The Development and Implementation of a Protocol for the Characterisation of Poor-quality Antimalarials (COPA)

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Abstract

This thesis describes the development and implementation of a protocol to characterise poor-quality antimalarials (COPA). The research focused on artemisinin-derived combination therapies (ACTs) of co-formulated artemether (ATM) and lumefantrine (LUM). These antimalarials are the most commonly procured ACTs used to combat a malaria infection. Therefore, the protocol to identify poor-quality treatments is highly relevant and applicable.

Chapter 1 provides an introduction into the paradigm of poor-quality antimalarials at the onset of the research project. A systematic literature review is presented[1], which quantitatively assesses relevant field surveys investigating the quality of antimalarials to determine why there is still a lack a consensus on characterising medicines as falsified, substandard, and degraded. While many field surveys did possess the analytical capability, there was a lack in accurate characterisation. The chapter addresses the importance of characterising the outcomes, specifically, how the characterisation can inform effective countermeasures and positive outcomes. The aims and overview of the COPA research are included at the conclusion of the chapter.

Chapter 2 describes the attempted application of liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the detection of ATM, LUM and their potential degradant products, including how in-source fragmentation and in-system precