.258
0.0	-181.0	-14.337	42.7	0.0	22.2	1.761
0.0	-174.6	-13.833	49.6	0.0	28.6	2.264
0.0	-168.3	-13.330	56.5	3.9	34.9	2.767
0.0	-161.9	-12.827	63.4	14.9	41.3	3.270
0.0	-155.6	-12.324	70.4	7.8	47.6	3.773
0.0	-149.2	-11.821	77.3	0.0	54.0	4.276
0.0	-142.9	-11.318	84.2	0.0	60.3	4.779
0.0	-136.5	-10.815	91.1	0.0	66.7	5.282
0.0	-130.2	-10.312	98.1	0.0	73.0	5.785
0.0	-123.8	-9.809	105.0	0.0	79.4	6.288
0.0	-117.5	-9.306	111.9	0.0	85.7	6.791
0.0	-111.1	-8.803	118.8	0.0	92.1	7.294
0.0	-104.8	-8.300	125.8	0.0	98.4	7.797
0.0	-98.4	-7.797	132.7	0.0	104.8	8.300
0.0	-92.1	-7.294	139.6	0.0	111.1	8.803
0.0	-85.7	-6.791	146.5	0.0	117.5	9.306
0.0	-79.4	-6.288	153.4	0.0	123.8	9.809
0.0	-73.0	-5.785	160.4	0.0	130.2	10.312
0.0	-66.7	-5.282	167.3	0.0	136.5	10.815
0.0	-60.3	-4.779	174.2	0.0	142.9	11.318
0.0	-54.0	-4.276	181.1	0.0	149.2	11.821
0.0	-47.6	-3.773	188.1	0.0	155.6	12.324
0.0	-41.3	-3.270	195.0	0.0	161.9	12.827
0.0	-34.9	-2.767	201.9	0.0	168.3	13.330
0.0	-28.6	-2.264	208.8	0.0	174.6	13.833
0.0	-22.2	-1.761	215.8	0.0	181.0	14.337
0.0	-15.9	-1.258	222.7	0.0	187.3	14.840
0.0	-9.5	-0.755	229.6	0.0	193.7	15.343
0.0	-3.2	-0.252	236.5	0.0	200.0	15.846



Malvern Instruments Ltd, Malvern UK +44 1684 892456

ZETASIZER

Zeta Potential Report

PS0411-01
TMC particle 3:1 , without Tween 80
Jaco van Heerden

Sample

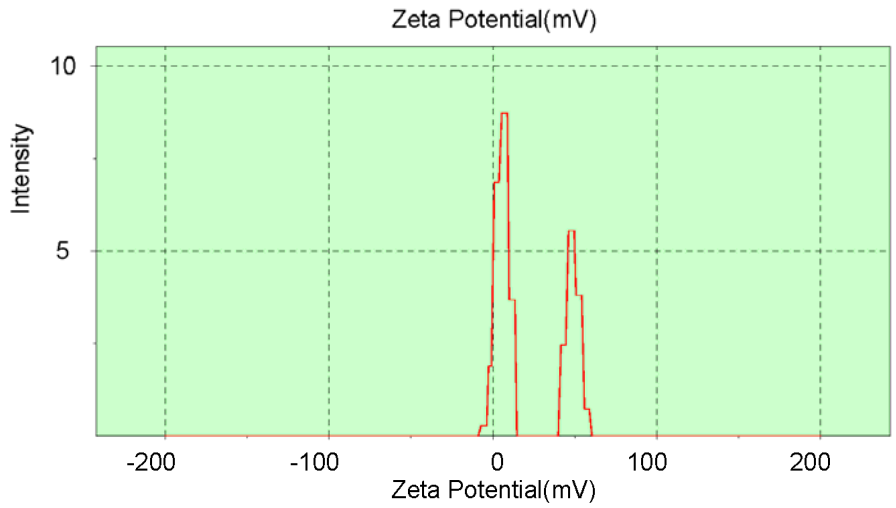
Record Number:	5
Filename:	19MAY.zet
File Path:	G:\MALVERN\ZETASI~1\19MAY
Dielectric Constant:	79.7
pH:	N/A
Viscosity (cP):	0.890
Date (DMY):	23/05/11
Time:	15:39:43

System

Instrument Type:	Zetasizer 2000
Temperature (°C):	25.0
Count rate (kCps):	3939.2
Cell Type:	Capillary cell
Cell Position (%):	50.00
Cell field (V/cm):	29.0
Current (mA):	5.7

Result

Zeta Potential (mV):	22.3	Mobility (umcm/V.s):	1.717
StDev (mV):	21.4	StDev (umcm/V.s):	1.695
Conductivity (mS/cm):	1.95	F(ka):	1.50



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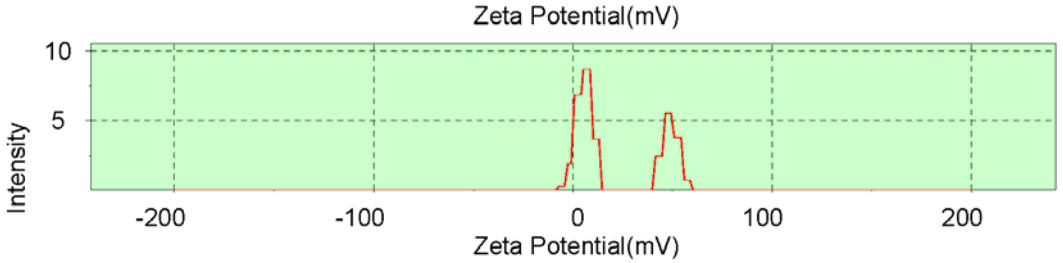
PS0411-01
TMC particle 3:1 , without Tween 80
Jaco van Heerden
File data from G:\MALVERN\ZETASI~1\19MAY2~1\19MAY.zet Record 5
Zetasizer 2000Data type 1 256

Int.	Zeta(mV)	Mob(µmcm/Vs)	Freq.(Hz)	Int.	Zeta(mV)	Mob(µmcm/V
0.0	-200.0	-15.855	17.8	23.4	3.2	0.252
0.0	-193.7	-15.352	24.7	29.8	9.5	0.755
0.0	-187.3	-14.849	31.6	0.0	15.9	1.258
0.0	-181.0	-14.345	38.5	0.0	22.2	1.762
0.0	-174.6	-13.842	45.5	0.0	28.6	2.265
0.0	-168.3	-13.339	52.4	0.0	34.9	2.768
0.0	-161.9	-12.835	59.3	8.3	41.3	3.272
0.0	-155.6	-12.332	66.3	18.9	47.6	3.775
0.0	-149.2	-11.829	73.2	13.0	54.0	4.278
0.0	-142.9	-11.325	80.1	0.0	60.3	4.782
0.0	-136.5	-10.822	87.0	0.0	66.7	5.285
0.0	-130.2	-10.319	94.0	0.0	73.0	5.788
0.0	-123.8	-9.815	100.9	0.0	79.4	6.292
0.0	-117.5	-9.312	107.8	0.0	85.7	6.795
0.0	-111.1	-8.809	114.8	0.0	92.1	7.298
0.0	-104.8	-8.305	121.7	0.0	98.4	7.802
0.0	-98.4	-7.802	128.6	0.0	104.8	8.305
0.0	-92.1	-7.298	135.5	0.0	111.1	8.809
0.0	-85.7	-6.795	142.5	0.0	117.5	9.312
0.0	-79.4	-6.292	149.4	0.0	123.8	9.815
0.0	-73.0	-5.788	156.3	0.0	130.2	10.319
0.0	-66.7	-5.285	163.3	0.0	136.5	10.822
0.0	-60.3	-4.782	170.2	0.0	142.9	11.325
0.0	-54.0	-4.278	177.1	0.0	149.2	11.829
0.0	-47.6	-3.775	184.0	0.0	155.6	12.332
0.0	-41.3	-3.272	191.0	0.0	161.9	12.835
0.0	-34.9	-2.768	197.9	0.0	168.3	13.339
0.0	-28.6	-2.265	204.8	0.0	174.6	13.842
0.0	-22.2	-1.762	211.8	0.0	181.0	14.345
0.0	-15.9	-1.258	218.7	0.0	187.3	14.849
0.0	-9.5	-0.755	225.6	0.0	193.7	15.352
6.5	-3.2	-0.252	232.5	0.0	200.0	15.855



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ZETASIZER

Zeta Potential Report

PS0411-01
TMC particle 3:1 , without Tween 80
Jaco van Heerden

Sample

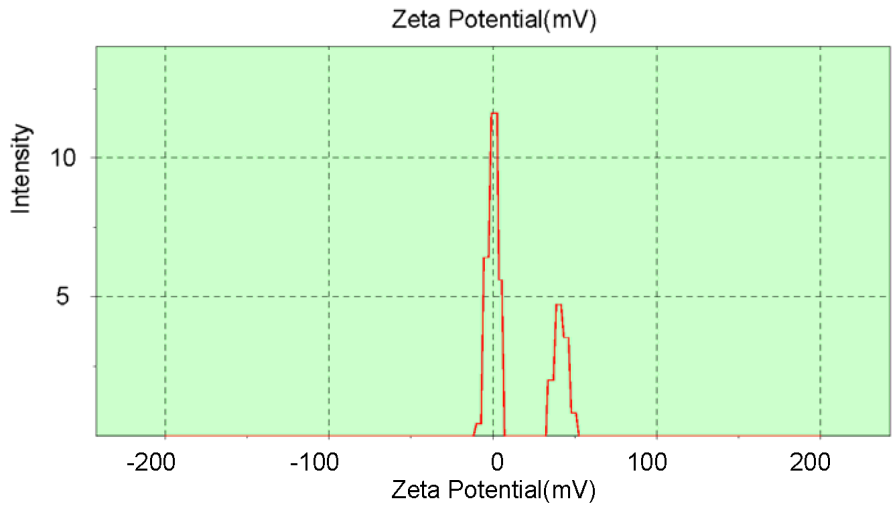
Record Number:	6
Filename:	19MAY.zet
File Path:	G:\MALVERN\ZETASI~1\19MAY
Dielectric Constant:	79.7
pH:	N/A
Viscosity (cP):	0.890
Date (DMY):	23/05/11
Time:	15:40:42

System

Instrument Type:	Zetasizer 2000
Temperature (°C):	25.0
Count rate (kCps):	3417.7
Cell Type:	Capillary cell
Cell Position (%):	50.00
Cell field (V/cm):	29.0
Current (mA):	5.7

Result

Zeta Potential (mV):	11.8	Mobility (umcm/V.s):	0.872
StDev (mV):	19.2	StDev (umcm/V.s):	1.519
Conductivity (mS/cm):	1.97	F(ka):	1.50



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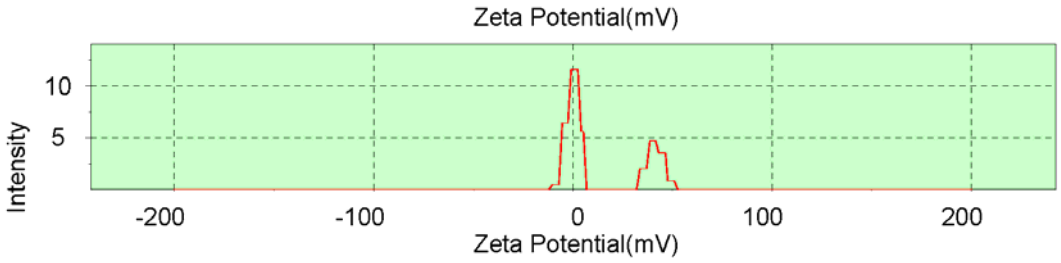
Int.	Zeta(mV)	Mob(µmcm/Vs)	Freq.(Hz)	Int.	Zeta(mV)	Mob(µmcm/V
0.0	-200.0	-15.855	25.8	28.1	3.2	0.252
0.0	-193.7	-15.351	32.7	0.0	9.5	0.755
0.0	-187.3	-14.848	39.6	0.0	15.9	1.258
0.0	-181.0	-14.345	46.6	0.0	22.2	1.762
0.0	-174.6	-13.841	53.5	0.0	28.6	2.265
0.0	-168.3	-13.338	60.4	10.0	34.9	2.768
0.0	-161.9	-12.835	67.3	23.6	41.3	3.272
0.0	-155.6	-12.331	74.3	4.1	47.6	3.775
0.0	-149.2	-11.828	81.2	0.0	54.0	4.278
0.0	-142.9	-11.325	88.1	0.0	60.3	4.782
0.0	-136.5	-10.821	95.1	0.0	66.7	5.285
0.0	-130.2	-10.318	102.0	0.0	73.0	5.788
0.0	-123.8	-9.815	108.9	0.0	79.4	6.291
0.0	-117.5	-9.311	115.8	0.0	85.7	6.795
0.0	-111.1	-8.808	122.8	0.0	92.1	7.298
0.0	-104.8	-8.305	129.7	0.0	98.4	7.801
0.0	-98.4	-7.801	136.6	0.0	104.8	8.305
0.0	-92.1	-7.298	143.5	0.0	111.1	8.808
0.0	-85.7	-6.795	150.5	0.0	117.5	9.311
0.0	-79.4	-6.291	157.4	0.0	123.8	9.815
0.0	-73.0	-5.788	164.3	0.0	130.2	10.318
0.0	-66.7	-5.285	171.3	0.0	136.5	10.821
0.0	-60.3	-4.782	178.2	0.0	142.9	11.325
0.0	-54.0	-4.278	185.1	0.0	149.2	11.828
0.0	-47.6	-3.775	192.0	0.0	155.6	12.331
0.0	-41.3	-3.272	199.0	0.0	161.9	12.835
0.0	-34.9	-2.768	205.9	0.0	168.3	13.338
0.0	-28.6	-2.265	212.8	0.0	174.6	13.841
0.0	-22.2	-1.762	219.8	0.0	181.0	14.345
0.0	-15.9	-1.258	226.7	0.0	187.3	14.848
2.3	-9.5	-0.755	233.6	0.0	193.7	15.351
32.0	-3.2	-0.252	240.5	0.0	200.0	15.855



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ZETASIZER

Zeta Potential Report

PS0411-02
TMC particles 3:1, With Tween 80
Jaco van Heerden

Sample

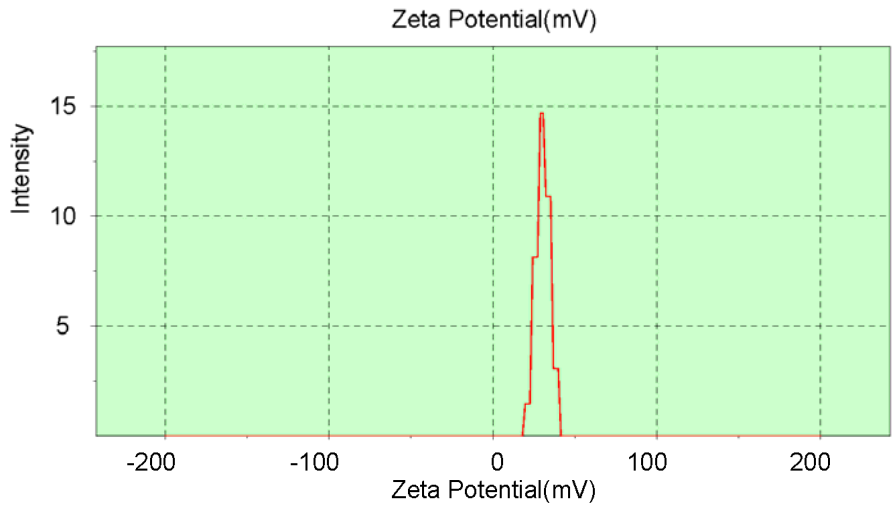
Record Number:	7
Filename:	19MAY.zet
File Path:	G:\MALVERN\ZETASI~1\19MAY
Dielectric Constant:	79.7
pH:	N/A
Viscosity (cP):	0.890
Date (DMY):	23/05/11
Time:	15:49:25

System

Instrument Type:	Zetasizer 2000
Temperature (°C):	25.0
Count rate (kCps):	3131.4
Cell Type:	Capillary cell
Cell Position (%):	50.00
Cell field (V/cm):	29.0
Current (mA):	6.1

Result

Zeta Potential (mV):	30.6	Mobility (umcm/V.s):	2.434
StDev (mV):	1.6	StDev (umcm/V.s):	0.126
Conductivity (mS/cm):	2.11	F(ka):	1.50



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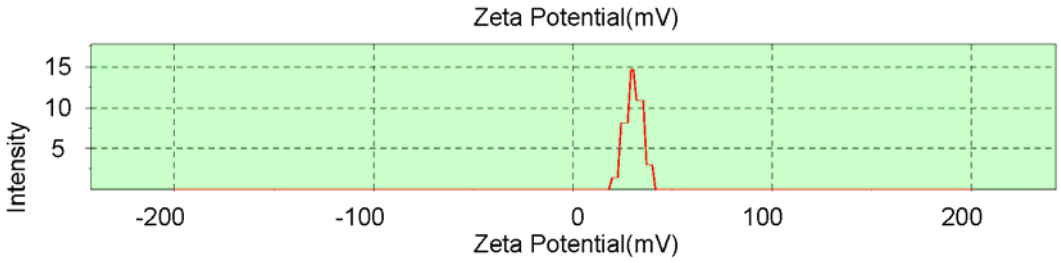
Int.	Zeta(mV)	Mob(µmcm/Vs)	Freq.(Hz)	Int.	Zeta(mV)	Mob(µmcm/V
0.0	-200.0	-15.856	479.8	0.0	3.2	0.252
0.0	-193.7	-15.353	472.9	0.0	9.5	0.755
0.0	-187.3	-14.849	466.0	0.0	15.9	1.258
0.0	-181.0	-14.346	459.1	5.4	22.2	1.762
0.0	-174.6	-13.843	452.2	54.3	28.6	2.265
0.0	-168.3	-13.339	445.2	40.3	34.9	2.769
0.0	-161.9	-12.836	438.3	0.0	41.3	3.272
0.0	-155.6	-12.333	431.4	0.0	47.6	3.775
0.0	-149.2	-11.829	424.5	0.0	54.0	4.279
0.0	-142.9	-11.326	417.6	0.0	60.3	4.782
0.0	-136.5	-10.822	410.7	0.0	66.7	5.285
0.0	-130.2	-10.319	403.7	0.0	73.0	5.789
0.0	-123.8	-9.816	396.8	0.0	79.4	6.292
0.0	-117.5	-9.312	389.9	0.0	85.7	6.795
0.0	-111.1	-8.809	383.0	0.0	92.1	7.299
0.0	-104.8	-8.306	376.1	0.0	98.4	7.802
0.0	-98.4	-7.802	369.2	0.0	104.8	8.306
0.0	-92.1	-7.299	362.3	0.0	111.1	8.809
0.0	-85.7	-6.795	355.3	0.0	117.5	9.312
0.0	-79.4	-6.292	348.4	0.0	123.8	9.816
0.0	-73.0	-5.789	341.5	0.0	130.2	10.319
0.0	-66.7	-5.285	334.6	0.0	136.5	10.822
0.0	-60.3	-4.782	327.7	0.0	142.9	11.326
0.0	-54.0	-4.279	320.8	0.0	149.2	11.829
0.0	-47.6	-3.775	313.9	0.0	155.6	12.333
0.0	-41.3	-3.272	306.9	0.0	161.9	12.836
0.0	-34.9	-2.769	300.0	0.0	168.3	13.339
0.0	-28.6	-2.265	293.1	0.0	174.6	13.843
0.0	-22.2	-1.762	286.2	0.0	181.0	14.346
0.0	-15.9	-1.258	279.3	0.0	187.3	14.849
0.0	-9.5	-0.755	272.4	0.0	193.7	15.353
0.0	-3.2	-0.252	265.5	0.0	200.0	15.856



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ZETASIZER

Zeta Potential Report

PS0411-02
TMC particles 3:1, With Tween 80
Jaco van Heerden

Sample

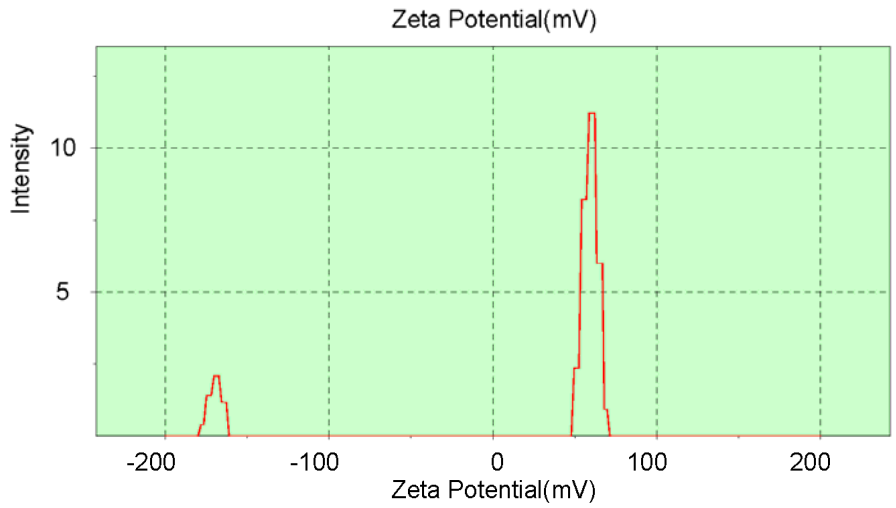
Record Number:	8
Filename:	19MAY.zet
File Path:	G:\MALVERN\ZETASI~1\19MAY
Dielectric Constant:	79.7
pH:	N/A
Viscosity (cP):	0.891
Date (DMY):	23/05/11
Time:	15:50:24

System

Instrument Type:	Zetasizer 2000
Temperature (°C):	25.0
Count rate (kCps):	2854.9
Cell Type:	Capillary cell
Cell Position (%):	50.00
Cell field (V/cm):	29.0
Current (mA):	6.2

Result

Zeta Potential (mV):	23.6	Mobility (umcm/V.s):	1.849
StDev (mV):	82.4	StDev (umcm/V.s):	6.530
Conductivity (mS/cm):	2.12	F(ka):	1.50



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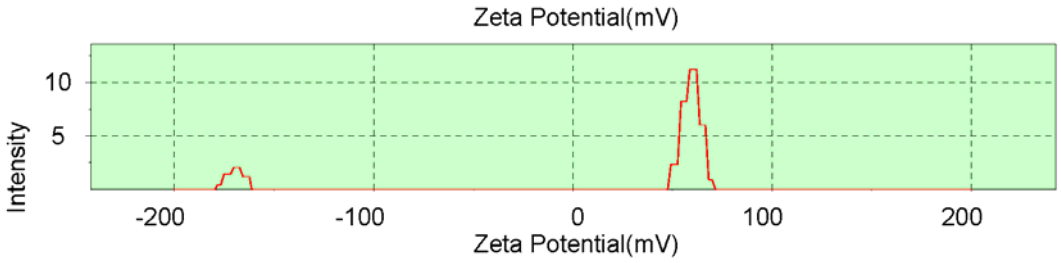
Int.	Zeta(mV)	Mob(µmcm/Vs)	Freq.(Hz)	Int.	Zeta(mV)	Mob(µmcm/V
0.0	-200.0	-15.852	0.0	0.0	3.2	0.252
0.0	-193.7	-15.349	0.0	0.0	9.5	0.755
0.0	-187.3	-14.846	3.7	0.0	15.9	1.258
0.0	-181.0	-14.342	10.6	0.0	22.2	1.761
4.7	-174.6	-13.839	17.5	0.0	28.6	2.265
6.9	-168.3	-13.336	24.4	0.0	34.9	2.768
3.9	-161.9	-12.833	31.4	0.0	41.3	3.271
0.0	-155.6	-12.329	38.3	0.0	47.6	3.774
0.0	-149.2	-11.826	45.2	27.3	54.0	4.278
0.0	-142.9	-11.323	52.1	37.3	60.3	4.781
0.0	-136.5	-10.820	59.1	20.0	66.7	5.284
0.0	-130.2	-10.316	66.0	0.0	73.0	5.787
0.0	-123.8	-9.813	72.9	0.0	79.4	6.291
0.0	-117.5	-9.310	79.9	0.0	85.7	6.794
0.0	-111.1	-8.807	86.8	0.0	92.1	7.297
0.0	-104.8	-8.303	93.7	0.0	98.4	7.800
0.0	-98.4	-7.800	100.6	0.0	104.8	8.303
0.0	-92.1	-7.297	107.6	0.0	111.1	8.807
0.0	-85.7	-6.794	114.5	0.0	117.5	9.310
0.0	-79.4	-6.291	121.4	0.0	123.8	9.813
0.0	-73.0	-5.787	128.3	0.0	130.2	10.316
0.0	-66.7	-5.284	135.3	0.0	136.5	10.820
0.0	-60.3	-4.781	142.2	0.0	142.9	11.323
0.0	-54.0	-4.278	149.1	0.0	149.2	11.826
0.0	-47.6	-3.774	156.0	0.0	155.6	12.329
0.0	-41.3	-3.271	163.0	0.0	161.9	12.833
0.0	-34.9	-2.768	169.9	0.0	168.3	13.336
0.0	-28.6	-2.265	176.8	0.0	174.6	13.839
0.0	-22.2	-1.761	183.8	0.0	181.0	14.342
0.0	-15.9	-1.258	190.7	0.0	187.3	14.846
0.0	-9.5	-0.755	197.6	0.0	193.7	15.349
0.0	-3.2	-0.252	204.5	0.0	200.0	15.852



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ZETASIZER

Zeta Potential Report

PS0411-02
TMC particles 3:1, With Tween 80
Jaco van Heerden

Sample

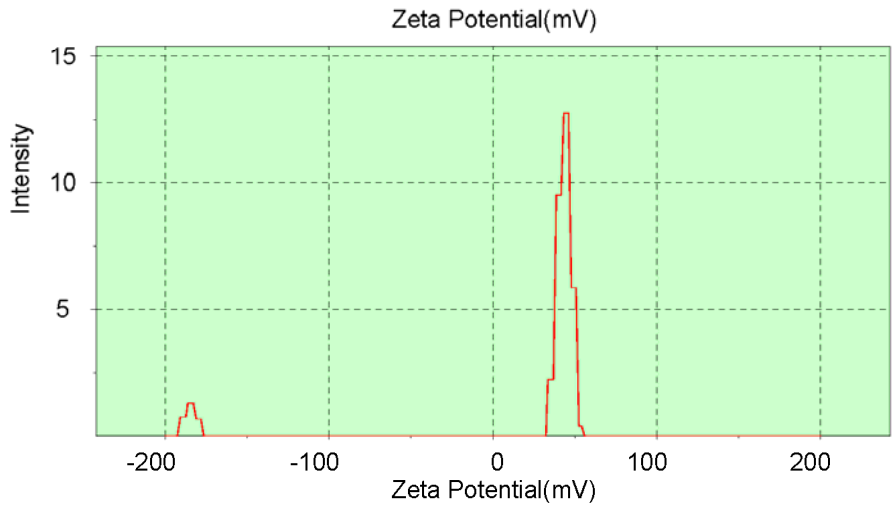
Record Number:	9
Filename:	19MAY.zet
File Path:	G:\MALVERN\ZETASI~1\19MAY
Dielectric Constant:	79.7
pH:	N/A
Viscosity (cP):	0.890
Date (DMY):	23/05/11
Time:	15:51:23

System

Instrument Type:	Zetasizer 2000
Temperature (°C):	25.0
Count rate (kCps):	2345.5
Cell Type:	Capillary cell
Cell Position (%):	50.00
Cell field (V/cm):	29.0
Current (mA):	6.2

Result

Zeta Potential (mV):	22.4	Mobility (umcm/V.s):	1.728
StDev (mV):	65.6	StDev (umcm/V.s):	5.202
Conductivity (mS/cm):	2.13	F(ka):	1.50



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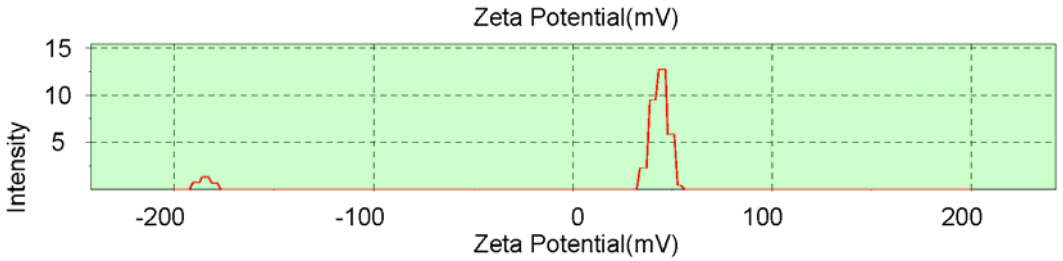
Int.	Zeta(mV)	Mob(µmcm/Vs)	Freq.(Hz)	Int.	Zeta(mV)	Mob(µmcm/V
0.0	-200.0	-15.853	6.9	0.0	3.2	0.252
0.0	-193.7	-15.350	13.9	0.0	9.5	0.755
3.9	-187.3	-14.846	20.8	0.0	15.9	1.258
3.4	-181.0	-14.343	27.7	0.0	22.2	1.761
0.0	-174.6	-13.840	34.6	0.0	28.6	2.265
0.0	-168.3	-13.337	41.6	11.6	34.9	2.768
0.0	-161.9	-12.833	48.5	49.0	41.3	3.271
0.0	-155.6	-12.330	55.4	30.1	47.6	3.775
0.0	-149.2	-11.827	62.3	2.0	54.0	4.278
0.0	-142.9	-11.324	69.2	0.0	60.3	4.781
0.0	-136.5	-10.820	76.2	0.0	66.7	5.284
0.0	-130.2	-10.317	83.1	0.0	73.0	5.788
0.0	-123.8	-9.814	90.0	0.0	79.4	6.291
0.0	-117.5	-9.310	96.9	0.0	85.7	6.794
0.0	-111.1	-8.807	103.9	0.0	92.1	7.297
0.0	-104.8	-8.304	110.8	0.0	98.4	7.801
0.0	-98.4	-7.801	117.7	0.0	104.8	8.304
0.0	-92.1	-7.297	124.6	0.0	111.1	8.807
0.0	-85.7	-6.794	131.5	0.0	117.5	9.310
0.0	-79.4	-6.291	138.5	0.0	123.8	9.814
0.0	-73.0	-5.788	145.4	0.0	130.2	10.317
0.0	-66.7	-5.284	152.3	0.0	136.5	10.820
0.0	-60.3	-4.781	159.2	0.0	142.9	11.324
0.0	-54.0	-4.278	166.2	0.0	149.2	11.827
0.0	-47.6	-3.775	173.1	0.0	155.6	12.330
0.0	-41.3	-3.271	180.0	0.0	161.9	12.833
0.0	-34.9	-2.768	186.9	0.0	168.3	13.337
0.0	-28.6	-2.265	193.8	0.0	174.6	13.840
0.0	-22.2	-1.761	200.8	0.0	181.0	14.343
0.0	-15.9	-1.258	207.7	0.0	187.3	14.846
0.0	-9.5	-0.755	214.6	0.0	193.7	15.350
0.0	-3.2	-0.252	221.5	0.0	200.0	15.853



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ZETASIZER

Zeta Potential Report

PS0411-03
TMC particles 3:1, without Tween80 and with ultrasonic rod
Jaco van Heerden

Sample

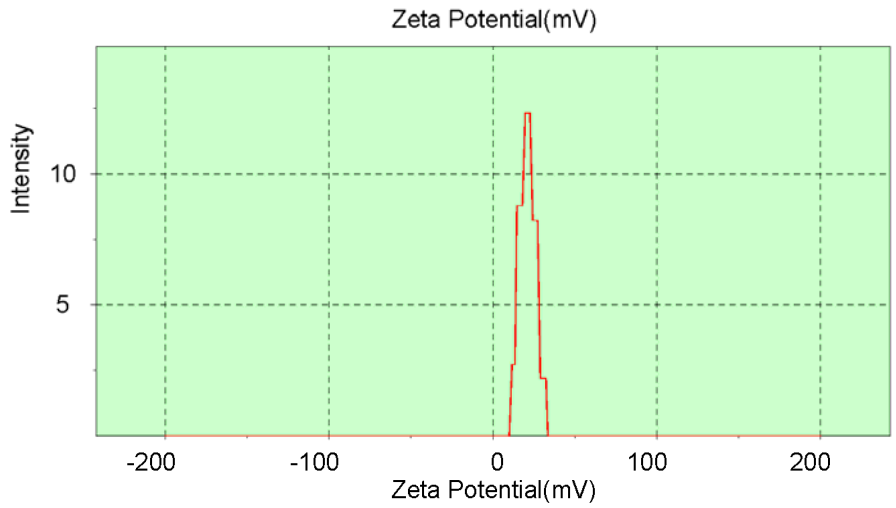
Record Number:	10
Filename:	19MAY.zet
File Path:	G:\MALVERN\ZETASI~1\19MAY
Dielectric Constant:	79.7
pH:	N/A
Viscosity (cP):	0.891
Date (DMY):	23/05/11
Time:	16:00:09

System

Instrument Type:	Zetasizer 2000
Temperature (°C):	25.0
Count rate (kCps):	2887.6
Cell Type:	Capillary cell
Cell Position (%):	50.00
Cell field (V/cm):	29.0
Current (mA):	7.8

Result

Zeta Potential (mV):	21.2	Mobility (umcm/V.s):	1.626
StDev (mV):	1.6	StDev (umcm/V.s):	0.126
Conductivity (mS/cm):	2.68	F(ka):	1.50



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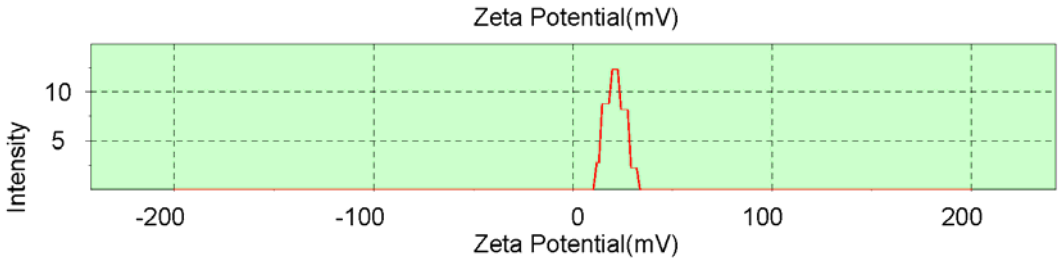
Int.	Zeta(mV)	Mob(µmcm/Vs)	Freq.(Hz)	Int.	Zeta(mV)	Mob(µmcm/V
0.0	-200.0	-15.848	32.2	0.0	3.2	0.252
0.0	-193.7	-15.345	39.1	0.0	9.5	0.755
0.0	-187.3	-14.842	46.0	37.7	15.9	1.258
0.0	-181.0	-14.339	52.9	52.8	22.2	1.761
0.0	-174.6	-13.836	59.8	9.5	28.6	2.264
0.0	-168.3	-13.332	66.7	0.0	34.9	2.767
0.0	-161.9	-12.829	73.6	0.0	41.3	3.270
0.0	-155.6	-12.326	80.6	0.0	47.6	3.773
0.0	-149.2	-11.823	87.5	0.0	54.0	4.276
0.0	-142.9	-11.320	94.4	0.0	60.3	4.780
0.0	-136.5	-10.817	101.3	0.0	66.7	5.283
0.0	-130.2	-10.314	108.2	0.0	73.0	5.786
0.0	-123.8	-9.811	115.1	0.0	79.4	6.289
0.0	-117.5	-9.308	122.1	0.0	85.7	6.792
0.0	-111.1	-8.804	129.0	0.0	92.1	7.295
0.0	-104.8	-8.301	135.9	0.0	98.4	7.798
0.0	-98.4	-7.798	142.8	0.0	104.8	8.301
0.0	-92.1	-7.295	149.7	0.0	111.1	8.804
0.0	-85.7	-6.792	156.6	0.0	117.5	9.308
0.0	-79.4	-6.289	163.6	0.0	123.8	9.811
0.0	-73.0	-5.786	170.5	0.0	130.2	10.314
0.0	-66.7	-5.283	177.4	0.0	136.5	10.817
0.0	-60.3	-4.780	184.3	0.0	142.9	11.320
0.0	-54.0	-4.276	191.2	0.0	149.2	11.823
0.0	-47.6	-3.773	198.1	0.0	155.6	12.326
0.0	-41.3	-3.270	205.0	0.0	161.9	12.829
0.0	-34.9	-2.767	212.0	0.0	168.3	13.332
0.0	-28.6	-2.264	218.9	0.0	174.6	13.836
0.0	-22.2	-1.761	225.8	0.0	181.0	14.339
0.0	-15.9	-1.258	232.7	0.0	187.3	14.842
0.0	-9.5	-0.755	239.6	0.0	193.7	15.345
0.0	-3.2	-0.252	246.5	0.0	200.0	15.848



Malvern Instruments Ltd, Malvern UK +44 1684 892456

ZETASIZER

Zeta Potential Report

PS0411-03
TMC particles 3:1, without Tween80 and with ultrasonic rod
Jaco van Heerden

Sample

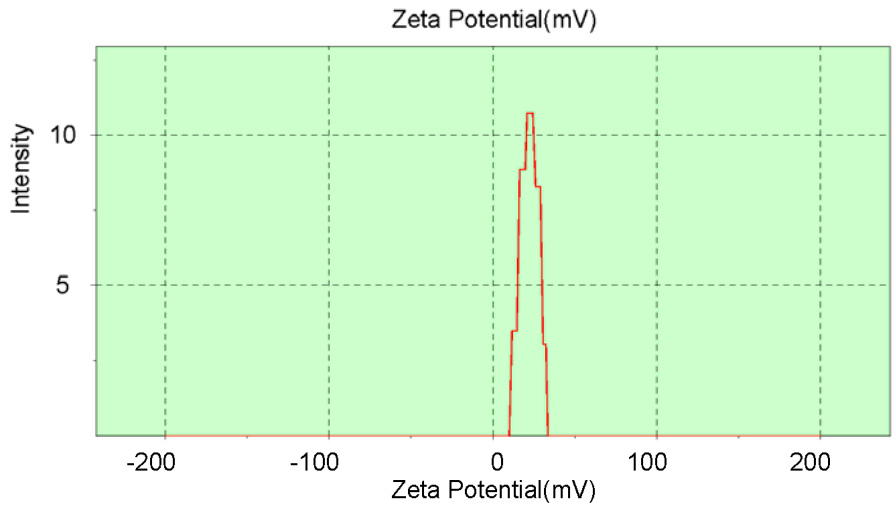
Record Number:	11
Filename:	19MAY.zet
File Path:	G:\MALVERN\ZETASI~1\19MAY
Dielectric Constant:	79.7
pH:	N/A
Viscosity (cP):	0.890
Date (DMY):	23/05/11
Time:	16:01:09

System

Instrument Type:	Zetasizer 2000
Temperature (°C):	25.0
Count rate (kCps):	3127.5
Cell Type:	Capillary cell
Cell Position (%):	50.00
Cell field (V/cm):	29.1
Current (mA):	7.8

Result

Zeta Potential (mV):	22.1	Mobility (umcm/V.s):	1.700
StDev (mV):	1.6	StDev (umcm/V.s):	0.125
Conductivity (mS/cm):	2.70	F(ka):	1.50



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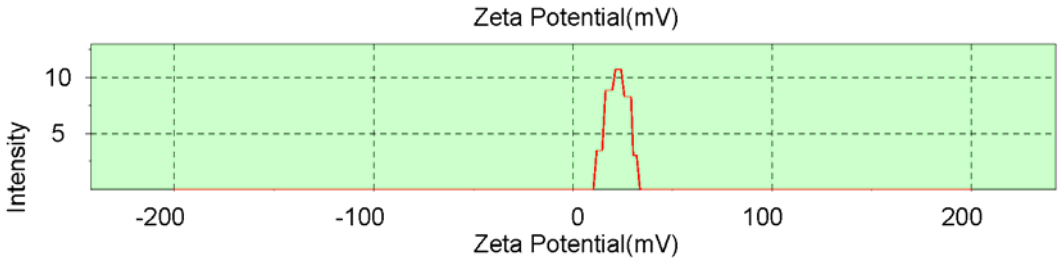
Int.	Zeta(mV)	Mob(µmcm/Vs)	Freq.(Hz)	Int.	Zeta(mV)	Mob(µmcm/V
0.0	-200.0	-15.863	30.5	0.0	3.2	0.252
0.0	-193.7	-15.360	37.4	0.0	9.5	0.755
0.0	-187.3	-14.856	44.4	31.8	15.9	1.259
0.0	-181.0	-14.353	51.3	38.5	22.2	1.763
0.0	-174.6	-13.849	58.2	29.7	28.6	2.266
0.0	-168.3	-13.345	65.2	0.0	34.9	2.770
0.0	-161.9	-12.842	72.1	0.0	41.3	3.273
0.0	-155.6	-12.338	79.1	0.0	47.6	3.777
0.0	-149.2	-11.835	86.0	0.0	54.0	4.281
0.0	-142.9	-11.331	92.9	0.0	60.3	4.784
0.0	-136.5	-10.827	99.9	0.0	66.7	5.288
0.0	-130.2	-10.324	106.8	0.0	73.0	5.791
0.0	-123.8	-9.820	113.7	0.0	79.4	6.295
0.0	-117.5	-9.317	120.7	0.0	85.7	6.799
0.0	-111.1	-8.813	127.6	0.0	92.1	7.302
0.0	-104.8	-8.309	134.5	0.0	98.4	7.806
0.0	-98.4	-7.806	141.5	0.0	104.8	8.309
0.0	-92.1	-7.302	148.4	0.0	111.1	8.813
0.0	-85.7	-6.799	155.4	0.0	117.5	9.317
0.0	-79.4	-6.295	162.3	0.0	123.8	9.820
0.0	-73.0	-5.791	169.2	0.0	130.2	10.324
0.0	-66.7	-5.288	176.2	0.0	136.5	10.827
0.0	-60.3	-4.784	183.1	0.0	142.9	11.331
0.0	-54.0	-4.281	190.0	0.0	149.2	11.835
0.0	-47.6	-3.777	197.0	0.0	155.6	12.338
0.0	-41.3	-3.273	203.9	0.0	161.9	12.842
0.0	-34.9	-2.770	210.8	0.0	168.3	13.345
0.0	-28.6	-2.266	217.8	0.0	174.6	13.849
0.0	-22.2	-1.763	224.7	0.0	181.0	14.353
0.0	-15.9	-1.259	231.7	0.0	187.3	14.856
0.0	-9.5	-0.755	238.6	0.0	193.7	15.360
0.0	-3.2	-0.252	245.5	0.0	200.0	15.863



Malvern Instruments Ltd, Malvern UK +44 1684 892456

ZETASIZER

Zeta Potential Report

PS0411-03
TMC particles 3:1, without Tween80 and with ultrasonic rod
Jaco van Heerden

Sample

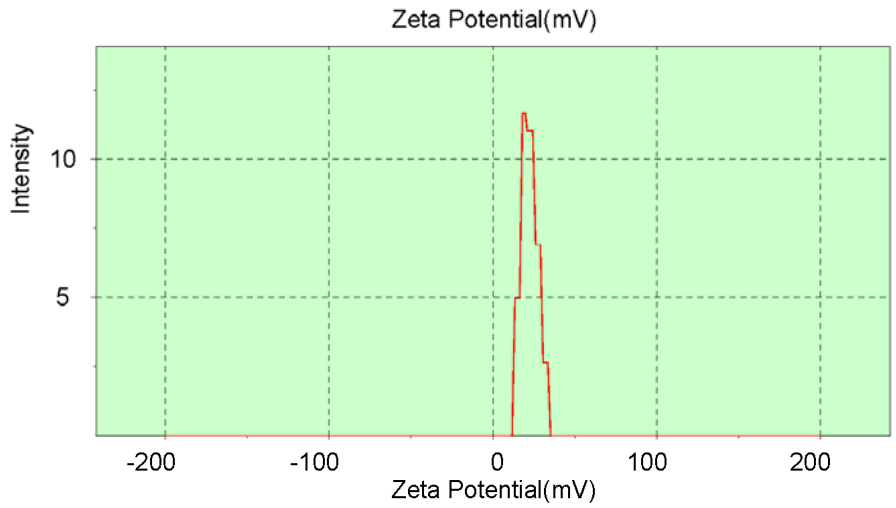
Record Number:	12
Filename:	19MAY.zet
File Path:	G:\MALVERN\ZETASI~1\19MAY
Dielectric Constant:	79.7
pH:	N/A
Viscosity (cP):	0.891
Date (DMY):	23/05/11
Time:	16:02:08

System

Instrument Type:	Zetasizer 2000
Temperature (°C):	25.0
Count rate (kCps):	3156.4
Cell Type:	Capillary cell
Cell Position (%):	50.00
Cell field (V/cm):	29.0
Current (mA):	7.9

Result

Zeta Potential (mV):	22.2	Mobility (umcm/V.s):	1.732
StDev (mV):	1.6	StDev (umcm/V.s):	0.126
Conductivity (mS/cm):	2.72	F(ka):	1.50



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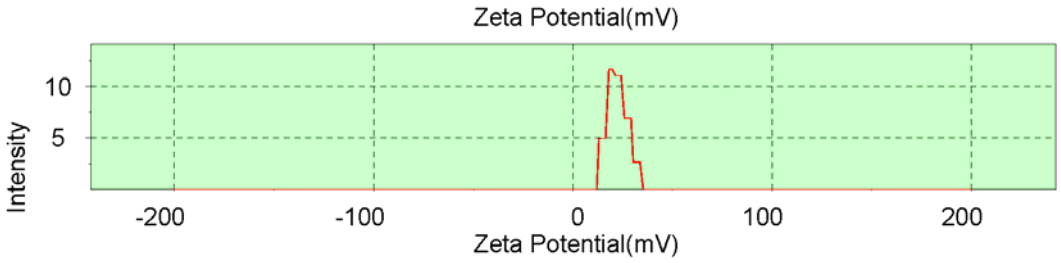
Int.	Zeta(mV)	Mob(µmcm/Vs)	Freq.(Hz)	Int.	Zeta(mV)	Mob(µmcm/V
0.0	-200.0	-15.848	30.3	0.0	3.2	0.252
0.0	-193.7	-15.345	37.2	0.0	9.5	0.755
0.0	-187.3	-14.842	44.1	21.7	15.9	1.258
0.0	-181.0	-14.339	51.0	48.2	22.2	1.761
0.0	-174.6	-13.836	57.9	30.1	28.6	2.264
0.0	-168.3	-13.332	64.9	0.0	34.9	2.767
0.0	-161.9	-12.829	71.8	0.0	41.3	3.270
0.0	-155.6	-12.326	78.7	0.0	47.6	3.773
0.0	-149.2	-11.823	85.6	0.0	54.0	4.276
0.0	-142.9	-11.320	92.5	0.0	60.3	4.780
0.0	-136.5	-10.817	99.4	0.0	66.7	5.283
0.0	-130.2	-10.314	106.3	0.0	73.0	5.786
0.0	-123.8	-9.811	113.2	0.0	79.4	6.289
0.0	-117.5	-9.308	120.1	0.0	85.7	6.792
0.0	-111.1	-8.804	127.1	0.0	92.1	7.295
0.0	-104.8	-8.301	134.0	0.0	98.4	7.798
0.0	-98.4	-7.798	140.9	0.0	104.8	8.301
0.0	-92.1	-7.295	147.8	0.0	111.1	8.804
0.0	-85.7	-6.792	154.7	0.0	117.5	9.308
0.0	-79.4	-6.289	161.6	0.0	123.8	9.811
0.0	-73.0	-5.786	168.5	0.0	130.2	10.314
0.0	-66.7	-5.283	175.4	0.0	136.5	10.817
0.0	-60.3	-4.780	182.3	0.0	142.9	11.320
0.0	-54.0	-4.276	189.3	0.0	149.2	11.823
0.0	-47.6	-3.773	196.2	0.0	155.6	12.326
0.0	-41.3	-3.270	203.1	0.0	161.9	12.829
0.0	-34.9	-2.767	210.0	0.0	168.3	13.332
0.0	-28.6	-2.264	216.9	0.0	174.6	13.836
0.0	-22.2	-1.761	223.8	0.0	181.0	14.339
0.0	-15.9	-1.258	230.7	0.0	187.3	14.842
0.0	-9.5	-0.755	237.6	0.0	193.7	15.345
0.0	-3.2	-0.252	244.5	0.0	200.0	15.848



Malvern Instruments Ltd, Malvern UK +44 1684 892456

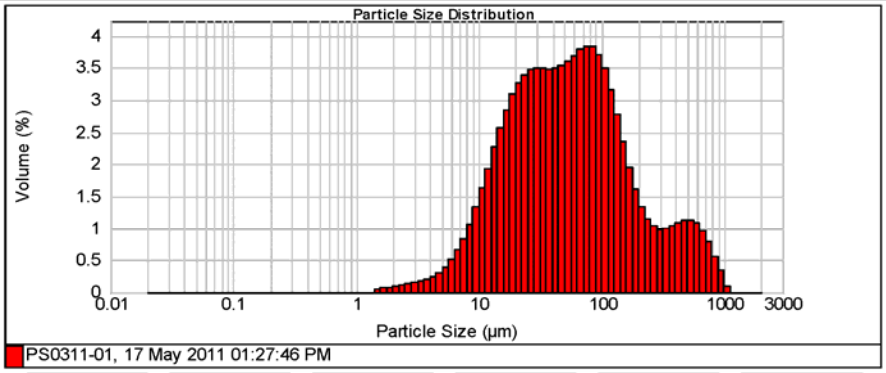
5. Mastersizer: TMC-TPP particles (TMC:TPP ratio – 5:1)

MASTERSIZER 2000

Result Analysis Report

Sample Name: PS0311-01	**SOP Name:** Jaco (TMC)	**Measured:** 17 May 2011 01:27:46 PM	
Sample Source & type:	**Measured by:** Jaco van Heerden	**Analysed:** 17 May 2011 01:27:47 PM	
Sample bulk lot ref:	**Result Source:** Measurement		
Particle Name: Titanium Dioxide	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.741	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 18.39 %
Dispersant Name: Alcohol	**Dispersant RI:** 1.320	**Weighted Residual:** 0.531 %	**Result Emulation:** Off
Concentration: 0.0415 %Vol	**Span :** 5.247	**Uniformity:** 1.64	**Result units:** Volume
Specific Surface Area: 0.213 m²/g	**Surface Weighted Mean D[3,2]:** 28.136 um	**Vol. Weighted Mean D[4,3]:** 111.520 um	

d(0.1): 12.596 um **d(0.5):** 52.858 um **d(0.9):** 289.946 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 %
0.020	0.00	0.142	0.00	1.002	0.00	7.096	0.84	50.238	3.60	355.656	1.04
0.022	0.00	0.159	0.00	1.125	0.00	7.962	1.07	56.368	3.69	399.052	1.09
0.025	0.00	0.178	0.00	1.262	0.00	8.934	1.33	63.246	3.78	447.744	1.14
0.028	0.00	0.200	0.00	1.416	0.06	10.024	1.63	70.963	3.84	502.377	1.14
0.032	0.00	0.224	0.00	1.589	0.07	11.247	1.94	79.621	3.83	563.677	1.08
0.036	0.00	0.252	0.00	1.783	0.08	12.619	2.26	89.337	3.71	632.456	0.97
0.040	0.00	0.283	0.00	2.000	0.10	14.159	2.57	100.237	3.49	709.627	0.78
0.045	0.00	0.317	0.00	2.244	0.11	15.887	2.85	112.468	3.17	796.214	0.56
0.050	0.00	0.356	0.00	2.518	0.13	17.825	3.09	126.191	2.77	893.367	0.34
0.056	0.00	0.399	0.00	2.825	0.15	20.000	3.27	141.589	2.35	1002.374	0.09
0.063	0.00	0.448	0.00	3.170	0.17	22.440	3.40	158.866	1.96	1124.683	0.00
0.071	0.00	0.502	0.00	3.557	0.21	25.179	3.47	178.250	1.62	1261.915	0.00
0.080	0.00	0.564	0.00	3.991	0.25	28.251	3.49	200.000	1.35	1415.892	0.00
0.089	0.00	0.632	0.00	4.477	0.31	31.698	3.49	224.404	1.16	1588.656	0.00
0.100	0.00	0.710	0.00	5.024	0.40	35.566	3.48	251.785	1.04	1782.502	0.00
0.112	0.00	0.796	0.00	5.637	0.51	39.905	3.49	282.508	0.99	2000.000	
0.126	0.00	0.893	0.00	6.325	0.66	44.774	3.49	316.979	1.00		
0.142		1.002		7.096		50.238	3.53	355.656			

Operator notes:

Malvern Instruments Ltd.
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007548

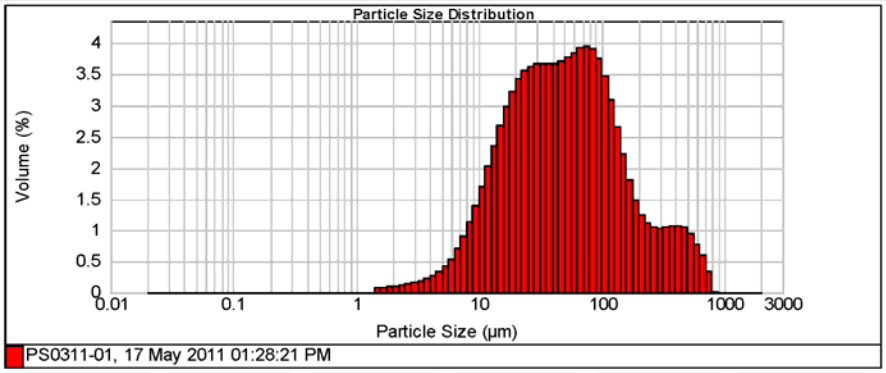
File name: 17 May 2011
Record Number: 1

MASTERSIZER 2000

Result Analysis Report

Sample Name: PS0311-01	**SOP Name:** Jaco (TMC)	**Measured:** 17 May 2011 01:28:21 PM	
Sample Source & type:	**Measured by:** Jaco van Heerden	**Analysed:** 17 May 2011 01:28:22 PM	
Sample bulk lot ref:	**Result Source:** Measurement		
Particle Name: Titanium Dioxide	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.741	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 18.32 %
Dispersant Name: Alcohol	**Dispersant RI:** 1.320	**Weighted Residual:** 0.485 %	**Result Emulation:** Off
Concentration: 0.0394 %Vol	**Span :** 4.380	**Uniformity:** 1.43	**Result units:** Volume
Specific Surface Area: 0.223 m²/g	**Surface Weighted Mean D[3,2]:** 26.942 um	**Vol. Weighted Mean D[4,3]:** 93.814 um	

d(0.1): 12.237 um **d(0.5):** 49.104 um **d(0.9):** 227.288 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 %
0.020	0.00	0.142	0.00	1.002	0.00	7.096	0.89	50.238	3.76	355.656	1.08
0.022	0.00	0.159	0.00	1.125	0.00	7.962	1.13	56.368	3.84	399.052	1.08
0.025	0.00	0.178	0.00	1.262	0.00	8.934	1.40	63.246	3.92	447.744	1.04
0.028	0.00	0.200	0.00	1.416	0.07	10.024	1.71	70.963	3.95	502.377	0.95
0.032	0.00	0.224	0.00	1.589	0.08	11.247	2.03	79.621	3.90	563.677	0.78
0.036	0.00	0.252	0.00	1.783	0.09	12.619	2.37	89.337	3.74	632.456	0.60
0.040	0.00	0.283	0.00	2.000	0.10	14.159	2.69	100.237	3.47	709.627	0.34
0.045	0.00	0.317	0.00	2.244	0.12	15.887	2.98	112.468	3.10	796.214	0.01
0.050	0.00	0.356	0.00	2.518	0.14	17.825	3.23	126.191	2.67	893.367	0.00
0.056	0.00	0.399	0.00	2.825	0.16	20.000	3.42	141.589	2.22	1002.374	0.00
0.063	0.00	0.448	0.00	3.170	0.18	22.440	3.55	158.866	1.82	1124.683	0.00
0.071	0.00	0.502	0.00	3.557	0.22	25.179	3.62	178.250	1.49	1261.915	0.00
0.080	0.00	0.564	0.00	3.991	0.27	28.251	3.65	200.000	1.26	1415.892	0.00
0.089	0.00	0.632	0.00	4.477	0.33	31.698	3.65	224.404	1.11	1588.656	0.00
0.100	0.00	0.710	0.00	5.024	0.42	35.566	3.65	251.785	1.04	1782.502	0.00
0.112	0.00	0.796	0.00	5.637	0.54	39.905	3.66	282.508	1.03	2000.000	0.00
0.126	0.00	0.893	0.00	6.325	0.70	44.774	3.70	316.979	1.05		
0.142	0.00	1.002	0.00	7.096		50.238		355.656			

Operator notes:

Malvern Instruments Ltd.
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007548

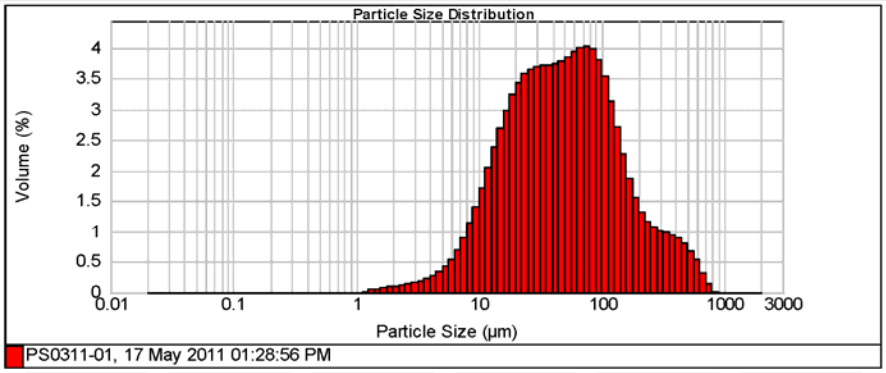
File name: 17 May 2011
Record Number: 2

MASTERSIZER 2000

Result Analysis Report

Sample Name: PS0311-01	**SOP Name:** Jaco (TMC)	**Measured:** 17 May 2011 01:28:56 PM	
Sample Source & type:	**Measured by:** Jaco van Heerden	**Analysed:** 17 May 2011 01:28:57 PM	
Sample bulk lot ref:	**Result Source:** Measurement		
Particle Name: Titanium Dioxide	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.741	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 18.34 %
Dispersant Name: Alcohol	**Dispersant RI:** 1.320	**Weighted Residual:** 0.491 %	**Result Emulation:** Off
Concentration: 0.0385 %Vol	**Span :** 3.875	**Uniformity:** 1.3	**Result units:** Volume
Specific Surface Area: 0.228 m²/g	**Surface Weighted Mean D[3,2]:** 26.325 um	**Vol. Weighted Mean D[4,3]:** 86.443 um	

d(0.1): 12.134 um **d(0.5):** 48.247 um **d(0.9):** 199.112 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 %
0.020	0.00	0.142	0.00	1.002	0.00	7.096	0.90	50.238	3.85	355.656	0.95
0.022	0.00	0.159	0.00	1.125	0.01	7.962	1.14	56.368	3.94	399.052	0.90
0.025	0.00	0.178	0.00	1.262	0.05	8.934	1.41	63.246	4.01	447.744	0.81
0.028	0.00	0.200	0.00	1.416	0.06	10.024	1.72	70.963	4.03	502.377	0.69
0.032	0.00	0.224	0.00	1.589	0.08	11.247	2.05	79.621	3.97	563.677	0.55
0.036	0.00	0.252	0.00	1.783	0.09	12.619	2.38	89.337	3.81	632.456	0.32
0.040	0.00	0.283	0.00	2.000	0.10	14.159	2.70	100.237	3.53	709.627	0.15
0.045	0.00	0.317	0.00	2.244	0.12	15.887	2.99	112.468	3.15	796.214	0.01
0.050	0.00	0.356	0.00	2.518	0.14	17.825	3.24	126.191	2.72	893.367	0.00
0.056	0.00	0.399	0.00	2.825	0.16	20.000	3.43	141.589	2.27	1002.374	0.00
0.063	0.00	0.448	0.00	3.170	0.19	22.440	3.57	158.866	1.88	1124.683	0.00
0.071	0.00	0.502	0.00	3.557	0.22	25.179	3.65	178.250	1.56	1261.915	0.00
0.080	0.00	0.564	0.00	3.991	0.27	28.251	3.69	200.000	1.33	1415.892	0.00
0.089	0.00	0.632	0.00	4.477	0.34	31.698	3.71	224.404	1.17	1588.656	0.00
0.100	0.00	0.710	0.00	5.024	0.43	35.566	3.71	251.785	1.07	1782.502	0.00
0.112	0.00	0.796	0.00	5.637	0.55	39.905	3.74	282.508	1.02	2000.000	0.00
0.126	0.00	0.893	0.00	6.325	0.70	44.774	3.78	316.979	0.98		
0.142	0.00	1.002	0.00	7.096		50.238		355.656			

Operator notes:

Malvern Instruments Ltd.
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007548

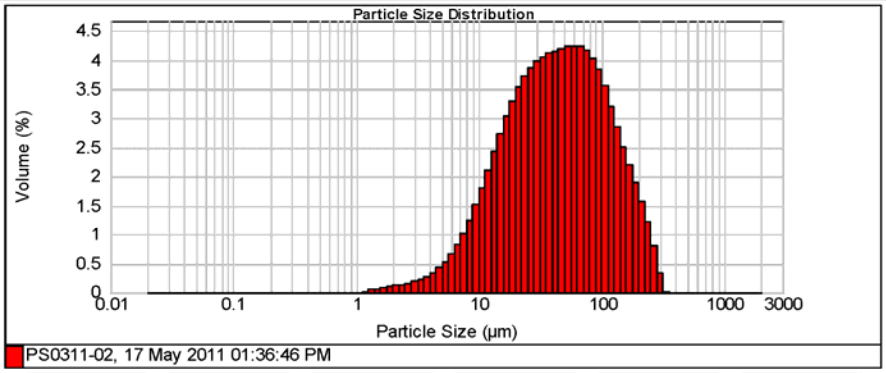
File name: 17 May 2011
Record Number: 3

MASTERSIZER 2000

Result Analysis Report

Sample Name: PS0311-02	**SOP Name:** Jaco (TMC)	**Measured:** 17 May 2011 01:36:46 PM	
Sample Source & type:	**Measured by:** Jaco van Heerden	**Analysed:** 17 May 2011 01:36:47 PM	
Sample bulk lot ref:	**Result Source:** Measurement		
Particle Name: Titanium Dioxide	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.741	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 17.36 %
Dispersant Name: Alcohol	**Dispersant RI:** 1.320	**Weighted Residual:** 2.335 %	**Result Emulation:** Off
Concentration: 0.0319 %Vol	**Span :** 3.060	**Uniformity:** 0.941	**Result units:** Volume
Specific Surface Area: 0.247 m²/g	**Surface Weighted Mean D[3,2]:** 24.275 um	**Vol. Weighted Mean D[4,3]:** 63.022 um	

d(0.1): 11.357 um **d(0.5):** 43.667 um **d(0.9):** 144.967 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 %
0.020	0.00	0.142	0.00	1.002	0.00	7.096	1.02	50.238	4.22	355.656	0.00
0.022	0.00	0.159	0.00	1.125	0.02	7.962	1.26	56.368	4.24	399.052	0.00
0.025	0.00	0.178	0.00	1.262	0.06	8.934	1.52	63.246	4.23	447.744	0.00
0.028	0.00	0.200	0.00	1.416	0.07	10.024	1.81	70.963	4.17	502.377	0.00
0.032	0.00	0.224	0.00	1.589	0.08	11.247	2.12	79.621	4.04	563.677	0.00
0.036	0.00	0.252	0.00	1.783	0.10	12.619	2.43	89.337	3.83	632.456	0.00
0.040	0.00	0.283	0.00	2.000	0.12	14.159	2.75	100.237	3.55	709.627	0.00
0.045	0.00	0.317	0.00	2.244	0.14	15.887	3.04	112.468	3.22	796.214	0.00
0.050	0.00	0.356	0.00	2.518	0.16	17.825	3.31	126.191	2.87	893.367	0.00
0.056	0.00	0.399	0.00	2.825	0.19	20.000	3.54	141.589	2.52	1002.374	0.00
0.063	0.00	0.448	0.00	3.170	0.23	22.440	3.73	158.866	2.20	1124.683	0.00
0.071	0.00	0.502	0.00	3.557	0.28	25.179	3.87	178.250	1.89	1261.915	0.00
0.080	0.00	0.564	0.00	3.991	0.34	28.251	3.98	200.000	1.58	1415.892	0.00
0.089	0.00	0.632	0.00	4.477	0.42	31.698	4.05	224.404	1.22	1588.656	0.00
0.100	0.00	0.710	0.00	5.024	0.53	35.566	4.11	251.785	0.80	1782.502	0.00
0.112	0.00	0.796	0.00	5.637	0.66	39.905	4.15	282.508	0.33	2000.000	0.00
0.126	0.00	0.893	0.00	6.325	0.83	44.774	4.19	316.979	0.01		
0.142	0.00	1.002	0.00	7.096		50.238		355.656			

Operator notes:

Malvern Instruments Ltd.
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007548

File name: 17 May 2011
Record Number: 4

MASTERSIZER 2000

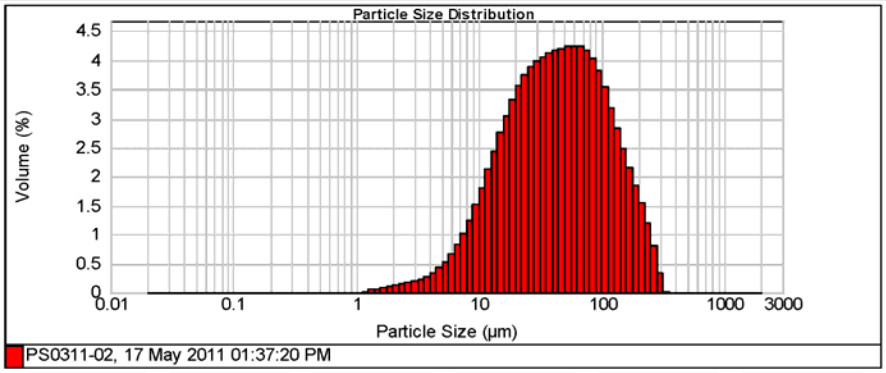
Result Analysis Report

Sample Name: PS0311-02	**SOP Name:** Jaco (TMC)	**Measured:** 17 May 2011 01:37:20 PM
Sample Source & type:	**Measured by:** Jaco van Heerden	**Analysed:** 17 May 2011 01:37:21 PM
Sample bulk lot ref:	**Result Source:** Measurement	

Particle Name: Titanium Dioxide	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.741	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 17.42 %
Dispersant Name: Alcohol	**Dispersant RI:** 1.320	**Weighted Residual:** 2.316 %	**Result Emulation:** Off

Concentration: 0.0320 %Vol	**Span :** 3.054	**Uniformity:** 0.941	**Result units:** Volume
Specific Surface Area: 0.248 m²/g	**Surface Weighted Mean D[3,2]:** 24.192 um	**Vol. Weighted Mean D[4,3]:** 62.733 um	

d(0.1): 11.342 um **d(0.5):** 43.450 um **d(0.9):** 144.052 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 %
0.020	0.00	0.142	0.00	1.002	0.00	7.096	1.02	50.238	4.23	355.656	0.00
0.022	0.00	0.159	0.00	1.125	0.02	7.962	1.26	56.368	4.25	399.052	0.00
0.025	0.00	0.178	0.00	1.262	0.06	8.934	1.52	63.246	4.23	447.744	0.00
0.028	0.00	0.200	0.00	1.416	0.07	10.024	1.82	70.963	4.17	502.377	0.00
0.032	0.00	0.224	0.00	1.589	0.08	11.247	2.13	79.621	4.03	563.677	0.00
0.036	0.00	0.252	0.00	1.783	0.10	12.619	2.45	89.337	3.82	632.456	0.00
0.040	0.00	0.283	0.00	2.000	0.12	14.159	2.76	100.237	3.54	709.627	0.00
0.045	0.00	0.317	0.00	2.244	0.14	15.887	3.06	112.468	3.20	796.214	0.00
0.050	0.00	0.356	0.00	2.518	0.16	17.825	3.33	126.191	2.84	893.367	0.00
0.056	0.00	0.399	0.00	2.825	0.19	20.000	3.55	141.589	2.48	1002.374	0.00
0.063	0.00	0.448	0.00	3.170	0.23	22.440	3.74	158.866	2.16	1124.683	0.00
0.071	0.00	0.502	0.00	3.557	0.28	25.179	3.88	178.250	1.85	1261.915	0.00
0.080	0.00	0.564	0.00	3.991	0.34	28.251	3.99	200.000	1.54	1415.892	0.00
0.089	0.00	0.632	0.00	4.477	0.42	31.698	4.06	224.404	1.20	1588.656	0.00
0.100	0.00	0.710	0.00	5.024	0.53	35.566	4.12	251.785	0.80	1782.502	0.00
0.112	0.00	0.796	0.00	5.637	0.66	39.905	4.16	282.508	0.34	2000.000	0.00
0.126	0.00	0.893	0.00	6.325	0.82	44.774	4.20	316.979	0.01		
0.142	0.00	1.002	0.00	7.096		50.238		355.656			

Operator notes:

Malvern Instruments Ltd.
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007548

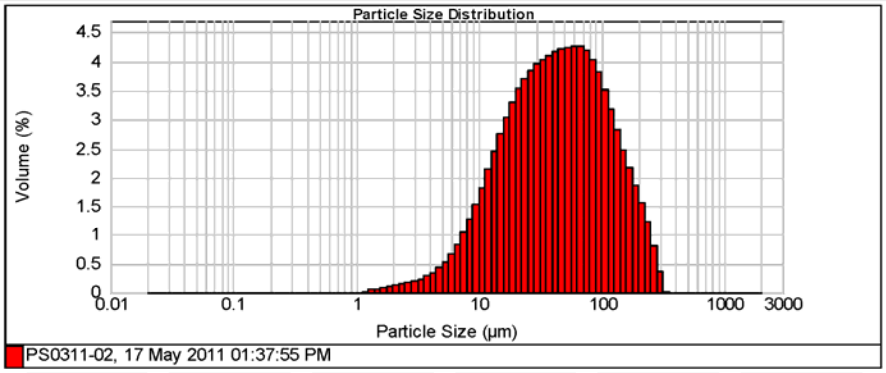
File name: 17 May 2011
Record Number: 5

MASTERSIZER 2000

Result Analysis Report

Sample Name: PS0311-02	**SOP Name:** Jaco (TMC)	**Measured:** 17 May 2011 01:37:55 PM	
Sample Source & type:	**Measured by:** Jaco van Heerden	**Analysed:** 17 May 2011 01:37:56 PM	
Sample bulk lot ref:	**Result Source:** Measurement		
Particle Name: Titanium Dioxide	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.741	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 17.43 %
Dispersant Name: Alcohol	**Dispersant RI:** 1.320	**Weighted Residual:** 2.318 %	**Result Emulation:** Off
Concentration: 0.0319 %Vol	**Span :** 3.063	**Uniformity:** 0.942	**Result units:** Volume
Specific Surface Area: 0.249 m²/g	**Surface Weighted Mean D[3,2]:** 24.124 um	**Vol. Weighted Mean D[4,3]:** 62.896 um	

d(0.1): 11.273 um **d(0.5):** 43.565 um **d(0.9):** 144.722 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 %
0.020	0.00	0.142	0.00	1.002	0.00	7.096	1.04	50.238	4.25	355.656	0.00
0.022	0.00	0.159	0.00	1.125	0.02	7.962	1.27	56.368	4.27	399.052	0.00
0.025	0.00	0.178	0.00	1.262	0.06	8.934	1.54	63.246	4.25	447.744	0.00
0.028	0.00	0.200	0.00	1.416	0.07	10.024	1.83	70.963	4.18	502.377	0.00
0.032	0.00	0.224	0.00	1.589	0.09	11.247	2.14	79.621	4.04	563.677	0.00
0.036	0.00	0.252	0.00	1.783	0.10	12.619	2.45	89.337	3.81	632.456	0.00
0.040	0.00	0.283	0.00	2.000	0.12	14.159	2.76	100.237	3.52	709.627	0.00
0.045	0.00	0.317	0.00	2.244	0.14	15.887	3.05	112.468	3.17	796.214	0.00
0.050	0.00	0.356	0.00	2.518	0.17	17.825	3.31	126.191	2.82	893.367	0.00
0.056	0.00	0.399	0.00	2.825	0.20	20.000	3.53	141.589	2.47	1002.374	0.00
0.063	0.00	0.448	0.00	3.170	0.23	22.440	3.71	158.866	2.16	1124.683	0.00
0.071	0.00	0.502	0.00	3.557	0.28	25.179	3.85	178.250	1.87	1261.915	0.00
0.080	0.00	0.564	0.00	3.991	0.34	28.251	3.96	200.000	1.57	1415.892	0.00
0.089	0.00	0.632	0.00	4.477	0.43	31.698	4.04	224.404	1.23	1588.656	0.00
0.100	0.00	0.710	0.00	5.024	0.53	35.566	4.10	251.785	0.82	1782.502	0.00
0.112	0.00	0.796	0.00	5.637	0.67	39.905	4.16	282.508	0.36	2000.000	0.00
0.126	0.00	0.893	0.00	6.325	0.84	44.774	4.21	316.979	0.02		
0.142	0.00	1.002	0.00	7.096		50.238		355.656			

Operator notes:

Malvern Instruments Ltd.
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007548

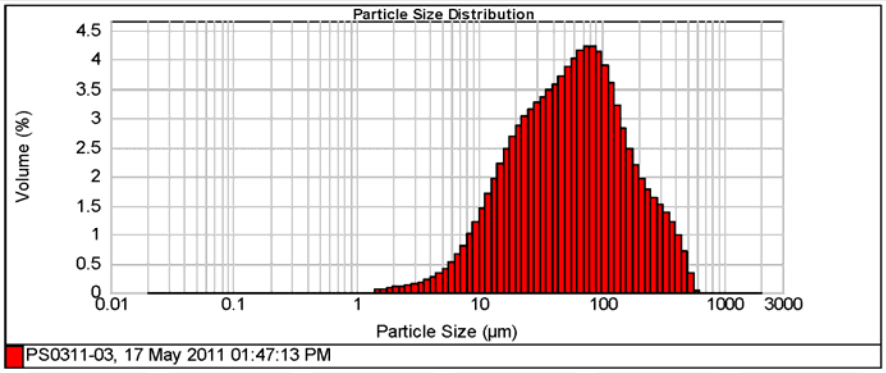
File name: 17 May 2011
Record Number: 6

MASTERSIZER 2000

Result Analysis Report

Sample Name: PS0311-03	**SOP Name:** Jaco (TMC)	**Measured:** 17 May 2011 01:47:13 PM	
Sample Source & type:	**Measured by:** Jaco van Heerden	**Analysed:** 17 May 2011 01:47:14 PM	
Sample bulk lot ref:	**Result Source:** Measurement		
Particle Name: Titanium Dioxide	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.741	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 17.98 %
Dispersant Name: Alcohol	**Dispersant RI:** 1.320	**Weighted Residual:** 1.138 %	**Result Emulation:** Off
Concentration: 0.0411 %Vol	**Span :** 3.594	**Uniformity:** 1.1	**Result units:** Volume
Specific Surface Area: 0.206 m²/g	**Surface Weighted Mean D[3,2]:** 29.129 um	**Vol. Weighted Mean D[4,3]:** 90.709 um	

d(0.1): 12.990 um **d(0.5):** 57.464 um **d(0.9):** 219.503 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 %
0.020	0.00	0.142	0.00	1.002	0.00	7.096	0.81	50.238	3.88	355.656	1.22
0.022	0.00	0.159	0.00	1.125	0.00	7.962	1.01	56.368	4.03	399.052	0.99
0.025	0.00	0.178	0.00	1.262	0.00	8.934	1.22	63.246	4.16	447.744	0.70
0.028	0.00	0.200	0.00	1.416	0.00	10.024	1.46	70.963	4.24	502.377	0.35
0.032	0.00	0.224	0.00	1.589	0.06	11.247	1.72	79.621	4.24	563.677	0.04
0.036	0.00	0.252	0.00	1.783	0.07	12.619	1.98	89.337	4.13	632.456	0.00
0.040	0.00	0.283	0.00	2.000	0.08	14.159	2.24	100.237	3.91	709.627	0.00
0.045	0.00	0.317	0.00	2.244	0.09	15.887	2.48	112.468	3.60	796.214	0.00
0.050	0.00	0.356	0.00	2.518	0.11	17.825	2.70	126.191	3.23	893.367	0.00
0.056	0.00	0.399	0.00	2.825	0.13	20.000	2.89	141.589	2.84	1002.374	0.00
0.063	0.00	0.448	0.00	3.170	0.16	22.440	3.04	158.866	2.49	1124.683	0.00
0.071	0.00	0.502	0.00	3.557	0.19	25.179	3.17	178.250	2.20	1261.915	0.00
0.080	0.00	0.564	0.00	3.991	0.22	28.251	3.28	200.000	1.96	1415.892	0.00
0.089	0.00	0.632	0.00	4.477	0.27	31.698	3.37	224.404	1.79	1588.656	0.00
0.100	0.00	0.710	0.00	5.024	0.33	35.566	3.48	251.785	1.64	1782.502	0.00
0.112	0.00	0.796	0.00	5.637	0.42	39.905	3.59	282.508	1.52	2000.000	0.00
0.126	0.00	0.893	0.00	6.325	0.52	44.774	3.72	316.979	1.39		
0.142	0.00	1.002	0.00	7.096	0.65	50.238		355.656			

Operator notes:

Malvern Instruments Ltd.
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007548

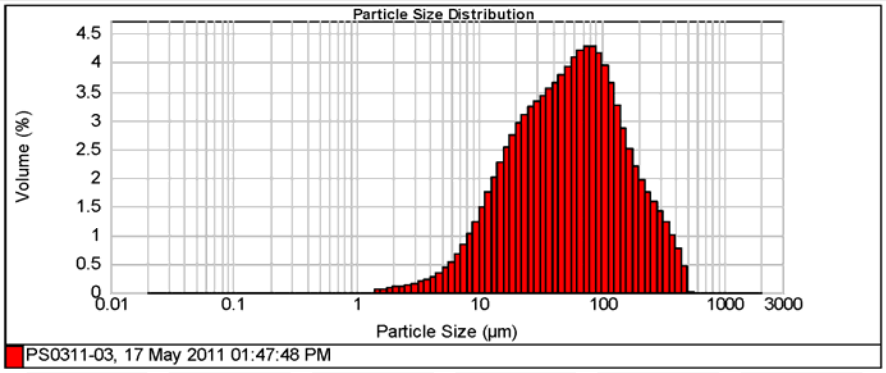
File name: 17 May 2011
Record Number: 7

MASTERSIZER 2000

Result Analysis Report

Sample Name: PS0311-03	**SOP Name:** Jaco (TMC)	**Measured:** 17 May 2011 01:47:48 PM	
Sample Source & type:	**Measured by:** Jaco van Heerden	**Analysed:** 17 May 2011 01:47:49 PM	
Sample bulk lot ref:	**Result Source:** Measurement		
Particle Name: Titanium Dioxide	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.741	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 17.92 %
Dispersant Name: Alcohol	**Dispersant RI:** 1.320	**Weighted Residual:** 1.184 %	**Result Emulation:** Off
Concentration: 0.0401 %Vol	**Span :** 3.392	**Uniformity:** 1.05	**Result units:** Volume
Specific Surface Area: 0.21 m²/g	**Surface Weighted Mean D[3,2]:** 28.573 um	**Vol. Weighted Mean D[4,3]:** 85.463 um	

d(0.1): 12.834 um **d(0.5):** 55.883 um **d(0.9):** 202.393 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 %
0.020	0.00	0.142	0.00	1.002	0.00	7.096	0.83	50.238	3.93	355.656	1.00
0.022	0.00	0.159	0.00	1.125	0.00	7.962	1.02	56.368	4.08	399.052	0.77
0.025	0.00	0.178	0.00	1.262	0.00	8.934	1.24	63.246	4.21	447.744	0.46
0.028	0.00	0.200	0.00	1.416	0.00	10.024	1.49	70.963	4.29	502.377	0.02
0.032	0.00	0.224	0.00	1.589	0.06	11.247	1.75	79.621	4.28	563.677	0.00
0.036	0.00	0.252	0.00	1.783	0.07	12.619	2.02	89.337	4.17	632.456	0.00
0.040	0.00	0.283	0.00	2.000	0.08	14.159	2.28	100.237	3.95	709.627	0.00
0.045	0.00	0.317	0.00	2.244	0.10	15.887	2.53	112.468	3.63	796.214	0.00
0.050	0.00	0.356	0.00	2.518	0.12	17.825	2.76	126.191	3.26	893.367	0.00
0.056	0.00	0.399	0.00	2.825	0.14	20.000	2.95	141.589	2.88	1002.374	0.00
0.063	0.00	0.448	0.00	3.170	0.16	22.440	3.11	158.866	2.52	1124.683	0.00
0.071	0.00	0.502	0.00	3.557	0.19	25.179	3.24	178.250	2.21	1261.915	0.00
0.080	0.00	0.564	0.00	3.991	0.23	28.251	3.34	200.000	1.97	1415.892	0.00
0.089	0.00	0.632	0.00	4.477	0.28	31.698	3.44	224.404	1.76	1588.656	0.00
0.100	0.00	0.710	0.00	5.024	0.34	35.566	3.54	251.785	1.58	1782.502	0.00
0.112	0.00	0.796	0.00	5.637	0.43	39.905	3.65	282.508	1.42	2000.000	0.00
0.126	0.00	0.893	0.00	6.325	0.53	44.774	3.78	316.979	1.23		
0.142	0.00	1.002	0.00	7.096	0.67	50.238		355.656			

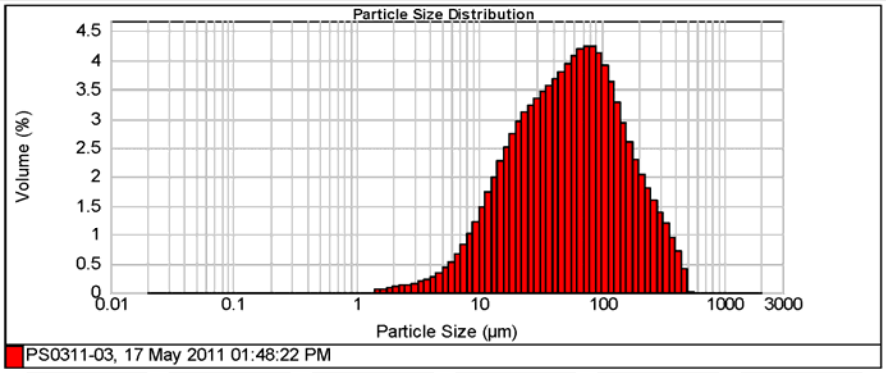
Operator notes:

MASTERSIZER 2000

Result Analysis Report

Sample Name: PS0311-03	**SOP Name:** Jaco (TMC)	**Measured:** 17 May 2011 01:48:22 PM	
Sample Source & type:	**Measured by:** Jaco van Heerden	**Analysed:** 17 May 2011 01:48:23 PM	
Sample bulk lot ref:	**Result Source:** Measurement		
Particle Name: Titanium Dioxide	**Accessory Name:** Hydro 2000SM (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 2.741	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 17.86 %
Dispersant Name: Alcohol	**Dispersant RI:** 1.320	**Weighted Residual:** 1.288 %	**Result Emulation:** Off
Concentration: 0.0399 %Vol	**Span :** 3.375	**Uniformity:** 1.05	**Result units:** Volume
Specific Surface Area: 0.21 m²/g	**Surface Weighted Mean D[3,2]:** 28.607 um	**Vol. Weighted Mean D[4,3]:** 85.180 um	

d(0.1): 12.866 um **d(0.5):** 55.851 um **d(0.9):** 201.358 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 %
0.020	0.00	0.142	0.00	1.002	0.00	7.096	0.82	50.238	3.94	355.656	0.96
0.022	0.00	0.159	0.00	1.125	0.00	7.962	1.02	56.368	4.07	399.052	0.71
0.025	0.00	0.178	0.00	1.262	0.00	8.934	1.23	63.246	4.19	447.744	0.41
0.028	0.00	0.200	0.00	1.416	0.06	10.024	1.48	70.963	4.25	502.377	0.02
0.032	0.00	0.224	0.00	1.589	0.07	11.247	1.74	79.621	4.24	563.677	0.00
0.036	0.00	0.252	0.00	1.783	0.08	12.619	2.00	89.337	4.13	632.456	0.00
0.040	0.00	0.283	0.00	2.000	0.10	14.159	2.27	100.237	3.93	709.627	0.00
0.045	0.00	0.317	0.00	2.244	0.12	15.887	2.52	112.468	3.63	796.214	0.00
0.050	0.00	0.356	0.00	2.518	0.14	17.825	2.75	126.191	3.29	893.367	0.00
0.056	0.00	0.399	0.00	2.825	0.16	20.000	2.95	141.589	2.93	1002.374	0.00
0.063	0.00	0.448	0.00	3.170	0.19	22.440	3.11	158.866	2.60	1124.683	0.00
0.071	0.00	0.502	0.00	3.557	0.23	25.179	3.25	178.250	2.30	1261.915	0.00
0.080	0.00	0.564	0.00	3.991	0.28	28.251	3.36	200.000	2.03	1415.892	0.00
0.089	0.00	0.632	0.00	4.477	0.34	31.698	3.46	224.404	1.81	1588.656	0.00
0.100	0.00	0.710	0.00	5.024	0.43	35.566	3.56	251.785	1.60	1782.502	0.00
0.112	0.00	0.796	0.00	5.637	0.53	39.905	3.67	282.508	1.40	2000.000	0.00
0.126	0.00	0.893	0.00	6.325	0.66	44.774	3.80	316.979	1.19		
0.142	0.00	1.002	0.00	7.096		50.238		355.656			

Operator notes:

Malvern Instruments Ltd.
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007548

File name: 17 May 2011
Record Number: 9

6. Zetasizer: TMC-TPP particles (TMC:TPP ratio – 5:1)

Zeta Potential Report

PS0311-01
TMC particles 5:1 (TMC:TPP)
Jaco van Heerden

Sample

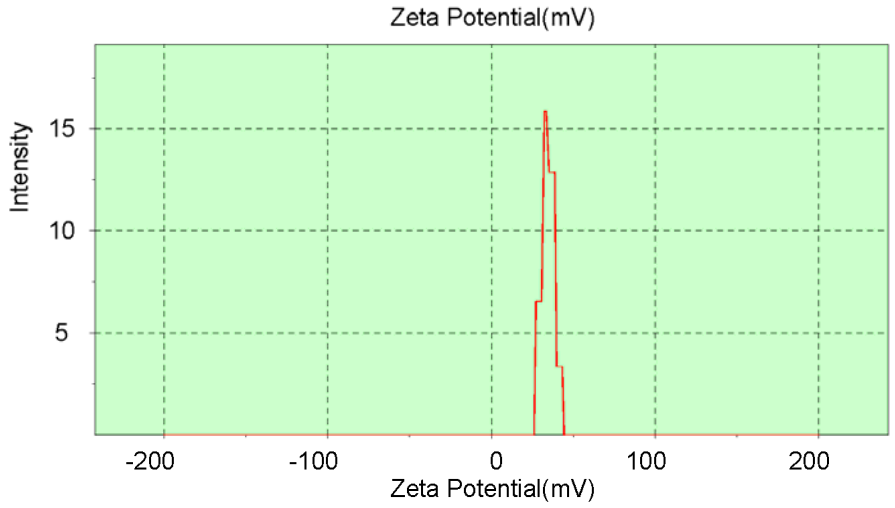
Record Number:	1
Filename:	ZSIZER.zet
File Path:	G:\MALVERN\ZETASI~1\17MAY
Dielectric Constant:	79.7
pH:	N/A
Viscosity (cP):	0.890
Date (DMY):	17/05/11
Time:	15:25:29

System

Instrument Type:	Zetasizer 2000
Temperature (°C):	25.0
Count rate (kCps):	2475.4
Cell Type:	Capillary cell
Cell Position (%):	50.00
Cell field (V/cm):	29.1
Current (mA):	0.8

Result

Zeta Potential (mV):	34.4	Mobility (umcm/V.s):	2.698
StDev (mV):	1.6	StDev (umcm/V.s):	0.125
Conductivity (mS/cm):	0.26	F(ka):	1.50



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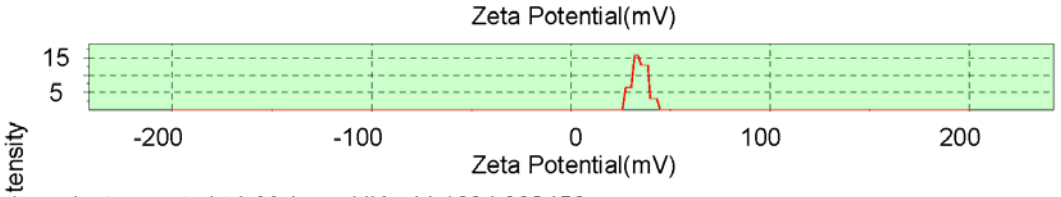
PS0311-01
TMC particles 5:1 (TMC:TPP)
Jaco van Heerden
File data from G:\MALVERN\ZETASI~1\17MAY2~1\ZSIZER.zet Record 1
Zetasizer 2000Data type 1 256

Int.	Zeta(mV)	Mob(µmcm/Vs)	Freq.(Hz)	Int.	Zeta(mV)	Mob(µmcm/V
0.0	-200.0	-15.858	33.3	0.0	3.2	0.252
0.0	-193.7	-15.354	40.2	0.0	9.5	0.755
0.0	-187.3	-14.851	47.2	0.0	15.9	1.259
0.0	-181.0	-14.348	54.1	0.0	22.2	1.762
0.0	-174.6	-13.844	61.1	25.4	28.6	2.265
0.0	-168.3	-13.341	68.0	61.6	34.9	2.769
0.0	-161.9	-12.837	74.9	13.1	41.3	3.272
0.0	-155.6	-12.334	81.9	0.0	47.6	3.776
0.0	-149.2	-11.830	88.8	0.0	54.0	4.279
0.0	-142.9	-11.327	95.8	0.0	60.3	4.783
0.0	-136.5	-10.824	102.7	0.0	66.7	5.286
0.0	-130.2	-10.320	109.7	0.0	73.0	5.789
0.0	-123.8	-9.817	116.6	0.0	79.4	6.293
0.0	-117.5	-9.313	123.5	0.0	85.7	6.796
0.0	-111.1	-8.810	130.5	0.0	92.1	7.300
0.0	-104.8	-8.306	137.4	0.0	98.4	7.803
0.0	-98.4	-7.803	144.4	0.0	104.8	8.306
0.0	-92.1	-7.300	151.3	0.0	111.1	8.810
0.0	-85.7	-6.796	158.3	0.0	117.5	9.313
0.0	-79.4	-6.293	165.2	0.0	123.8	9.817
0.0	-73.0	-5.789	172.2	0.0	130.2	10.320
0.0	-66.7	-5.286	179.1	0.0	136.5	10.824
0.0	-60.3	-4.783	186.0	0.0	142.9	11.327
0.0	-54.0	-4.279	193.0	0.0	149.2	11.830
0.0	-47.6	-3.776	199.9	0.0	155.6	12.334
0.0	-41.3	-3.272	206.9	0.0	161.9	12.837
0.0	-34.9	-2.769	213.8	0.0	168.3	13.341
0.0	-28.6	-2.265	220.8	0.0	174.6	13.844
0.0	-22.2	-1.762	227.7	0.0	181.0	14.348
0.0	-15.9	-1.259	234.6	0.0	187.3	14.851
0.0	-9.5	-0.755	241.6	0.0	193.7	15.354
0.0	-3.2	-0.252	248.5	0.0	200.0	15.858



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ZETASIZER

Zeta Potential Report

PS0311-01
TMC particles 5:1 (TMC:TPP)
Jaco van Heerden

Sample

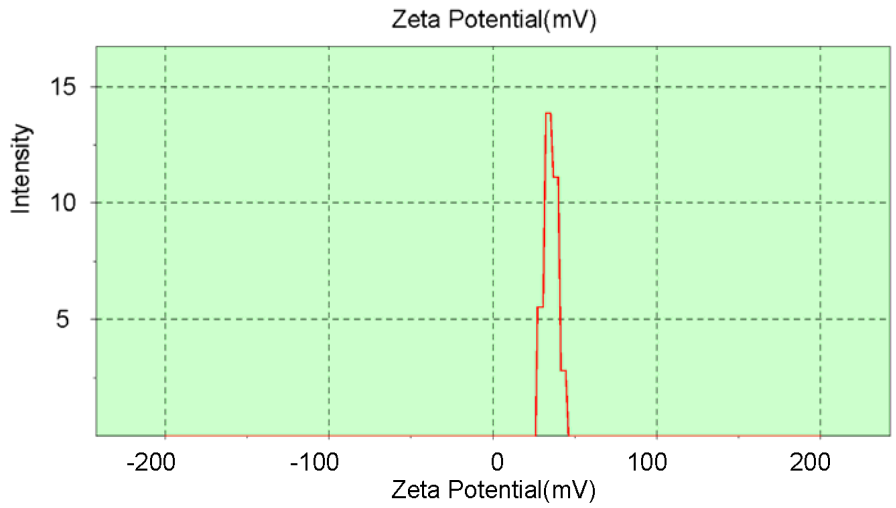
Record Number:	2
Filename:	ZSIZER.zet
File Path:	G:\MALVERN\ZETASI~1\17MAY
Dielectric Constant:	79.7
pH:	N/A
Viscosity (cP):	0.891
Date (DMY):	17/05/11
Time:	15:26:36

System

Instrument Type:	Zetasizer 2000
Temperature (°C):	25.0
Count rate (kCps):	2716.0
Cell Type:	Capillary cell
Cell Position (%):	50.00
Cell field (V/cm):	29.2
Current (mA):	0.7

Result

Zeta Potential (mV):	35.3	Mobility (umcm/V.s):	2.768
StDev (mV):	1.6	StDev (umcm/V.s):	0.125
Conductivity (mS/cm):	0.25	F(ka):	1.50



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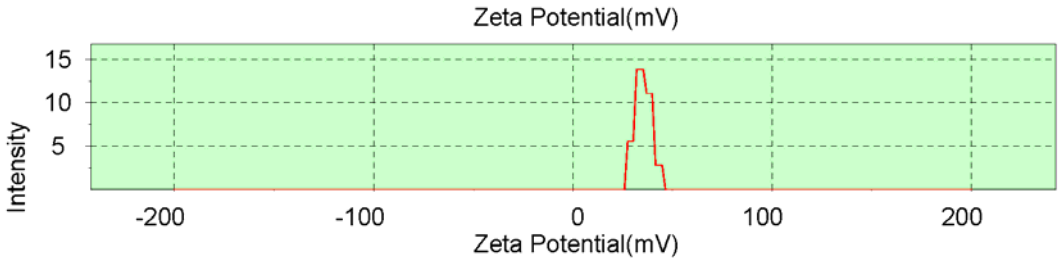
Int.	Zeta(mV)	Mob(µmcm/Vs)	Freq.(Hz)	Int.	Zeta(mV)	Mob(µmcm/V
0.0	-200.0	-15.850	31.8	0.0	3.2	0.252
0.0	-193.7	-15.347	38.8	0.0	9.5	0.755
0.0	-187.3	-14.844	45.7	0.0	15.9	1.258
0.0	-181.0	-14.341	52.7	0.0	22.2	1.761
0.0	-174.6	-13.838	59.6	25.0	28.6	2.264
0.0	-168.3	-13.335	66.6	62.4	34.9	2.768
0.0	-161.9	-12.831	73.6	12.6	41.3	3.271
0.0	-155.6	-12.328	80.5	0.0	47.6	3.774
0.0	-149.2	-11.825	87.5	0.0	54.0	4.277
0.0	-142.9	-11.322	94.4	0.0	60.3	4.780
0.0	-136.5	-10.819	101.4	0.0	66.7	5.283
0.0	-130.2	-10.315	108.3	0.0	73.0	5.787
0.0	-123.8	-9.812	115.3	0.0	79.4	6.290
0.0	-117.5	-9.309	122.3	0.0	85.7	6.793
0.0	-111.1	-8.806	129.2	0.0	92.1	7.296
0.0	-104.8	-8.303	136.2	0.0	98.4	7.799
0.0	-98.4	-7.799	143.1	0.0	104.8	8.303
0.0	-92.1	-7.296	150.1	0.0	111.1	8.806
0.0	-85.7	-6.793	157.1	0.0	117.5	9.309
0.0	-79.4	-6.290	164.0	0.0	123.8	9.812
0.0	-73.0	-5.787	171.0	0.0	130.2	10.315
0.0	-66.7	-5.283	177.9	0.0	136.5	10.819
0.0	-60.3	-4.780	184.9	0.0	142.9	11.322
0.0	-54.0	-4.277	191.9	0.0	149.2	11.825
0.0	-47.6	-3.774	198.8	0.0	155.6	12.328
0.0	-41.3	-3.271	205.8	0.0	161.9	12.831
0.0	-34.9	-2.768	212.7	0.0	168.3	13.335
0.0	-28.6	-2.264	219.7	0.0	174.6	13.838
0.0	-22.2	-1.761	226.6	0.0	181.0	14.341
0.0	-15.9	-1.258	233.6	0.0	187.3	14.844
0.0	-9.5	-0.755	240.6	0.0	193.7	15.347
0.0	-3.2	-0.252	247.5	0.0	200.0	15.850



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ZETASIZER

Zeta Potential Report

PS0311-01
TMC particles 5:1 (TMC:TPP)
Jaco van Heerden

Sample

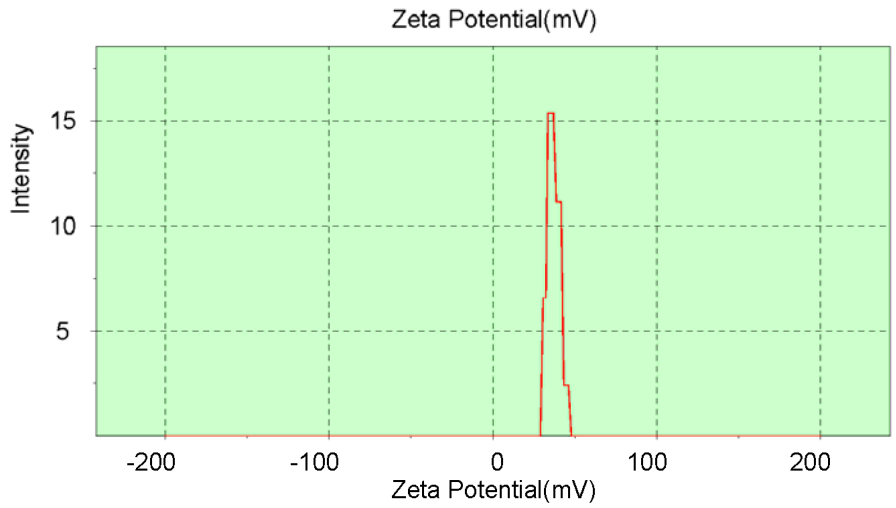
Record Number:	3
Filename:	ZSIZER.zet
File Path:	G:\MALVERN\ZETASI~1\17MAY
Dielectric Constant:	79.7
pH:	N/A
Viscosity (cP):	0.891
Date (DMY):	17/05/11
Time:	15:27:44

System

Instrument Type:	Zetasizer 2000
Temperature (°C):	25.0
Count rate (kCps):	2658.0
Cell Type:	Capillary cell
Cell Position (%):	50.00
Cell field (V/cm):	29.1
Current (mA):	0.8

Result

Zeta Potential (mV):	37.1	Mobility (umcm/V.s):	2.906
StDev (mV):	1.6	StDev (umcm/V.s):	0.125
Conductivity (mS/cm):	0.26	F(ka):	1.50



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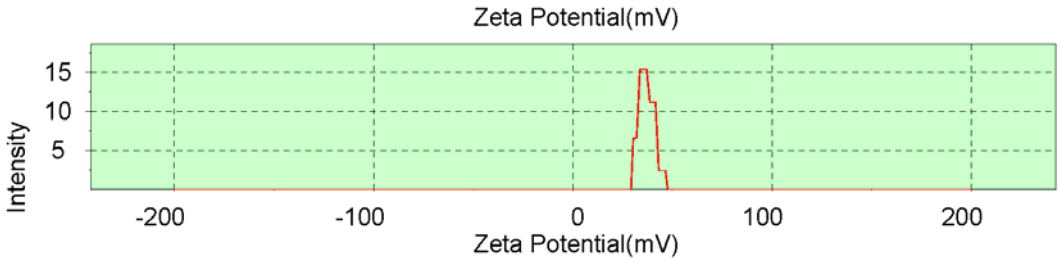
Int.	Zeta(mV)	Mob(µmcm/Vs)	Freq.(Hz)	Int.	Zeta(mV)	Mob(µmcm/V
0.0	-200.0	-15.845	30.6	0.0	3.2	0.252
0.0	-193.7	-15.342	37.5	0.0	9.5	0.755
0.0	-187.3	-14.839	44.5	0.0	15.9	1.258
0.0	-181.0	-14.336	51.4	0.0	22.2	1.761
0.0	-174.6	-13.833	58.3	0.0	28.6	2.264
0.0	-168.3	-13.330	65.3	57.9	34.9	2.767
0.0	-161.9	-12.827	72.2	42.1	41.3	3.270
0.0	-155.6	-12.324	79.1	0.0	47.6	3.773
0.0	-149.2	-11.821	86.1	0.0	54.0	4.276
0.0	-142.9	-11.318	93.0	0.0	60.3	4.779
0.0	-136.5	-10.815	99.9	0.0	66.7	5.282
0.0	-130.2	-10.312	106.9	0.0	73.0	5.785
0.0	-123.8	-9.809	113.8	0.0	79.4	6.288
0.0	-117.5	-9.306	120.7	0.0	85.7	6.791
0.0	-111.1	-8.803	127.7	0.0	92.1	7.294
0.0	-104.8	-8.300	134.6	0.0	98.4	7.797
0.0	-98.4	-7.797	141.5	0.0	104.8	8.300
0.0	-92.1	-7.294	148.5	0.0	111.1	8.803
0.0	-85.7	-6.791	155.4	0.0	117.5	9.306
0.0	-79.4	-6.288	162.3	0.0	123.8	9.809
0.0	-73.0	-5.785	169.3	0.0	130.2	10.312
0.0	-66.7	-5.282	176.2	0.0	136.5	10.815
0.0	-60.3	-4.779	183.1	0.0	142.9	11.318
0.0	-54.0	-4.276	190.1	0.0	149.2	11.821
0.0	-47.6	-3.773	197.0	0.0	155.6	12.324
0.0	-41.3	-3.270	203.9	0.0	161.9	12.827
0.0	-34.9	-2.767	210.9	0.0	168.3	13.330
0.0	-28.6	-2.264	217.8	0.0	174.6	13.833
0.0	-22.2	-1.761	224.7	0.0	181.0	14.336
0.0	-15.9	-1.258	231.7	0.0	187.3	14.839
0.0	-9.5	-0.755	238.6	0.0	193.7	15.342
0.0	-3.2	-0.252	245.5	0.0	200.0	15.845



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ZETASIZER

Zeta Potential Report

PS0311-02
TMC articles 5:1 (TMC:TPP) with Tween 80
Jaco van Heerden

Sample

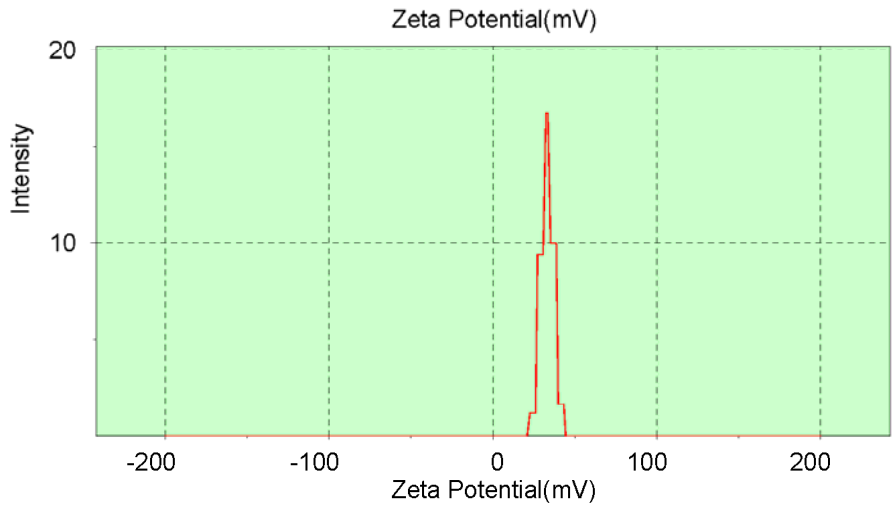
Record Number:	4
Filename:	ZSIZER.zet
File Path:	G:\MALVERN\ZETASI~1\17MAY
Dielectric Constant:	79.7
pH:	N/A
Viscosity (cP):	0.891
Date (DMY):	17/05/11
Time:	15:35:14

System

Instrument Type:	Zetasizer 2000
Temperature (°C):	25.0
Count rate (kCps):	2346.7
Cell Type:	Capillary cell
Cell Position (%):	50.00
Cell field (V/cm):	29.1
Current (mA):	1.6

Result

Zeta Potential (mV):	33.1	Mobility (umcm/V.s):	2.569
StDev (mV):	1.6	StDev (umcm/V.s):	0.125
Conductivity (mS/cm):	0.54	F(ka):	1.50



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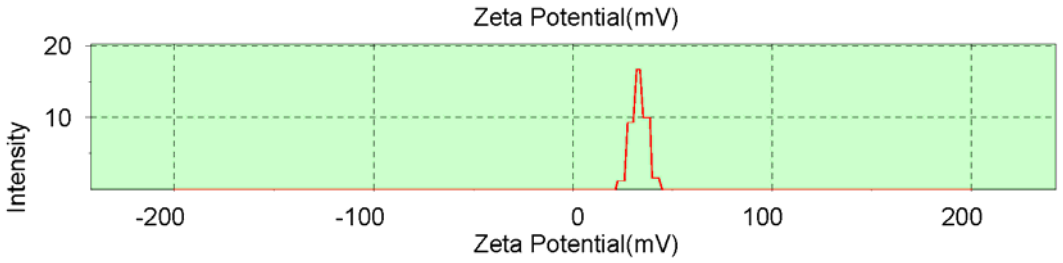
Int.	Zeta(mV)	Mob(µmcm/Vs)	Freq.(Hz)	Int.	Zeta(mV)	Mob(µmcm/V
0.0	-200.0	-15.850	33.5	0.0	3.2	0.252
0.0	-193.7	-15.347	40.5	0.0	9.5	0.755
0.0	-187.3	-14.844	47.4	0.0	15.9	1.258
0.0	-181.0	-14.341	54.3	4.1	22.2	1.761
0.0	-174.6	-13.838	61.3	32.5	28.6	2.264
0.0	-168.3	-13.335	68.2	57.8	34.9	2.768
0.0	-161.9	-12.831	75.1	5.6	41.3	3.271
0.0	-155.6	-12.328	82.1	0.0	47.6	3.774
0.0	-149.2	-11.825	89.0	0.0	54.0	4.277
0.0	-142.9	-11.322	96.0	0.0	60.3	4.780
0.0	-136.5	-10.819	102.9	0.0	66.7	5.283
0.0	-130.2	-10.315	109.8	0.0	73.0	5.787
0.0	-123.8	-9.812	116.8	0.0	79.4	6.290
0.0	-117.5	-9.309	123.7	0.0	85.7	6.793
0.0	-111.1	-8.806	130.6	0.0	92.1	7.296
0.0	-104.8	-8.303	137.6	0.0	98.4	7.799
0.0	-98.4	-7.799	144.5	0.0	104.8	8.303
0.0	-92.1	-7.296	151.4	0.0	111.1	8.806
0.0	-85.7	-6.793	158.4	0.0	117.5	9.309
0.0	-79.4	-6.290	165.3	0.0	123.8	9.812
0.0	-73.0	-5.787	172.2	0.0	130.2	10.315
0.0	-66.7	-5.283	179.2	0.0	136.5	10.819
0.0	-60.3	-4.780	186.1	0.0	142.9	11.322
0.0	-54.0	-4.277	193.0	0.0	149.2	11.825
0.0	-47.6	-3.774	200.0	0.0	155.6	12.328
0.0	-41.3	-3.271	206.9	0.0	161.9	12.831
0.0	-34.9	-2.768	213.9	0.0	168.3	13.335
0.0	-28.6	-2.264	220.8	0.0	174.6	13.838
0.0	-22.2	-1.761	227.7	0.0	181.0	14.341
0.0	-15.9	-1.258	234.7	0.0	187.3	14.844
0.0	-9.5	-0.755	241.6	0.0	193.7	15.347
0.0	-3.2	-0.252	248.5	0.0	200.0	15.850



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ZETASIZER

Zeta Potential Report

PS0311-02
TMC articles 5:1 (TMC:TPP) with Tween 80
Jaco van Heerden

Sample

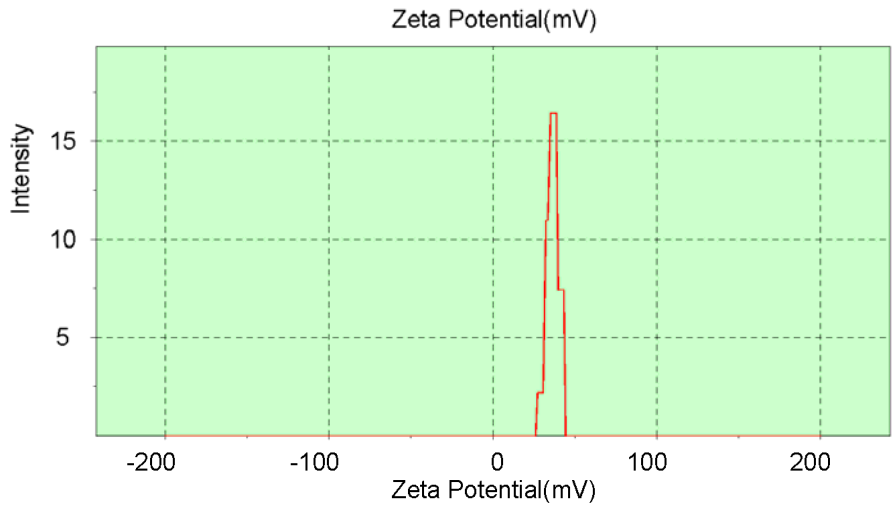
Record Number:	5
Filename:	ZSIZER.zet
File Path:	G:\MALVERN\ZETASI~1\17MAY
Dielectric Constant:	79.7
pH:	N/A
Viscosity (cP):	0.890
Date (DMY):	17/05/11
Time:	15:36:15

System

Instrument Type:	Zetasizer 2000
Temperature (°C):	25.0
Count rate (kCps):	2404.0
Cell Type:	Capillary cell
Cell Position (%):	50.00
Cell field (V/cm):	29.1
Current (mA):	1.6

Result

Zeta Potential (mV):	36.4	Mobility (umcm/V.s):	2.862
StDev (mV):	1.6	StDev (umcm/V.s):	0.125
Conductivity (mS/cm):	0.54	F(ka):	1.50



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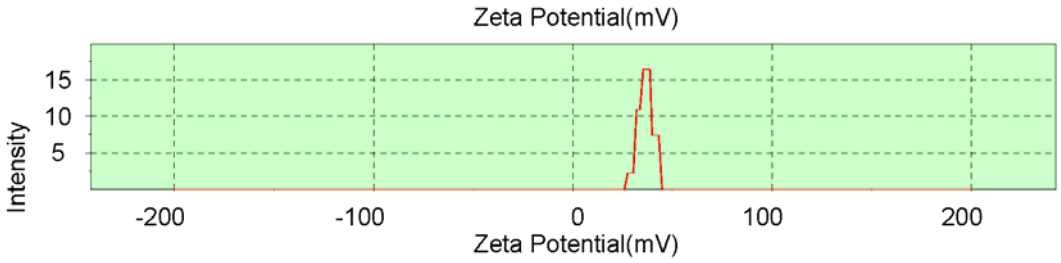
Int.	Zeta(mV)	Mob(µmcm/Vs)	Freq.(Hz)	Int.	Zeta(mV)	Mob(µmcm/V
0.0	-200.0	-15.855	33.3	0.0	3.2	0.252
0.0	-193.7	-15.351	40.3	0.0	9.5	0.755
0.0	-187.3	-14.848	47.2	0.0	15.9	1.258
0.0	-181.0	-14.345	54.2	0.0	22.2	1.762
0.0	-174.6	-13.841	61.1	10.6	28.6	2.265
0.0	-168.3	-13.338	68.0	53.3	34.9	2.768
0.0	-161.9	-12.835	75.0	36.1	41.3	3.272
0.0	-155.6	-12.331	81.9	0.0	47.6	3.775
0.0	-149.2	-11.828	88.9	0.0	54.0	4.278
0.0	-142.9	-11.325	95.8	0.0	60.3	4.782
0.0	-136.5	-10.821	102.7	0.0	66.7	5.285
0.0	-130.2	-10.318	109.7	0.0	73.0	5.788
0.0	-123.8	-9.815	116.6	0.0	79.4	6.291
0.0	-117.5	-9.311	123.6	0.0	85.7	6.795
0.0	-111.1	-8.808	130.5	0.0	92.1	7.298
0.0	-104.8	-8.305	137.5	0.0	98.4	7.801
0.0	-98.4	-7.801	144.4	0.0	104.8	8.305
0.0	-92.1	-7.298	151.3	0.0	111.1	8.808
0.0	-85.7	-6.795	158.3	0.0	117.5	9.311
0.0	-79.4	-6.291	165.2	0.0	123.8	9.815
0.0	-73.0	-5.788	172.2	0.0	130.2	10.318
0.0	-66.7	-5.285	179.1	0.0	136.5	10.821
0.0	-60.3	-4.782	186.1	0.0	142.9	11.325
0.0	-54.0	-4.278	193.0	0.0	149.2	11.828
0.0	-47.6	-3.775	199.9	0.0	155.6	12.331
0.0	-41.3	-3.272	206.9	0.0	161.9	12.835
0.0	-34.9	-2.768	213.8	0.0	168.3	13.338
0.0	-28.6	-2.265	220.8	0.0	174.6	13.841
0.0	-22.2	-1.762	227.7	0.0	181.0	14.345
0.0	-15.9	-1.258	234.6	0.0	187.3	14.848
0.0	-9.5	-0.755	241.6	0.0	193.7	15.351
0.0	-3.2	-0.252	248.5	0.0	200.0	15.855



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ZETASIZER

Zeta Potential Report

PS0311-02
TMC articles 5:1 (TMC:TPP) with Tween 80
Jaco van Heerden

Sample

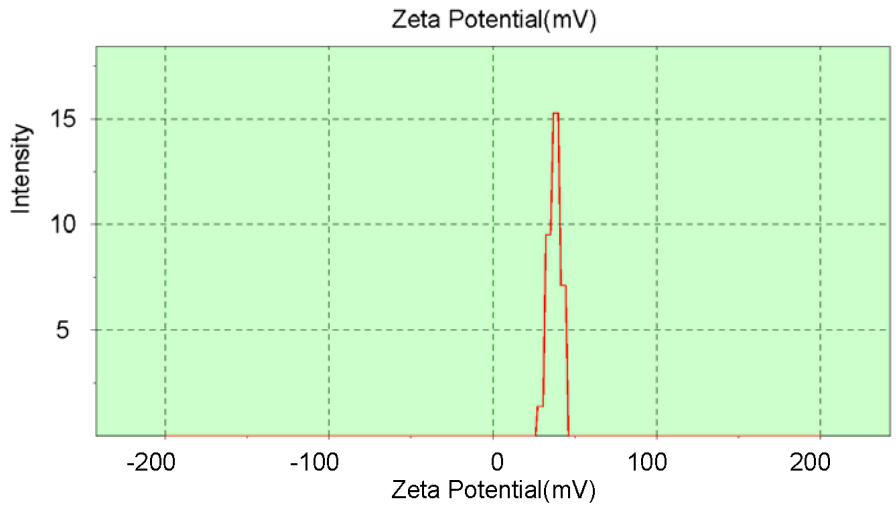
Record Number:	6
Filename:	ZSIZER.zet
File Path:	G:\MALVERN\ZETASI~1\17MAY
Dielectric Constant:	79.7
pH:	N/A
Viscosity (cP):	0.890
Date (DMY):	17/05/11
Time:	15:37:15

System

Instrument Type:	Zetasizer 2000
Temperature (°C):	25.0
Count rate (kCps):	2360.7
Cell Type:	Capillary cell
Cell Position (%):	50.00
Cell field (V/cm):	29.1
Current (mA):	1.6

Result

Zeta Potential (mV):	37.6	Mobility (umcm/V.s):	2.949
StDev (mV):	1.6	StDev (umcm/V.s):	0.125
Conductivity (mS/cm):	0.54	F(ka):	1.50



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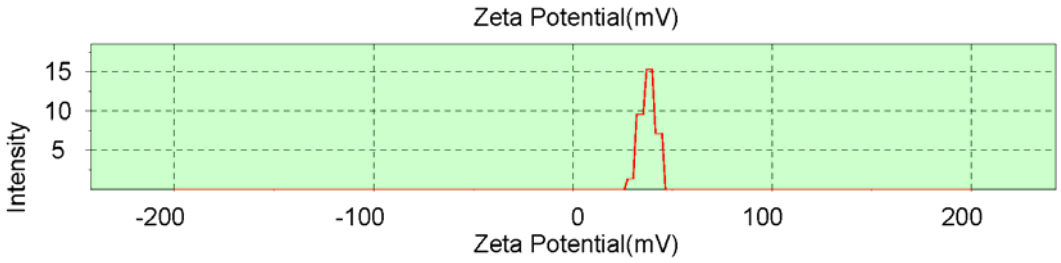
Int.	Zeta(mV)	Mob(µmcm/Vs)	Freq.(Hz)	Int.	Zeta(mV)	Mob(µmcm/V
0.0	-200.0	-15.859	32.3	0.0	3.2	0.252
0.0	-193.7	-15.355	39.2	0.0	9.5	0.755
0.0	-187.3	-14.852	46.2	0.0	15.9	1.259
0.0	-181.0	-14.348	53.1	0.0	22.2	1.762
0.0	-174.6	-13.845	60.0	7.7	28.6	2.266
0.0	-168.3	-13.341	67.0	52.9	34.9	2.769
0.0	-161.9	-12.838	73.9	39.5	41.3	3.272
0.0	-155.6	-12.334	80.9	0.0	47.6	3.776
0.0	-149.2	-11.831	87.8	0.0	54.0	4.279
0.0	-142.9	-11.328	94.8	0.0	60.3	4.783
0.0	-136.5	-10.824	101.7	0.0	66.7	5.286
0.0	-130.2	-10.321	108.7	0.0	73.0	5.790
0.0	-123.8	-9.817	115.6	0.0	79.4	6.293
0.0	-117.5	-9.314	122.5	0.0	85.7	6.797
0.0	-111.1	-8.810	129.5	0.0	92.1	7.300
0.0	-104.8	-8.307	136.4	0.0	98.4	7.803
0.0	-98.4	-7.803	143.4	0.0	104.8	8.307
0.0	-92.1	-7.300	150.3	0.0	111.1	8.810
0.0	-85.7	-6.797	157.3	0.0	117.5	9.314
0.0	-79.4	-6.293	164.2	0.0	123.8	9.817
0.0	-73.0	-5.790	171.1	0.0	130.2	10.321
0.0	-66.7	-5.286	178.1	0.0	136.5	10.824
0.0	-60.3	-4.783	185.0	0.0	142.9	11.328
0.0	-54.0	-4.279	192.0	0.0	149.2	11.831
0.0	-47.6	-3.776	198.9	0.0	155.6	12.334
0.0	-41.3	-3.272	205.9	0.0	161.9	12.838
0.0	-34.9	-2.769	212.8	0.0	168.3	13.341
0.0	-28.6	-2.266	219.8	0.0	174.6	13.845
0.0	-22.2	-1.762	226.7	0.0	181.0	14.348
0.0	-15.9	-1.259	233.6	0.0	187.3	14.852
0.0	-9.5	-0.755	240.6	0.0	193.7	15.355
0.0	-3.2	-0.252	247.5	0.0	200.0	15.859



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ZETASIZER

Zeta Potential Report

PS0311-03
TMC particles 5:1 (TMC:TPP) sonder Tween 80 en 10 min ultrasonic rod
Jaco van Heerden

Sample

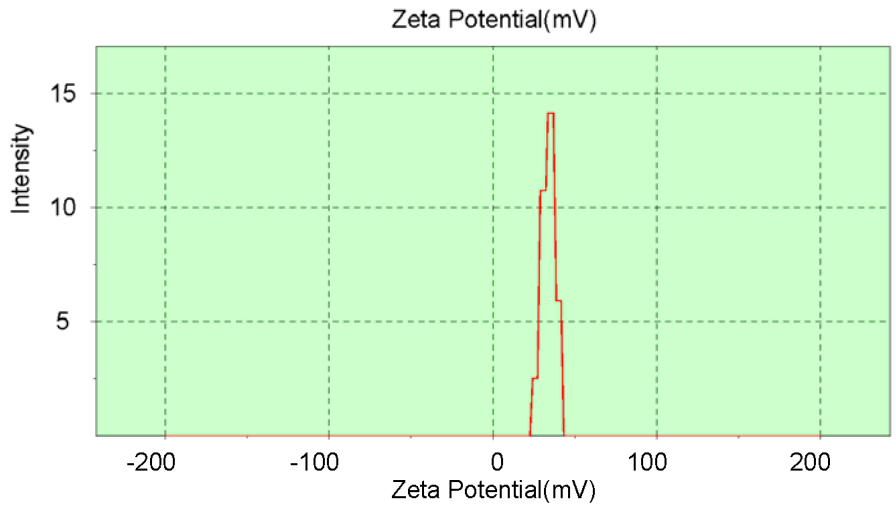
Record Number:	7
Filename:	ZSIZER.zet
File Path:	G:\MALVERN\ZETASI~1\17MAY
Dielectric Constant:	79.7
pH:	N/A
Viscosity (cP):	0.891
Date (DMY):	17/05/11
Time:	15:45:46

System

Instrument Type:	Zetasizer 2000
Temperature (°C):	25.0
Count rate (kCps):	2207.5
Cell Type:	Capillary cell
Cell Position (%):	50.00
Cell field (V/cm):	29.1
Current (mA):	2.0

Result

Zeta Potential (mV):	33.5	Mobility (umcm/V.s):	2.584
StDev (mV):	1.6	StDev (umcm/V.s):	0.125
Conductivity (mS/cm):	0.69	F(ka):	1.50



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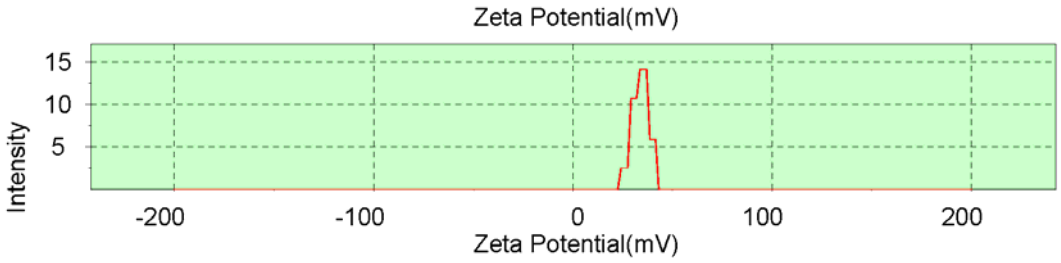
Int.	Zeta(mV)	Mob(µmcm/Vs)	Freq.(Hz)	Int.	Zeta(mV)	Mob(µmcm/V
0.0	-200.0	-15.851	31.5	0.0	3.2	0.252
0.0	-193.7	-15.348	38.5	0.0	9.5	0.755
0.0	-187.3	-14.845	45.4	0.0	15.9	1.258
0.0	-181.0	-14.342	52.3	0.0	22.2	1.761
0.0	-174.6	-13.838	59.3	34.9	28.6	2.264
0.0	-168.3	-13.335	66.2	45.9	34.9	2.768
0.0	-161.9	-12.832	73.1	19.2	41.3	3.271
0.0	-155.6	-12.329	80.1	0.0	47.6	3.774
0.0	-149.2	-11.826	87.0	0.0	54.0	4.277
0.0	-142.9	-11.322	93.9	0.0	60.3	4.781
0.0	-136.5	-10.819	100.9	0.0	66.7	5.284
0.0	-130.2	-10.316	107.8	0.0	73.0	5.787
0.0	-123.8	-9.813	114.8	0.0	79.4	6.290
0.0	-117.5	-9.309	121.7	0.0	85.7	6.793
0.0	-111.1	-8.806	128.6	0.0	92.1	7.297
0.0	-104.8	-8.303	135.6	0.0	98.4	7.800
0.0	-98.4	-7.800	142.5	0.0	104.8	8.303
0.0	-92.1	-7.297	149.4	0.0	111.1	8.806
0.0	-85.7	-6.793	156.4	0.0	117.5	9.309
0.0	-79.4	-6.290	163.3	0.0	123.8	9.813
0.0	-73.0	-5.787	170.2	0.0	130.2	10.316
0.0	-66.7	-5.284	177.2	0.0	136.5	10.819
0.0	-60.3	-4.781	184.1	0.0	142.9	11.322
0.0	-54.0	-4.277	191.0	0.0	149.2	11.826
0.0	-47.6	-3.774	198.0	0.0	155.6	12.329
0.0	-41.3	-3.271	204.9	0.0	161.9	12.832
0.0	-34.9	-2.768	211.9	0.0	168.3	13.335
0.0	-28.6	-2.264	218.8	0.0	174.6	13.838
0.0	-22.2	-1.761	225.7	0.0	181.0	14.342
0.0	-15.9	-1.258	232.7	0.0	187.3	14.845
0.0	-9.5	-0.755	239.6	0.0	193.7	15.348
0.0	-3.2	-0.252	246.5	0.0	200.0	15.851



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ZETASIZER

Zeta Potential Report

PS0311-03
TMC particles 5:1 (TMC:TPP) sonder Tween 80 en 10 min ultrasonic rod
Jaco van Heerden

Sample

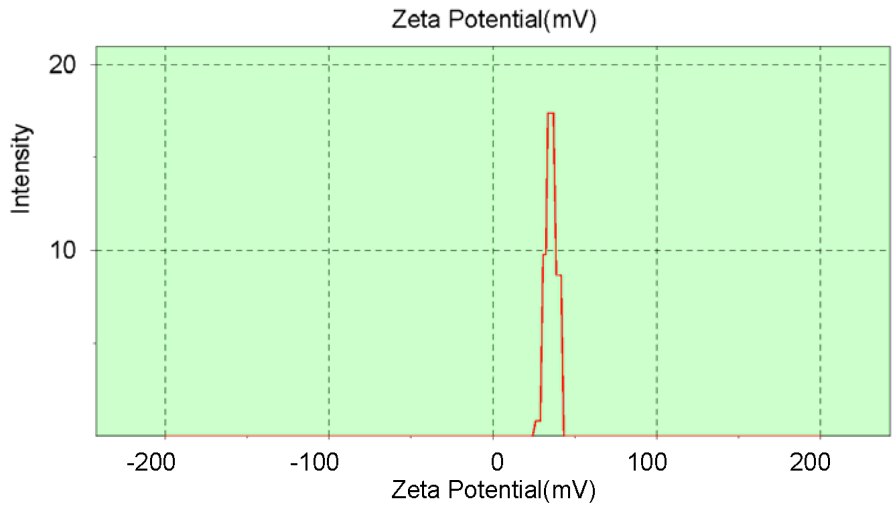
Record Number:	8
Filename:	ZSIZER.zet
File Path:	G:\MALVERN\ZETASI~1\17MAY
Dielectric Constant:	79.7
pH:	N/A
Viscosity (cP):	0.891
Date (DMY):	17/05/11
Time:	15:47:03

System

Instrument Type:	Zetasizer 2000
Temperature (°C):	25.0
Count rate (kCps):	2141.2
Cell Type:	Capillary cell
Cell Position (%):	50.00
Cell field (V/cm):	29.1
Current (mA):	2.0

Result

Zeta Potential (mV):	35.5	Mobility (umcm/V.s):	2.797
StDev (mV):	1.6	StDev (umcm/V.s):	0.125
Conductivity (mS/cm):	0.69	F(ka):	1.50



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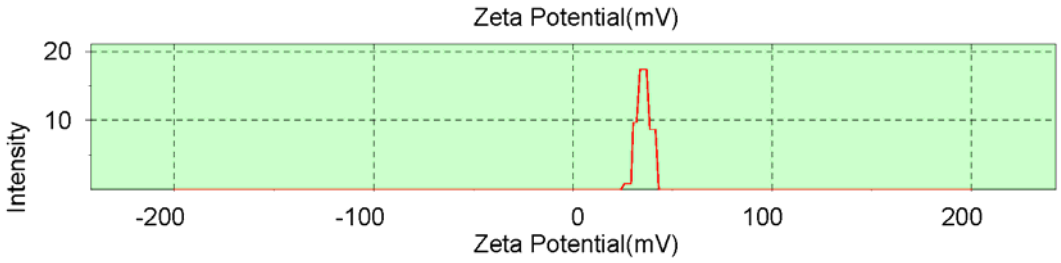
Int.	Zeta(mV)	Mob(µmcm/Vs)	Freq.(Hz)	Int.	Zeta(mV)	Mob(µmcm/V
0.0	-200.0	-15.848	30.7	0.0	3.2	0.252
0.0	-193.7	-15.345	37.6	0.0	9.5	0.755
0.0	-187.3	-14.842	44.6	0.0	15.9	1.258
0.0	-181.0	-14.339	51.5	0.0	22.2	1.761
0.0	-174.6	-13.836	58.4	3.0	28.6	2.264
0.0	-168.3	-13.332	65.4	64.8	34.9	2.767
0.0	-161.9	-12.829	72.3	32.2	41.3	3.270
0.0	-155.6	-12.326	79.2	0.0	47.6	3.773
0.0	-149.2	-11.823	86.2	0.0	54.0	4.276
0.0	-142.9	-11.320	93.1	0.0	60.3	4.780
0.0	-136.5	-10.817	100.0	0.0	66.7	5.283
0.0	-130.2	-10.314	106.9	0.0	73.0	5.786
0.0	-123.8	-9.811	113.9	0.0	79.4	6.289
0.0	-117.5	-9.308	120.8	0.0	85.7	6.792
0.0	-111.1	-8.804	127.7	0.0	92.1	7.295
0.0	-104.8	-8.301	134.7	0.0	98.4	7.798
0.0	-98.4	-7.798	141.6	0.0	104.8	8.301
0.0	-92.1	-7.295	148.5	0.0	111.1	8.804
0.0	-85.7	-6.792	155.4	0.0	117.5	9.308
0.0	-79.4	-6.289	162.4	0.0	123.8	9.811
0.0	-73.0	-5.786	169.3	0.0	130.2	10.314
0.0	-66.7	-5.283	176.2	0.0	136.5	10.817
0.0	-60.3	-4.780	183.2	0.0	142.9	11.320
0.0	-54.0	-4.276	190.1	0.0	149.2	11.823
0.0	-47.6	-3.773	197.0	0.0	155.6	12.326
0.0	-41.3	-3.270	204.0	0.0	161.9	12.829
0.0	-34.9	-2.767	210.9	0.0	168.3	13.332
0.0	-28.6	-2.264	217.8	0.0	174.6	13.836
0.0	-22.2	-1.761	224.7	0.0	181.0	14.339
0.0	-15.9	-1.258	231.7	0.0	187.3	14.842
0.0	-9.5	-0.755	238.6	0.0	193.7	15.345
0.0	-3.2	-0.252	245.5	0.0	200.0	15.848



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ZETASIZER

Zeta Potential Report

PS0311-03
TMC particles 5:1 (TMC:TPP) sonder Tween 80 en 10 min ultrasonic rod
Jaco van Heerden

Sample

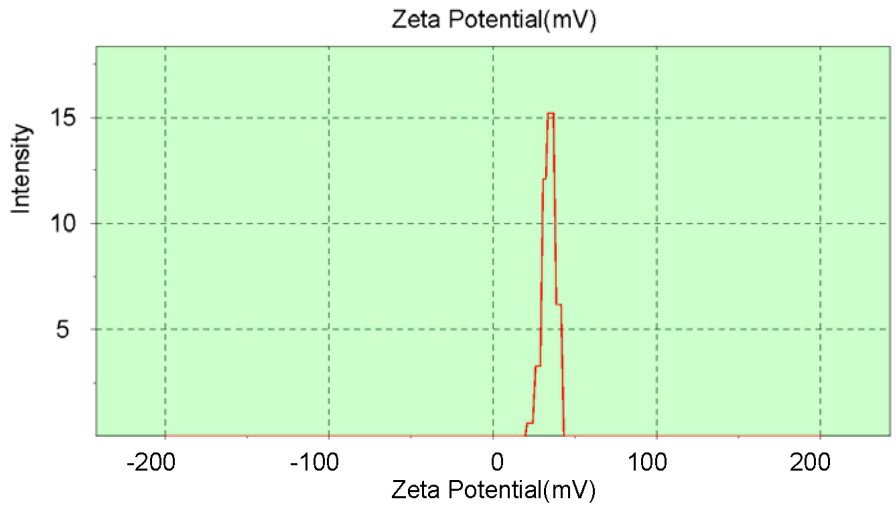
Record Number:	9
Filename:	ZSIZER.zet
File Path:	G:\MALVERN\ZETASI~1\17MAY
Dielectric Constant:	79.7
pH:	N/A
Viscosity (cP):	0.891
Date (DMY):	17/05/11
Time:	15:48:21

System

Instrument Type:	Zetasizer 2000
Temperature (°C):	25.0
Count rate (kCps):	2198.2
Cell Type:	Capillary cell
Cell Position (%):	50.00
Cell field (V/cm):	29.1
Current (mA):	2.0

Result

Zeta Potential (mV):	34.0	Mobility (umcm/V.s):	2.630
StDev (mV):	1.6	StDev (umcm/V.s):	0.125
Conductivity (mS/cm):	0.69	F(ka):	1.50



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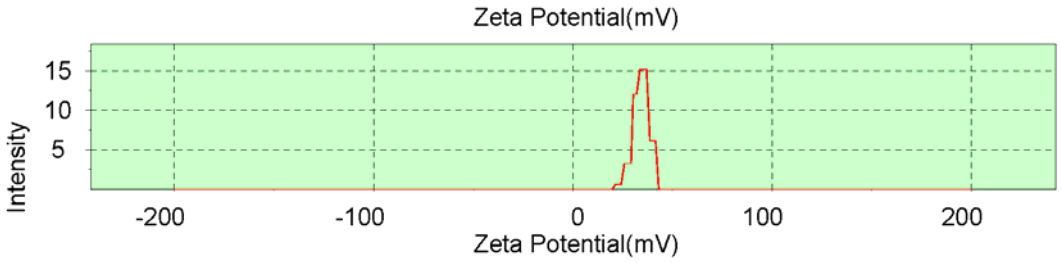
Int.	Zeta(mV)	Mob(µmcm/Vs)	Freq.(Hz)	Int.	Zeta(mV)	Mob(µmcm/V
0.0	-200.0	-15.848	30.6	0.0	3.2	0.252
0.0	-193.7	-15.345	37.5	0.0	9.5	0.755
0.0	-187.3	-14.842	44.4	0.0	15.9	1.258
0.0	-181.0	-14.339	51.4	2.4	22.2	1.761
0.0	-174.6	-13.836	58.3	13.1	28.6	2.264
0.0	-168.3	-13.332	65.2	60.1	34.9	2.767
0.0	-161.9	-12.829	72.2	24.4	41.3	3.270
0.0	-155.6	-12.326	79.1	0.0	47.6	3.773
0.0	-149.2	-11.823	86.0	0.0	54.0	4.276
0.0	-142.9	-11.320	93.0	0.0	60.3	4.780
0.0	-136.5	-10.817	99.9	0.0	66.7	5.283
0.0	-130.2	-10.314	106.8	0.0	73.0	5.786
0.0	-123.8	-9.811	113.8	0.0	79.4	6.289
0.0	-117.5	-9.308	120.7	0.0	85.7	6.792
0.0	-111.1	-8.804	127.6	0.0	92.1	7.295
0.0	-104.8	-8.301	134.6	0.0	98.4	7.798
0.0	-98.4	-7.798	141.5	0.0	104.8	8.301
0.0	-92.1	-7.295	148.5	0.0	111.1	8.804
0.0	-85.7	-6.792	155.4	0.0	117.5	9.308
0.0	-79.4	-6.289	162.3	0.0	123.8	9.811
0.0	-73.0	-5.786	169.3	0.0	130.2	10.314
0.0	-66.7	-5.283	176.2	0.0	136.5	10.817
0.0	-60.3	-4.780	183.1	0.0	142.9	11.320
0.0	-54.0	-4.276	190.1	0.0	149.2	11.823
0.0	-47.6	-3.773	197.0	0.0	155.6	12.326
0.0	-41.3	-3.270	203.9	0.0	161.9	12.829
0.0	-34.9	-2.767	210.9	0.0	168.3	13.332
0.0	-28.6	-2.264	217.8	0.0	174.6	13.836
0.0	-22.2	-1.761	224.7	0.0	181.0	14.339
0.0	-15.9	-1.258	231.7	0.0	187.3	14.842
0.0	-9.5	-0.755	238.6	0.0	193.7	15.345
0.0	-3.2	-0.252	245.5	0.0	200.0	15.848



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Annexure:

Bioanalytical report: Bioavailability evaluation of a reference dapsone and dapsone-TMC formulation in mice

Bioavailability evaluation of a reference dapsone and TMC dapsone formulation in mice

Bioanalytical report

Contact information

Dr Lubbe Wiesner
lubbe.wiesner@uct.ac.za

Proposed Timelines

Final report: 30 June 2014

Instrumentation

Agilent 1200 series HPLC system

Applied Biosystems API 5500 Q-Trap triple quadrupole mass spectrometer

Sample Storage

- Reference standards at 20 °C,
- Reference standard stock solutions at -80 °C,
- Study samples at -80 °C, and
- Freshly prepared calibration standards and quality controls were used.

Table of Contents

1 Introduction

The bioavailability of the test compound, dapsone mixed with N-trimethyl chitosan chloride (TMC) nanoparticles in a 1:1 (w/w) ratio, relative to free dapsone was evaluated in a mouse model. The animals utilized were male C57/BL6 mice, weighing approximately 20-25g.

The reference dapsone and TMC dapsone formulations were administered orally (N=5) at 3 mg/kg in a mixture of phosphate buffer saline (PBS) and dimethyl sulphoxide (90:10; v/v). The dose was prepared according to the average weight of the animals and an assumed volume of 200 µl of vehicle. Volumes were adjusted to dose the compound at 3 mg/kg for each animal's individual weight.

Whole mouse blood sample was collected at a predetermined sampling time points (0.17, 0.5, 1, 2, 4, 8, and 24 h) via tail vein bleeding into 1.5 ml heparinised microfuge tubes and were kept on ice. Blood samples were transferred to a -80 °C freezer within 60 minutes after collection for storage until analysis was carried out.

A sensitive and accurate LC-MS/MS method was developed to analyse the study samples.

Concentration vs. time graphs were constructed and the bioavailability of dapsone-TMC was determined relative to the free dapsone using the following formula.

$$relative\ bioavailability = \frac{AUC_{test}}{AUC_{ref}} \times \frac{Dose_{ref}}{Dose_{test}};$$

Where test = dapsone-TMC, and ref = free dapsone.

2 Methodology

A sensitive and selective LC-MS/MS method was developed to analyze the test compound in mice whole blood.

2.1 Mass spectrometry

Detection of dapsone (figure 1a) and the internal standard (dapsone-d4; figure 1b) was performed on an AB Sciex API 5500 mass spectrometer (ESI in the positive ion mode, MRM) and the settings on the apparatus are summarised in tables 1 and 2.

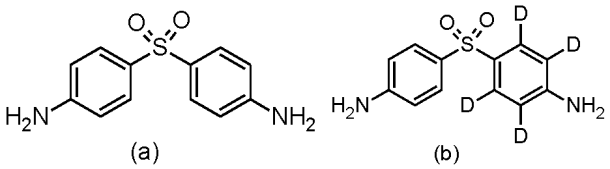


Figure 1: The structures of (a) dapsone, and (b) dapsone-d4

Table 1: Ionization source setting

Electro Spray Ionisation Settings	Value
Nebulizer gas (Gas 1) (arbitrary unit)	70
Turbo gas (Gas 2) (arbitrary unit)	65
CUR (curtain gas) (arbitrary unit)	30
CAD (collision gas) (arbitrary unit)	Medium
TEM (source temperature) (°C)	400
IS (Ion Spray Voltage) (V)	2500

Table 2: MS/MS detector setting

MS/MS Settings	Dapsone	ISTD
Protonated molecular mass (*m/z*)	249	253
Product ion molecular mass (*m/z*)	156	156
Dwell time (ms)	200	200
DP (declustering potential) (V)	100	140
EP (entrance potential) (V)	10	10
CE (collision energy) (eV)	20	20
CXP (collision cell exit potential) (V)	15	15
Scan Type	MRM	MRM
Polarity	Positive	Positive
Pause time	5ms	5ms

2.1.1 Mass spectra

Mass transitions of the protonated precursor ions to the product ions for dapsone and the ISTD

Transition of the protonated precursor ion m/z 249, and m/z 253 to the product ion m/z 156 for dapsone and the internal standard dapsone-d4, respectively were monitored at unit resolution in the multiple reaction monitoring (MRM) mode (see the infusion product ion mass spectrum of dapsone and the internal standard in Figure 2 and 3, respectively).

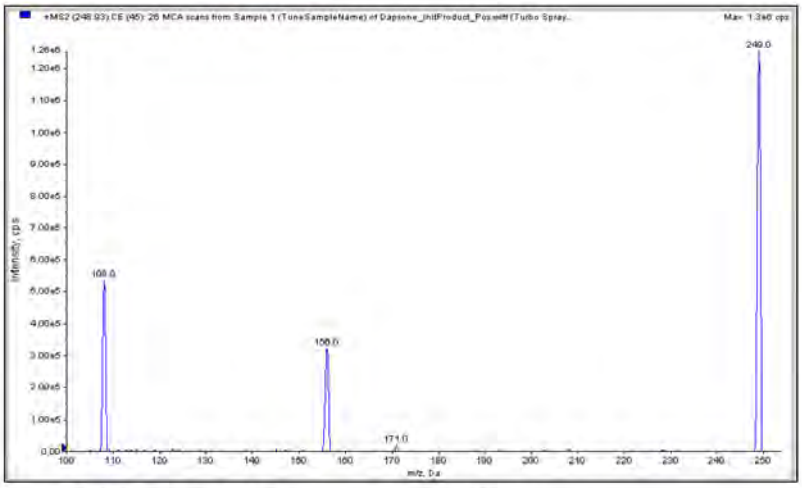


Figure 2: Infusion product ion mass spectrum of dapsone

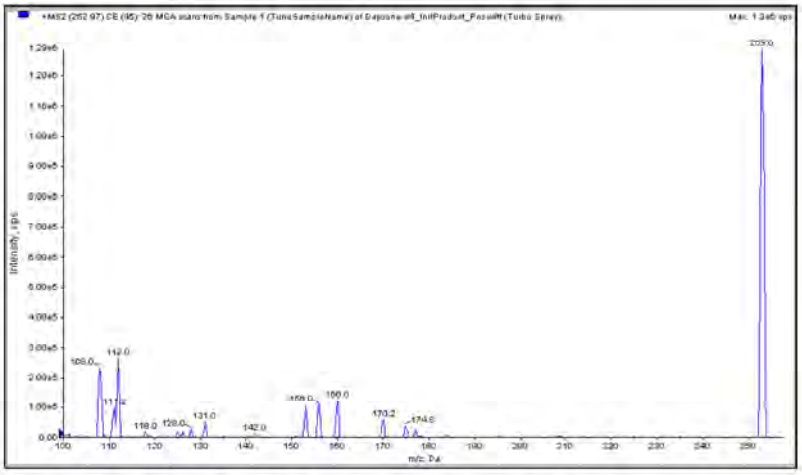


Figure 3: Infusion product ion mass spectrum of dapsone-d4

2.2 Chromatography

Chromatography was performed on a Phenomenex Luna C18 [2]-HST (2.5µm, 30x2 mm) analytical column using an Agilent 1200 series HPLC. The mobile phase consisted of a mixture of A and B at 60:40 v/v; where mobile phase A was a 5 mM ammonium acetate solution in water (pH 9.0) and B was a mixture of acetonitrile and 5 mM ammonium acetate solution in water (95:5; v/v) pH adjusted to 9.0. The mobile phase was delivered at a constant flow rate of 0.2 ml/min. The column was kept in a column compartment at 20 °C. An autosampler injected 1 µl onto the HPLC column. The injection needle was rinsed with mobile phase before each injection for 10 seconds using the flush port wash station. The samples were cooled to 4 °C while awaiting injection.

2.3. Preparation of calibration standards

A primary stock solution of dapsone at 1 mg/ml was prepared in DMSO. Fifty microliters of the primary stock solution was diluted with water to a final volume of 516 µl obtaining a secondary stock solution of 88.339 µg/ml, which was used to spike S1 (ULOQ at 5000 ng/ml). Five hundred microliters of mouse blood was spiked with 30 µl of the secondary stock solution to obtain S1. S1 was further diluted with blank mouse blood to obtain the required calibration standards as presented in table 3.

Table 3: Preparation of dapsone stock solution for spiking S1 in mouse blood

Preparation of Primary (1°SS) Stock Solution for Spiking Secondary Stock Solution

Solvent used	SG solvent	Mass Analyte (mg)	Mass Solvent (g)	Volume Solvent (ml)	Concentration of Analyte in 1°SS (µg/ml)
DMSO	1.100	1.040	1.144	1.040	1000.00

Preparation of Secondary (2°SS) Stock Solution for Spiking S1

Solvent used	SG solvent	Volume of 1°SS spiked (µl)	Mass Solvent (g)	Volume Solvent (ml)	Volume of 2°SS spiked (µl)	Concentration of Analyte in 2°SS (µg/ml)
Water	1.000	50	0.516	0.516	30	88.33922

Sample ID	Source Solution	A (ml)	B (ml)	C (ng/ml)
S1	*Stock Solution*	0.500		5000
S2	*S1*	0.200	0.050	1000
S3	*S2*	0.150	0.050	250.0
S4	*S3*	0.200	0.050	50.00
S5	*S4*	0.200	0.050	10.00
S6	*S5*	0.200	0.050	2.000

Key:
A = Volume of blank matrix
B = Volume of spiked matrix added to blank matrix
C = Concentration of analyte in matrix

2.4. Preparation of quality control samples

Stock solutions prepared in table 3 were used for spiking QC samples.

Table 4: Preparation of dapsone stock solution for spiking Q1 in mouse blood

Preparation of Primary (1°SS) Stock Solution for Spiking Secondary Stock Solution

Solvent used	SG solvent	Mass Analyte (mg)	Mass Solvent (g)	Volume Solvent (ml)	Concentration of Analyte in 1°SS (µg/ml)
DMSO	1.100	1.040	1.144	1.040	1000.00

Preparation of Secondary (2°SS) Stock Solution for Spiking Q1

Solvent used	SG solvent	Volume of 1°SS spiked (µl)	Mass Solvent (g)	Volume Solvent (ml)	Volume of 2°SS spiked (µl)	Concentration of Analyte in 2°SS (µg/ml)
Water	1.000	50	0.516	0.516	25	88.33922

Sample ID	Source Solution	A (ml)	B (ml)	C (ng/ml)
Q1	*Stock Solution*	0.527		4001
Q2	*Q1*	0.200	0.050	800.2
Q3	*Q2*	0.350	0.050	100.0
Q4	*Q3*	0.366	0.050	12.02
Q5	*Q4*	0.150	0.150	6.011
Q6	*Q4*	0.200	0.100	2.004

Key:
A = Volume of blank matrix
B = Volume of spiked matrix added to blank matrix
C = Concentration of analyte in matrix

2.5. Sample preparation

Blood samples were completely thawed unassisted at room temperature and briefly vortex mixed. *In vivo* mouse blood samples were assayed together with the calibration standards and quality control samples (STDs and QC samples were assayed in duplicates). The analyte and ISTD were isolated using protein precipitation as follows:

Extraction procedure:

1. Aliquot 20 µl of blood sample into a 1.5 ml microfuge tube.
2. Add ice cold acetonitrile containing 0.1 % ammonium hydroxide (80 µl), spiked with 375 ng/ml dapsone-d4 as an internal standard.
3. Vortex for one minute and ultrasonicate for 5 min, followed by brief vortex mixing.
4. Centrifuge at 13000 rpm for five minutes.
5. Transfer the supernatant to a 96-well plate and inject 1 µl onto the HPLC column.

3 Results

3.1 Calibration curve

The calibration standards and quality control samples were analysed in duplicate with the study samples. A representative calibration curve is presented in figure 4. The accuracy and precision statistics of this calibration curve is presented in table 5. Representative chromatograms are presented in figures 5 (STD 6), 6 (Mouse 1, 1 h post dose sample), 7 (blank) and 8 Zero sample (a blank containing the internal standard).

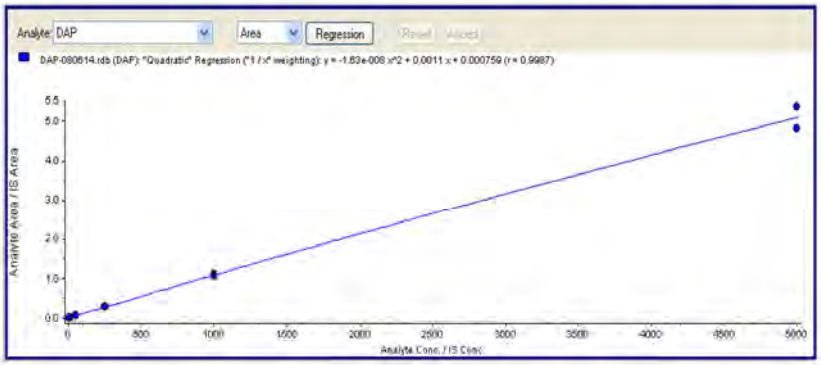


Figure 4: Representative calibration curve of dapsone

Table 5: Accuracy and precision data of a representative calibration curve

Expected Concentration (ng/ml)	STD	Number Of Values Used	Mean	%CV	%Accuracy
2.000	STD 6	2 of 2	2.016	0.4254	100.8
10.00	STD 5	2 of 2	9.994	1.533	99.9
50.00	STD 4	2 of 2	49.46	0.2753	98.9
250.0	STD 3	2 of 2	250.9	0.6714	100.4
1000	STD 2	2 of 2	999.5	37.53	99.9
5000	STD 1	1 of 1	4700	N/A	94.0
5000	STD 1	1 of 1	5302	N/A	106.0

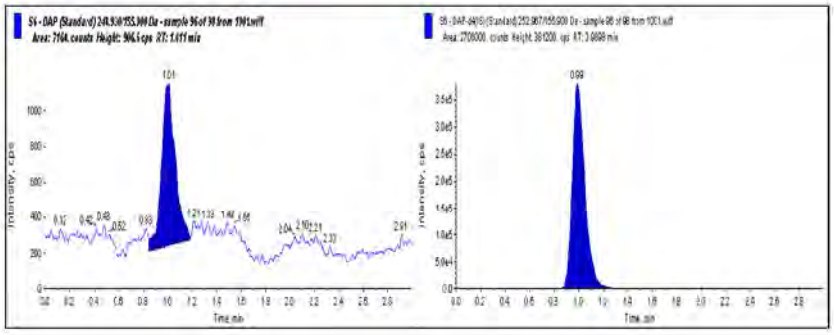


Figure 5: Representative chromatogram of STD 6 (2 ng/ml)

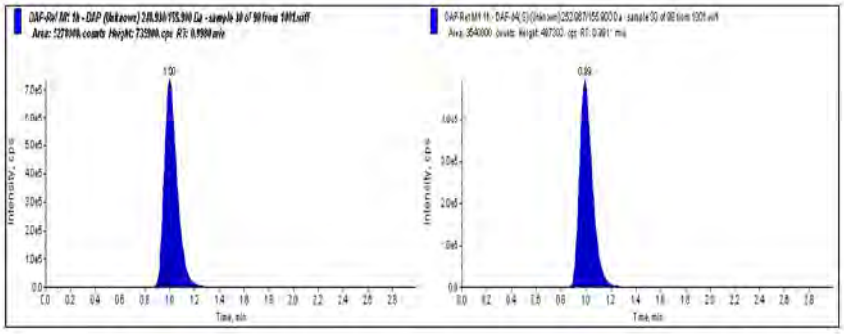


Figure 6: Representative chromatogram of Mouse 1 (1 h post dose sample)

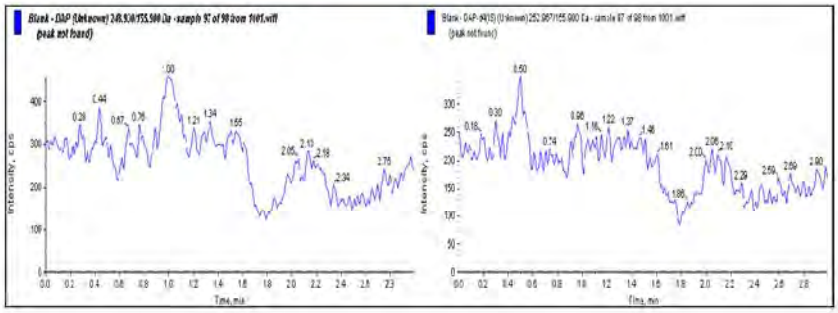


Figure 7: Representative chromatogram of a blank sample

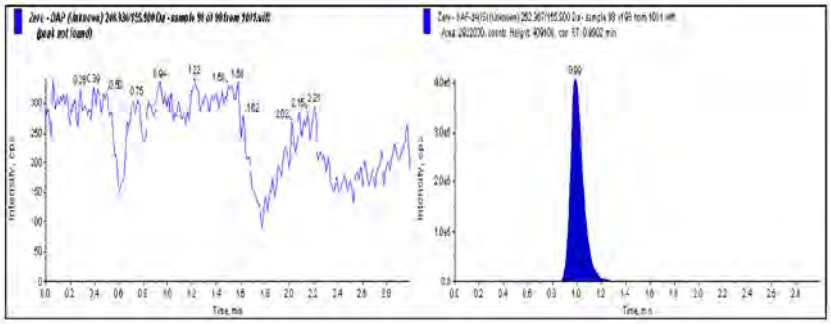


Figure 8: Representative chromatogram of a zero sample

3.2 Oral dose experiment

Both dapsone and dapsone-TMC oral dose samples were analysed in the same batch. The calculated concentrations of dapsone and dapsone-TMC are presented in table 6 and 7, respectively; while the concentration-time profiles in figure 9.

Table 6: Calculated blood concentration of dapsone

Dapsone (3 mg/kg oral) 080614																	
Time (h)	Conc. (ng/ml)								Conc. (μM)								
	M1	M2	M3	M4	M5	Mean	STDEV	SEM	M1	M2	M3	M4	M5	Mean	STDEV	SEM
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.0	0.0	0.00
0.17	851.2	763.6	1044	776.0	1086	904.2	151	67.6	3.4	3.1	4.2	3.1	4.4	3.6	0.6	0.27
0.5	1045	1092	1360	950.7	1153	1120	153	68.3	4.2	4.4	5.5	3.8	4.6	4.5	0.6	0.28
1	1383	1237	1440	1109	1155	1265	143	63.8	5.6	5.0	5.8	4.5	4.7	5.1	0.6	0.26
2	1400	2242	1331	1026	1048	1409	494	221	5.6	9.0	5.4	4.1	4.2	5.7	2.0	0.89
4	932.3	748.2	836.8	819.7	701.4	807.7	88.5	39.5	3.8	3.0	3.4	3.3	2.8	3.3	0.4	0.16
8	464.6	336.2	401.2	419.4	387.0	401.7	46.8	20.9	1.9	1.4	1.6	1.7	1.6	1.6	0.2	0.08
24	16.58	10.15	11.17	12.49	23.55	14.79	5.47	2.44	0.07	0.04	0.04	0.05	0.09	0.06	0.02	0.01

Table 7: Calculated blood concentration of dapsone-TMC

Dapsone-TMC (3 mg/kg oral) 080614																
Time (h)	Conc. (ng/ml)								Conc. (μM)							
	M1	M2	M3	M4	M5	Mean	STDEV	SEM	M1	M2	M3	M4	M5	Mean	STDEV	SEM
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.0	0.0	0.0
0.17	725.2	1056	1212	545.4	1085	924.7	278	124	2.9	4.3	4.9	2.2	4.4	3.7	1.1	0.5
0.5	1045	1465	1557	727.8	1275	1214	335	150	4.2	5.9	6.3	2.9	5.1	4.9	1.3	0.6
1	1321	1831	1524	830.5	1361	1374	364	162	5.3	7.4	6.1	3.3	5.5	5.5	1.5	0.7
2	1368	1775	1348	828.1	1312	1326	336	150	5.5	7.1	5.4	3.3	5.3	5.3	1.4	0.6
4	965.6	1867	894.5	883.3	898.2	1102	429	192	3.9	7.5	3.6	3.6	3.6	4.4	1.7	0.8
8	512.0	810.6	436.3	437.4	444.7	528.2	161	71.9	2.1	3.3	1.8	1.8	1.8	2.1	0.6	0.3
24	16.13	44.75	13.56	31.30	14.54	24.06	13.6	6.09	0.06	0.18	0.05	0.13	0.06	0.10	0.05	0.02

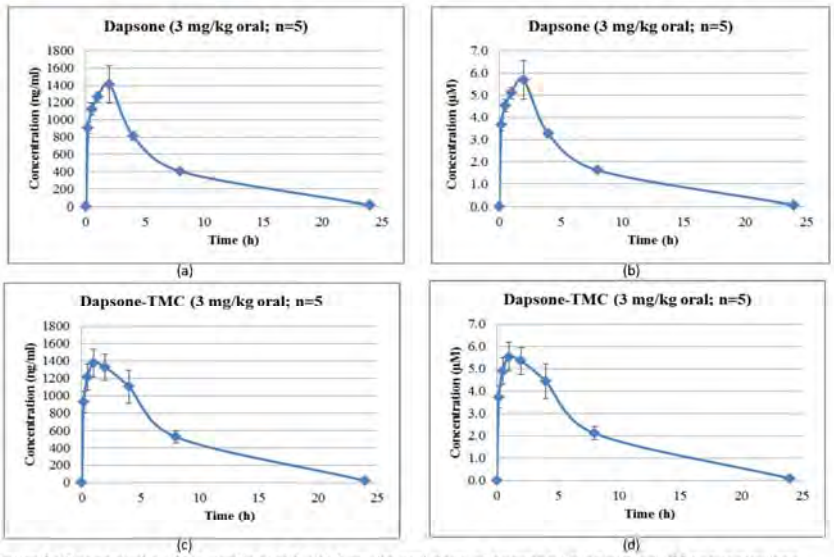


Figure 9: Concentration-time profiles (a) & (b) 3 mg/kg oral dose of dapsone (in ng/ml and μM concentrations, respectively); (c) & (d) 3 mg/kg oral dose of dapsone-TMC (in ng/ml and μM concentrations, respectively).

4 Pharmacokinetic properties of dapsone and dapsone-TMC

The pharmacokinetic properties of dapsone and dapsone-TMC were evaluated using Summit PK software. The bioavailability of dapsone-TMC (3 mg/kg oral dose, equivalent to 1.5 mg/kg dapsone) relative to dapsone (3 mg/kg oral dose) was found to be 244% (n=5). The pharmacokinetic properties are presented in table 8.

Table 8: Pharmacokinetic properties of dapsone

PK-Properties of Dapsone		
Parameters	**Oral dose**	
	Dapsone	**Dapsone-TMC**
Nominal Dose (mg/kg)	3	3 (equivalent to 1.5 mg/kg Dapsone)
Apparent $t_{1/2}$ (h)	3.4	3.6
C_{max} (µM)	5.7	5.5
T_{max} (h)	2	1
$AUC_{0-\infty}$ (min. µmol/L)	2510	3060
Bioavailability (%)	N/A	244 (188 - 370)
Results are mean of n=5		

5 Discussion

5.1 Calibration curve

A calibration graph was constructed using a quadratic regression curve fit with weighting factor 1/x based on a peak area ratio as the response type. The accuracy and precision of the standards used were within 20%.

5.2 Oral dose experiment

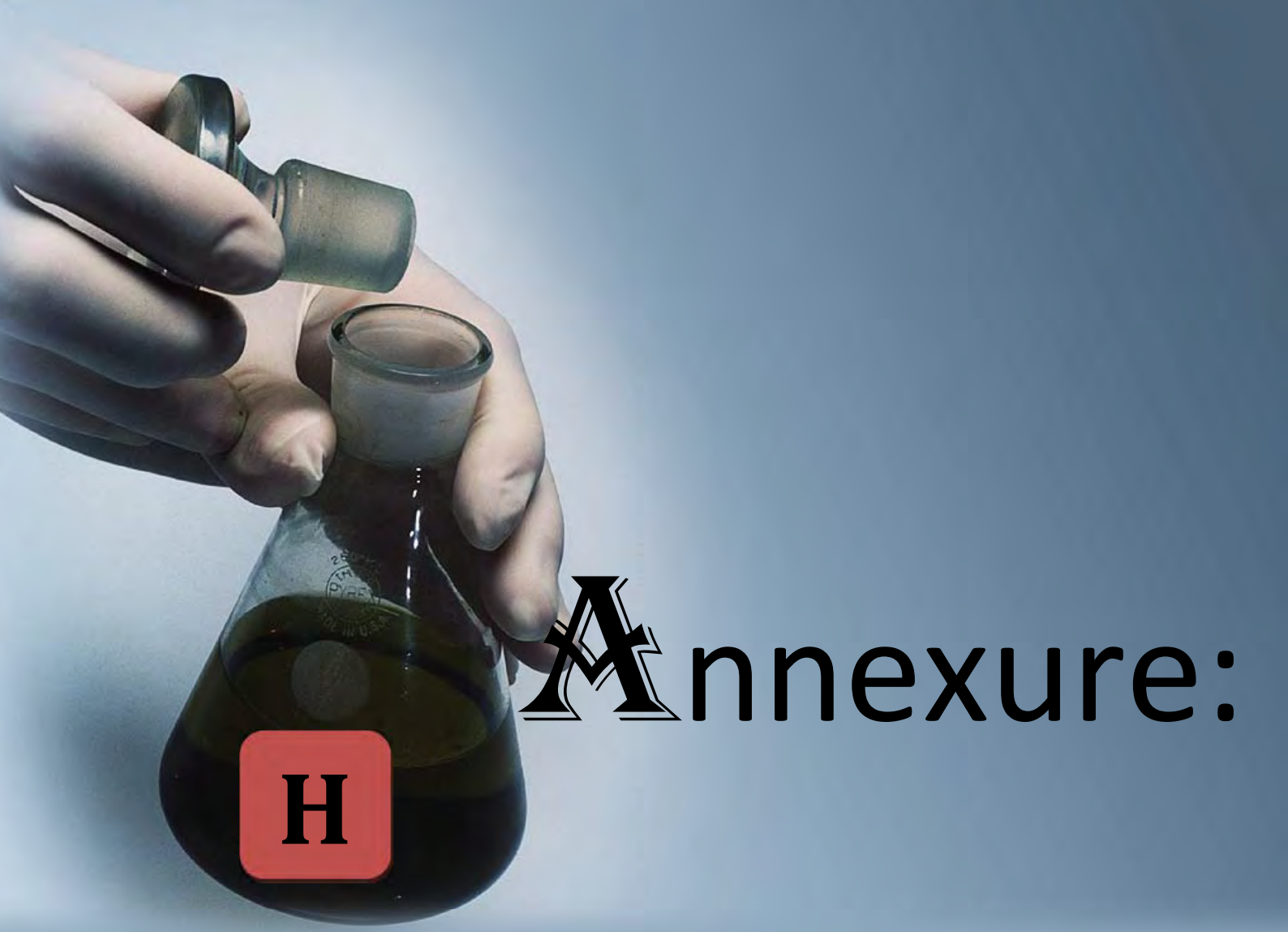
The drug blood levels were above the lower limit of quantification (2 ng/ml) up to 24 hours. The TMC nanoparticle formulation of dapsone was absorbed faster, i.e. the maximum concentration was attained at 1h post dose relative to that of

dapsone 2h. Both showed a moderate elimination half-life of 3.4 to 3.6 h. Mixing dapsone with TMC nanoparticle formulation has enhanced its absorption, and as a result its relative bioavailability too (BA = 244%).

6 Final discussion

A precise and accurate LC-MS/MS method was developed for dapsone and was used to analyse study samples. The test compound was evaluated for its relative bioavailability after oral administration. The bioavailability of the test compound was enhanced as the result of mixing it with TMC.

Study co-ordinator	Dr Lubbe Wiesner
Study performed by:	Dr Efrem Teclehaymanot Abay
Written by:	Dr Efrem Teclehaymanot Abay



Annexure:

Bioanalytical report: Bioavailability evaluation of a reference proguanil and proguanil-TMC formulation in mice

Bioavailability evaluation of a reference proguanil and TMC proguanil formulation in mice

Bioanalytical report

Contact information

Dr Lubbe Wiesner
lubbe.wiesner@uct.ac.za

Proposed Timelines

Final report: 30 June 2014

Instrumentation

Agilent 1200 series HPLC system

Applied Biosystems API 3200 triple quadrupole mass spectrometer

Sample Storage

- Reference standards at 20 °C,
- Reference standard stock solutions at -80 °C,
- Study samples at -80 °C, and
- Freshly prepared calibration standards and quality controls were used.

Table of Contents

1 Introduction

The bioavailability of the test compound, proguanil mixed with N-trimethyl chitosan chloride (TMC) nanoparticles in a 1:1 (w/w) ratio and its metabolite, relative to free proguanil was evaluated in a mouse model. The animals utilized were male C57/BL6 mice, weighing approximately 20-25g.

The reference proguanil and TMC proguanil formulations were administered orally (N=5) at 16 mg/kg in a mixture of phosphate buffer saline (PBS) and dimethyl sulphoxide (90:10; v/v). The dose was prepared according to the average weight of the animals and an assumed volume of 200 µl of vehicle. Volumes were adjusted to dose the compound at 16 mg/kg for each animal's individual weight.

Whole mouse blood samples were collected at predetermined sampling times (0.5, 1, 2, 4, 8, and 24 h) via tail vein bleeding into 1.5 ml heparinised microfuge tubes and kept on ice. Blood samples were transferred to a -80 °C freezer within 60 minutes after collection for storage until analysis is carried out.

A sensitive and accurate LC-MS/MS method was developed to analyse the study samples.

Concentration vs. time graphs were constructed and the bioavailability of proguanil-TMC was determined relative to the free proguanil using the following formula.

$$relative\ bioavailability = \frac{AUC_{test}}{AUC_{ref}} \times \frac{Dose_{ref}}{Dose_{test}};$$

Where test = proguanil-TMC, and ref = free proguanil.

2 Methodology

A sensitive and selective LC-MS/MS method was developed to analyze the test compound in mice whole blood.

2.1 Mass spectrometry

Detection of proguanil (figure 1a), cycloguanil (figure 1b) and the internal standard (priguanil-d4; figure 1c) was performed on an AB Sciex API 3200 mass spectrometer (ESI in the positive ion mode, MRM) and the settings on the apparatus are summarised in tables 1 and 2.

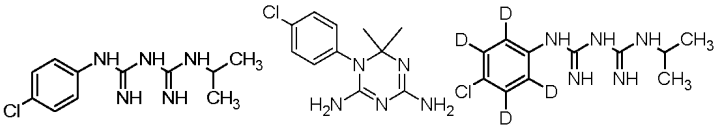


Figure 1: The structures of (a) proguanil, (b) cycloguanil, and (c) proguanil-d4

Table 1: Ionization source setting

Electro Spray Ionisation Settings	Value
Nebulizer gas (Gas 1) (arbitrary unit)	30
Turbo gas (Gas 2) (arbitrary unit)	70
CUR (curtain gas) (arbitrary unit)	10
CAD (collision gas) (arbitrary unit)	5
TEM (source temperature) (°C)	500
IS (Ion Spray Voltage) (V)	1500

Table 2: MS/MS detector setting

MS/MS Settings	Proguanil	Cycloguanil	ISTD
Protonated molecular mass (*m/z*)	254	252	258
Product ion molecular mass (*m/z*)	170	195	174
Dwell time (ms)	200	200	200
DP (declustering potential) (V)	45	45	40
EP (entrance potential) (V)	7	11	7
CE (collision energy) (eV)	25	25	25
CEP (collision cell entrance potential)(V)	30	30	25
CXP (collision cell exit potential) (V)	10	2	6
Scan Type	MRM	MRM	MRM
Polarity	Positive	Positive	Positive
Pause time	5ms	5ms	5ms

2.1.1 Mass spectra

Mass transition of the protonated precursor ion to the product ion for dapsone and the ISTD

Transitions of the protonated precursor ions at m/z 254, m/z 252 and m/z 258 to the product ions at m/z 170, m/z 195 and m/z 174 for proguanil, cycloguanil and the internal standard proguanil-d4, respectively were monitored at unit resolution in the multiple reaction monitoring (MRM) mode (see the infusion product ion mass spectrum of proguanil, cycloguanil and the internal standard in Figure 2, 3 and 4, respectively).

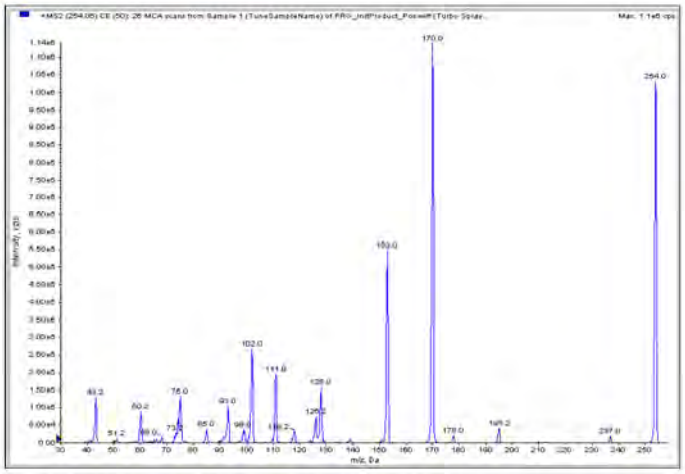


Figure 2: Infusion product ion mass spectrum of proguanil

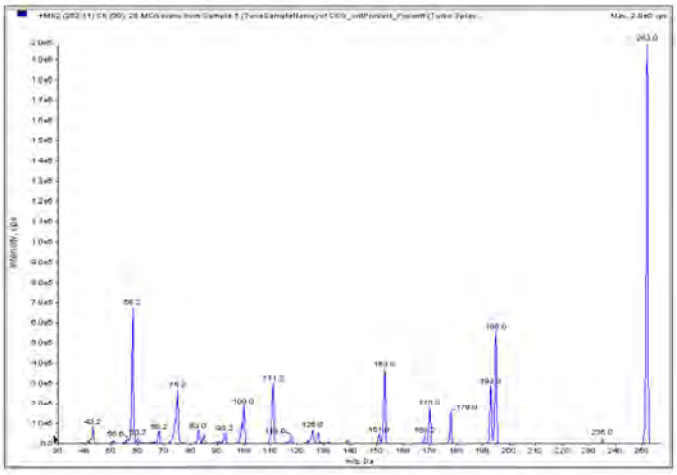


Figure 3: Infusion product ion mass spectrum of cycloguanil

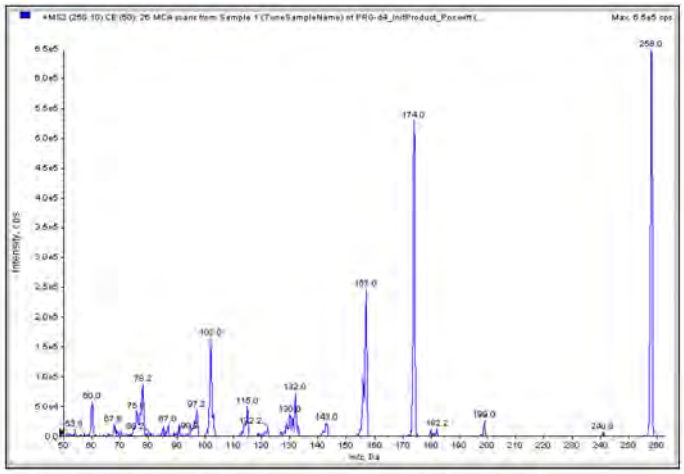


Figure 4: Infusion product ion mass spectrum of proguanil-d4

2.2 Chromatography

Chromatography was performed on an Agilent Eclipse Plus C18 (3.5µm, 50x2.1 mm) analytical column using an Agilent 1100 series HPLC. The mobile phase consisted of a mixture of A and B at 75:25 v/v; where mobile phase A was 0.1 % formic acid in water and B was 0.1 % formic acid in acetonitrile. The mobile phase was delivered at a constant flow rate of 0.45 ml/min. The column was kept in a column compartment at 20 °C. An autosampler injected 5 µl onto the HPLC column. The injection needle was rinsed with mobile phase before each injection for 10 seconds using the flush port wash station. The samples were cooled to 5 °C while awaiting injection.

2.3. Preparation of calibration standards

A primary stock solution of proguanil at 1 mg/ml and cycloguanil at 1.743 mg/ml in DMSO were prepared. Fifty microliters of the primary stock solution of proguanil was diluted with water to a final volume of 516 µl obtaining a secondary stock solution of 88.339 µg/ml, and 30 µl of the primary stock solution of cycloguanil was diluted with water to a final volume of 562 µl obtaining a secondary stock solution of 88.328 µg/ml, which were used to spike S1 (ULOQ at 5003 ng/ml). Five hundred and one microliters of mouse blood was spiked with 32 µl of each of the secondary stock solutions to obtain S1. S1 was further diluted with blank mouse blood to obtain the required calibration standards as presented in table 3a and 3b.

Table 3a: Preparation of proguanil stock solution for spiking S1 in mouse blood

Preparation of Primary (1°SS) Stock Solution for Spiking Secondary Stock Solution

Solvent used	SG solvent	Mass Analyte (mg)	Mass Solvent (g)	Volume Solvent (ml)	Concentration of Analyte in 1°SS (µg/ml)
DMSO	1.100	0.822	0.904	0.822	1000.00

Preparation of Secondary (2°SS) Stock Solution for Spiking S1

Solvent used	SG solvent	Volume of 1°SS spiked (µl)	Mass Solvent (g)	Volume Solvent (ml)	Volume of 2°SS spiked (µl)	Concentration of Analyte in 2°SS (µg/ml)
Water	1.000	50	0.516	0.516	32	88.33922

Sample ID	Source Solution	A (ml)	B (ml)	C (ng/ml)
S1	*Stock Solution*	0.501		5003
S2	*S1*	0.200	0.050	1001
S3	*S2*	0.150	0.050	250.2
S4	*S3*	0.200	0.050	50.03
S5	*S4*	0.200	0.050	10.01
S6	*S5*	0.200	0.050	2.001

Key:
A = Volume of blank matrix
B = Volume of spiked matrix added to blank matrix
C = Concentration of analyte in Matrix

Table 3b: Preparation of cycloguanil stock solution for spiking S1 in mouse blood

Preparation of Primary (1°SS) Stock Solution for Spiking Secondary Stock Solution

Solvent used	SG solvent	Mass Analyte (mg)	Mass Solvent (g)	Volume Solvent (ml)	Concentration of Analyte in 1°SS (µg/ml)
DMSO	1.100	1.743	1.100	1.000	1743.000

Preparation of Secondary (2°SS) Stock Solution for Spiking S1

Solvent used	SG solvent	Volume of 1°SS spiked (µl)	Mass Solvent (g)	Volume Solvent (ml)	Volume of 2°SS spiked (µl)	Concentration of Analyte in 2°SS (µg/ml)
Water	1.000	30.0	0.562	0.562	32	88.32770

Sample ID	Source Solution	A (ml)	B (ml)	C (ng/ml)
S1	*Stock Solution*	0.501		5003
S2	*S1*	0.200	0.050	1001
S3	*S2*	0.150	0.050	250.2
S4	*S3*	0.200	0.050	50.03
S5	*S4*	0.200	0.050	10.01
S6	*S5*	0.200	0.050	2.001

Key:
A = Volume of blank matrix
B = Volume of spiked matrix added to blank matrix
C = Concentration of analyte in Matrix

2.4. Preparation of quality control samples

Stock solutions prepared in table 3 were used for spiking QC samples.

Table 4a: Preparation of proguanil stock solution for spiking Q1 in mouse blood

Preparation of Primary (1°SS) Stock Solution for Spiking Secondary Stock Solution

Solvent used	SG solvent	Mass Analyte (mg)	Mass Solvent (g)	Volume Solvent (ml)	Concentration of Analyte in 1°SS (µg/ml)
DMSO	1.100	0.822	0.904	0.822	1000.00

Preparation of Secondary (2°SS) Stock Solution for Spiking Q1

Solvent used	SG solvent	Volume of 1°SS spiked (µl)	Mass Solvent (g)	Volume Solvent (ml)	Volume of 2°SS spiked (µl)	Concentration of Analyte in 2°SS (µg/ml)
Water	1.000	50	0.516	0.516	25	88.33922

Sample ID	Source Solution	A (ml)	B (ml)	C (ng/ml)
Q1	*Stock Solution*	0.495		4001
Q2	*Q1*	0.200	0.050	800.2
Q3	*Q2*	0.350	0.050	100.0
Q4	*Q3*	0.366	0.050	12.02
Q5	*Q4*	0.150	0.150	6.011
Q6	*Q4*	0.200	0.100	2.004

Key:
A = Volume of blank matrix
B = Volume of spiked matrix added to blank matrix
C = Concentration of analyte in matrix

Table 4a: Preparation of cycloguanil stock solution for spiking Q1 in mouse blood

Preparation of Primary (1°SS) Stock Solution for Spiking Secondary Stock Solution

Solvent used	SG solvent	Mass Analyte (mg)	Mass Solvent (g)	Volume Solvent (ml)	Concentration of Analyte in 1°SS (µg/ml)
DMSO	1.100	1.743	1.100	1.000	1743.00

Preparation of Secondary (2°SS) Stock Solution for Spiking Q1

Solvent used	SG solvent	Volume of 1°SS spiked (µl)	Mass Solvent (g)	Volume Solvent (ml)	Volume of 2°SS spiked (µl)	Concentration of Analyte in 2°SS (µg/ml)
Water	1.000	30.0	0.562	0.562	25	88.32770

Sample ID	Source Solution	A (ml)	B (ml)	C (ng/ml)
Q1	*Stock Solution*	0.495		4000
Q2	*Q1*	0.200	0.050	800.1
Q3	*Q2*	0.350	0.050	100.0
Q4	*Q3*	0.366	0.050	12.02
Q5	*Q4*	0.150	0.150	6.010
Q6	*Q4*	0.200	0.100	2.003

Key:
A = Volume of blank matrix
B = Volume of spiked matrix added to blank matrix
C = Concentration of analyte in matrix

2.5. Sample preparation

Blood samples were completely thawed unassisted at room temperature and briefly vortex mixed. *In vivo* mouse blood samples were assayed together with the calibration standards and quality control samples (STDs and QC samples were assayed in duplicates). The analyte and ISTD were isolated using protein precipitation as follows:

Extraction procedure:

1. Aliquot 20 µl of blood sample into a 1.5 ml microfuge tube.
2. Add 80 µl of ice cold 0.1% formic acid in methanol containing a 125 ng/ml proguanil-d4 as an internal standard.
3. Vortex for one minute and ultrasonicate for 5 min, followed by brief vortex mixing.
4. Centrifuge at 13000 rpm for five minutes.
5. Transfer the supernatant to a 96-well plate and inject 5 µl onto the HPLC column.

3 Results

3.1 Calibration curve

The calibration standards and quality control samples were analysed in duplicate with the study samples. A representative calibration curve for proguanil and cycloguanil are presented in figure 5 and 6, respectively. The accuracy and precision statistics of this calibration curve are presented in table 5 and 6 for proguanil and cycloguanil, respectively.

Representative chromatograms are presented in figures 7 (STD 6), 8 (Mouse 1, 1 h post dose sample), 9 (blank) and 10 Zero sample (a blank containing the internal standard) for proguanil; and figures 11 (STD 6), 12 (Mouse 1, 1 h post dose sample), 13 (blank) and 14 Zero sample (a blank containing the internal standard) for cycloguanil.

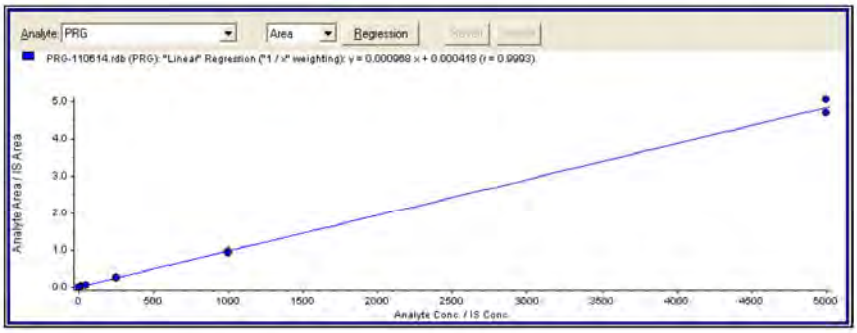


Figure 5: Representative calibration curve of proguanil

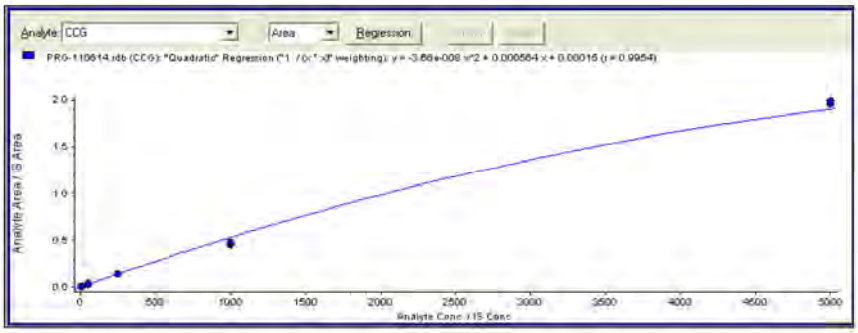


Figure 6: Representative calibration curve of cycloguanil

Table 5: Accuracy and precision data of a representative calibration curve of proguanil

Expected Concentration (ng/ml)	Sample name	Number Of Values Used	Mean	%CV	%Accuracy
2.001	S6	2 of 2	1.898	6.4	94.9
10.01	S5	2 of 2	10.68	11.8	106.7
50.03	S4	2 of 2	50.23	0.1	100.5
250.2	S3	2 of 2	249.5	7.0	99.7
1001	S2	2 of 2	978.6	3.9	97.8
5003	S1	2 of 2	5025	5.1	100.4

Table 6: Accuracy and precision data of a representative calibration curve of cycloguanil

Expected Concentration (ng/ml)	Sample name	Number Of Values Used	Mean	%CV	%Accuracy
2.001	S6	2 of 2	1.978	9.0	98.9
10.01	S5	2 of 2	10.41	5.1	104.0
50.03	S4	2 of 2	54.91	5.6	109.6
250.2	S3	2 of 2	242.3	4.2	96.9
1001	S2	2 of 2	864.5	3.4	86.4
5003	S1	2 of 2	5379	2.6	107.5

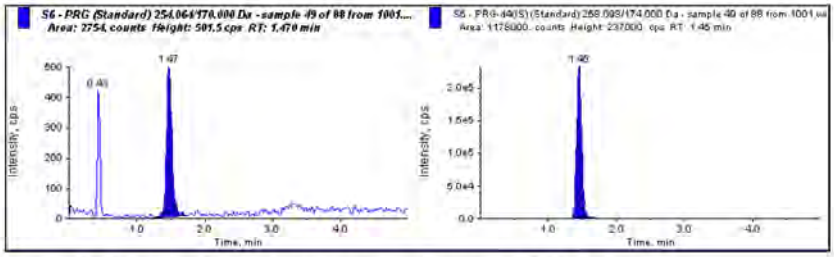


Figure 7: Representative chromatogram of proguanil STD 6 (2 ng/ml)

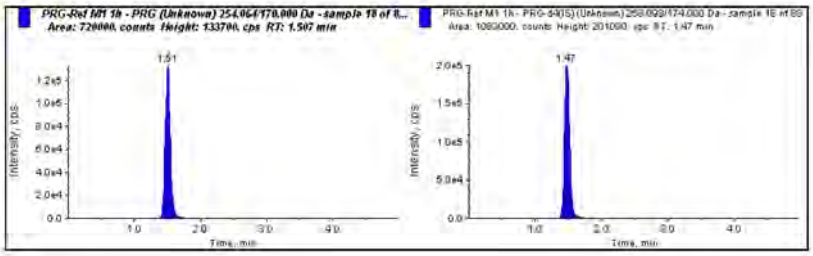
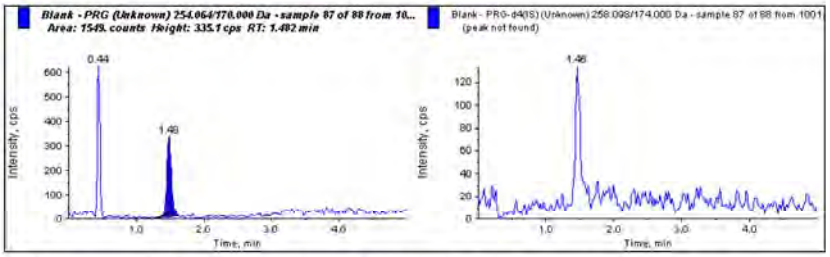


Figure 8: Representative chromatogram of Mouse 1 (1 h post dose proguanil sample)



Note: Carryover of ~ 50 % of the peak area at LLOQ (S6 = 2 ng/ml) and 0.04% of the upper limit of quantification (S1 = 5003 ng/ml) was noted. As the lowest drug blood concentration of the mouse sample obtained at 24 h post dose was ≥ 16 ng/ml, the carryover does not affect the integrity of the result. Thus the LLOQ was raised to the next level (S5 = 10 ng/ml).

Figure 9: Representative chromatogram of proguanil blank sample

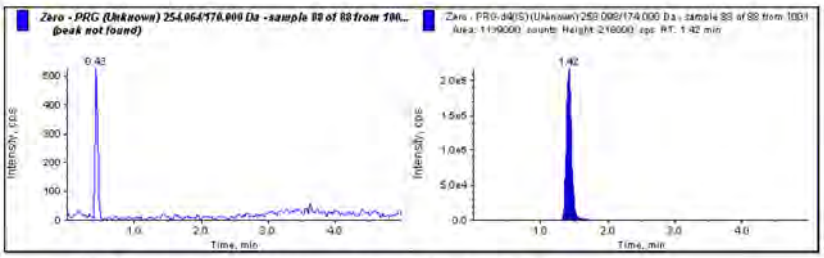


Figure 10: Representative chromatogram of proguanil zero sample

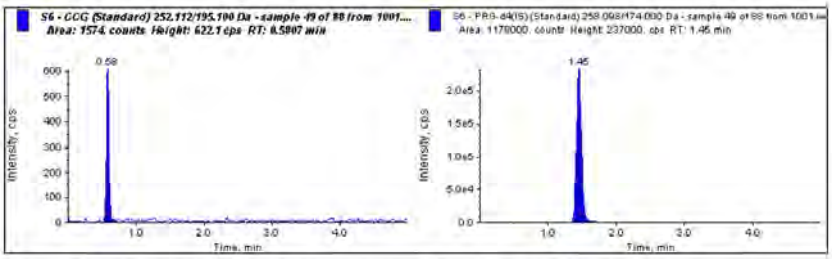


Figure 11: Representative chromatogram of cycloguanil STD 6 (2 ng/ml)

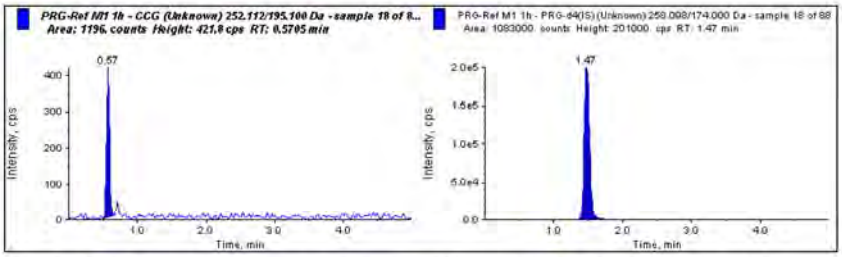


Figure 12: Representative chromatogram of Mouse 1 (1 h post dose sample) for cycloguanil

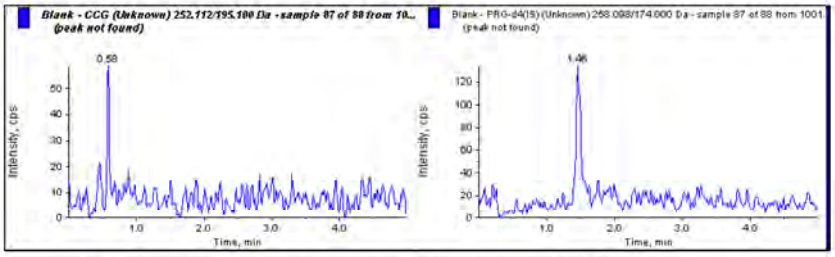


Figure 13: Representative chromatogram of cycloguanil blank sample

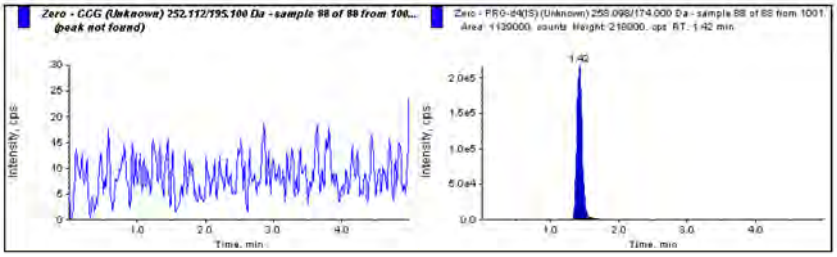


Figure 14: Representative chromatogram of cycloguanil zero sample.

3.2 Oral dose experiment

Both proguanil and proguanil-TMC oral dose samples were analysed in the same batch. The calculated concentrations of proguanil, proguanil-TMC and their metabolite are presented in tables 7, 8, 9 and 10, respectively; while the concentration-time profiles in figure 15 to 18.

Table 7: Calculated blood concentration of proguanil

Proguanil (16 mg/kg oral) 110614																
Time (h)	Conc. (ng/ml)								Conc. (µM)							
	M1	M2	M3	M4	M5	Mean	STDEV	SEM	M1	M2	M3	M4	M5	Mean	STDEV	SEM
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.5	484.0	641.0	599.0	416.0	642.0	556	102	45	1.91	2.53	2.36	1.64	2.53	2.19	0.40	0.18
1	687.0	649.0	495.0	355.0	561.0	549	132	59	2.71	2.56	1.95	1.40	2.21	2.17	0.52	0.23
2	934.0	780.0	870.0	504.0	1030	824	201	90	3.68	3.07	3.43	1.99	4.06	3.25	0.79	0.35
4	665.0	831.0	672.0	690.0	635.0	699	76.6	34	2.62	3.28	2.65	2.72	2.50	2.75	0.30	0.13
8	296.0	375.0	315.0	352.0	213.0	310	62.5	28	1.17	1.48	1.24	1.39	0.84	1.22	0.25	0.11
24	16.20	32.20	25.00	59.60	52.30	37.1	18.3	8.2	0.06	0.13	0.10	0.23	0.21	0.15	0.07	0.03

Table 8: Calculated blood concentration of proguanil-TMC

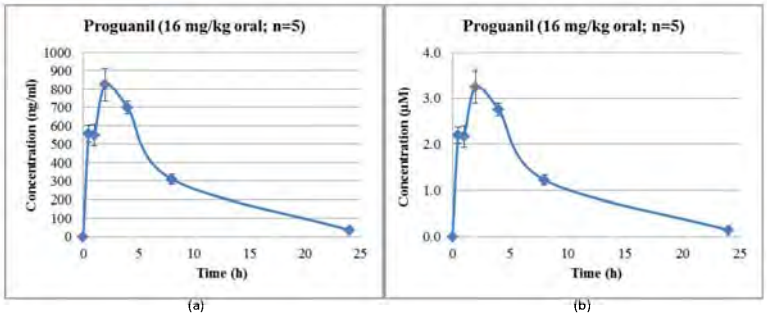
Proguanil-TMC (16 mg/kg oral; equivalent to 8 mg/kg proguanil) 110614																
Time (h)	Conc. (ng/ml)								Conc. (µM)							
	M1	M2	M3	M4	M5	Mean	STDEV	SEM	M1	M2	M3	M4	M5	Mean	STDEV	SEM
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.5	342.0	284.0	80.10	221.0	390.0	263	120	54	1.35	1.12	0.32	0.87	1.54	1.04	0.47	0.21
1	556.0	515.0	327.0	315.0	603.0	463	134	60	2.19	2.03	1.29	1.24	2.38	1.83	0.53	0.23
2	669.0	619.0	493.0	525.0	720.0	605	95.5	43	2.64	2.44	1.94	2.07	2.84	2.39	0.38	0.17
4	410.0	557.0	426.0	407.0	419.0	444	63.7	28	1.62	2.20	1.68	1.60	1.65	1.75	0.25	0.11
8	141.0	248.0	165.0	165.0	166.0	177	41.1	18	0.56	0.98	0.65	0.65	0.65	0.70	0.16	0.07
24	39.40	9.54	8.930	10.20	7.800	15.2	13.6	6.1	0.16	0.04	0.04	0.04	0.03	0.06	0.05	0.02

Table 9: Calculated blood concentration of cycloguanil obtained from 16 mg/kg proguanil oral dose

Cycloguanil (obtained from 16 mg/kg proguanil oral dose) 110614																
Time (h)	Conc. (ng/ml)								Conc. (µM)							
	M1	M2	M3	M4	M5	Mean	STDEV	SEM	M1	M2	M3	M4	M5	Mean	STDEV	SEM
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	7.23	5.02	6.62	2.13	6.56	5.51	2.06	0.92	0.028	0.020	0.026	0.008	0.026	0.022	0.008	0.004
4	11.0	11.6	10.6	8.27	7.83	9.86	1.70	0.76	0.043	0.046	0.042	0.033	0.031	0.039	0.007	0.003
8	8.31	10.9	8.58	8.39	5.16	8.27	2.04	0.91	0.033	0.043	0.034	0.033	0.020	0.033	0.008	0.004
24	3.05	5.95	3.50	4.35	3.24	4.02	1.19	0.53	0.012	0.023	0.014	0.017	0.013	0.016	0.005	0.002

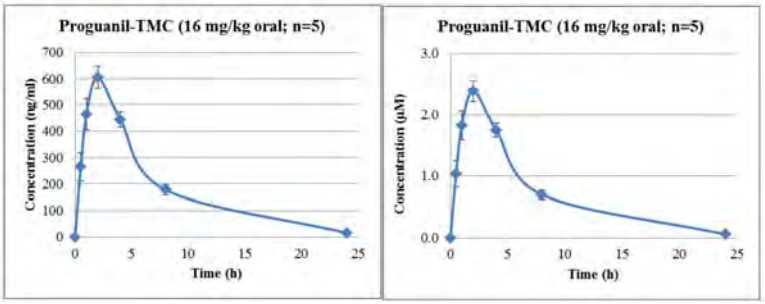
Table 10: Calculated blood concentration of cycloguanil obtained from 16 mg/kg proguanil-TMC oral dose

Cycloguanil (obtained from 16 mg/kg proguanil-TMC oral dose) 110614																
Time (h)	Conc. (ng/ml)								Conc. (µM)							
	M1	M2	M3	M4	M5	Mean	STDEV	SEM	M1	M2	M3	M4	M5	Mean	STDEV	SEM
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	6.07	4.45	4.00	4.66	7.37	5.31	1.39	0.62	0.024	0.018	0.016	0.018	0.029	0.021	0.005	0.002
4	7.70	7.60	7.82	8.62	8.96	8.14	0.61	0.27	0.030	0.030	0.031	0.034	0.035	0.032	0.002	0.001
8	5.64	6.74	6.44	6.30	6.84	6.39	0.47	0.21	0.022	0.027	0.025	0.025	0.027	0.025	0.002	0.001
24	3.47	2.60	2.99	2.31	3.61	3.00	0.55	0.25	0.014	0.010	0.012	0.009	0.014	0.012	0.002	0.001



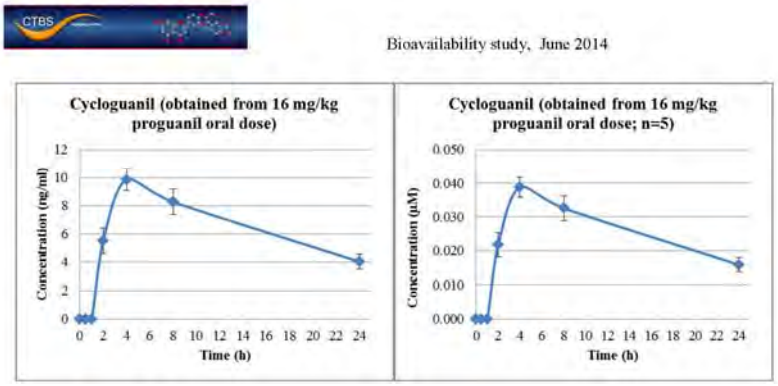
(a) (b)

Figure 15: Concentration-time profile of 16 mg/kg proguanil oral dose: (a) in ng/ml concentration, and (b) in µM concentration.



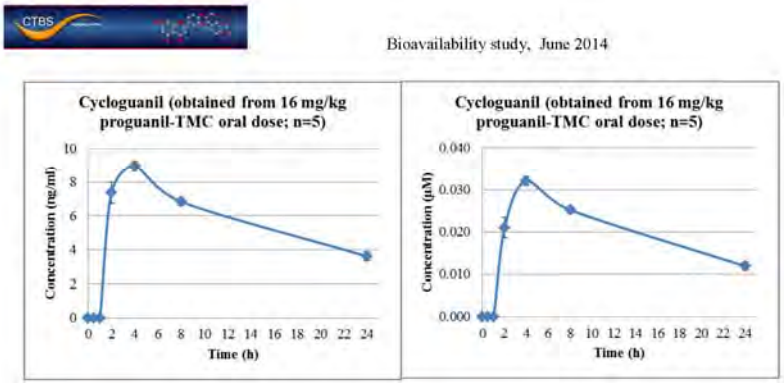
(a) (b)

Figure 16: Concentration-time profile of 16 mg/kg proguanil-TMC oral dose, equivalent to 8 mg/kg proguanil: (a) in ng/ml concentration, and (b) in µM concentration.



(a) (b)

Figure 17: Concentration-time profile of cycloguanil obtained from16 mg/kg proguanil oral dose: (a) in ng/ml concentration, and (b) in µM concentration.



(a) (b)

Figure 18: Concentration-time profile of cycloguanil obtained from 16 mg/kg proguanil-TMC oral dose, equivalent to 8 mg/kg proguanil: (a) in ng/ml concentration, and (b) in µM concentration.

4 Pharmacokinetic properties of proguanil, proguanil-TMC, and their metabolite

The pharmacokinetic properties of proguanil, proguanil-TMC, and their metabolites were evaluated using Summit PK software. The bioavailability of proguanil-TMC (16 mg/kg oral dose; equivalent to 8 mg/kg proguanil) relative to proguanil (16 mg/kg oral dose) was found to be 123% (n=5); whereas the bioavailability of cycloguanil obtained from 16 mg/kg poguanil-TMC oral dose was found to be 156%. The pharmacokinetic properties are presented in table 11 and 12 for proguanil and cycloguanil, respectively.

Table 11: Pharmacokinetic properties of proguanil

PK-Properties of Proguanil		
Parameters	**Oral dose**	
	Proguanil	**Proguanil-TMC**
Nominal Dose (mg/kg)	16	16 (equivalent to 8 mg/kg proguanil)
Apparent $t_{1/2}$ (h)	4.7	4.2
C_{max} (µM)	3.25	2.39
T_{max} (h)	2	2
$AUC_{0-\infty}$ (min. µmol/L)	1819	1117
Bioavailability (%)	N/A	123 (106 - 149)
Results are mean of n=5		

Table 12: Pharmacokinetic properties of cycloguanil

PK-Properties of Cycloguanil (obtained from proguanil oral dose)		
Parameters	**Oral dose**	
	Proguanil	**Proguanil-TMC**
Nominal Dose (mg/kg)	16	16 (equivalent to 8 mg/kg proguanil)
Apparent $t_{1/2}$ (h)	15.2	15.5
C_{max} (µM)	0.039	0.032
T_{max} (h)	2	2
$AUC_{0-\infty}$ (min. µmol/L)	4	4
Bioavailability (%)	N/A	156 (124 - 190)
Results are mean of n=5		

5 Discussion

5.1 Calibration curve

Calibration graph was constructed using a linear regression curve fit with weighting factor 1/x for proguanil, and a quadratic regression curve fit with weighting factor 1/x*x for cycloguanil based on a peak area ratio as the response type. The accuracy and precision of the standards used were within 20%. Good quality calibration curve was constructed with only 20 µl blood sample.

5.2 Oral dose experiment

The drug blood levels even after 24 h were above the lower limit of quantification (2 ng/ml). The TMC nanoparticle formulation of proguanil and proguanil were absorbed fast attaining maximum concentration at 2h post dose. Both proguanil and proguanil-TMC showed a moderate elimination half-life of 2.4 to 3.3 h, while their metabolites showed a long elimination half-life of about 15 h. Mixing proguanil with TMC nanoparticle formulation has enhanced its absorption, and as a result its relative bioavailability too (BA = 123%).

6 Final discussion

A precise and accurate LC-MS/MS method was developed for proguanil and its metabolite, and was used to analyse study samples. The test compound was evaluated for its relative bioavailability after oral administration. The bioavailability of the test compound was enhanced as the result of mixing it with TMC.

Study co-ordinator	Dr Lubbe Wiesner
Study performed by:	Dr Efrem Teclehaymanot Abay
Written by:	Dr Efrem Teclehaymanot Abay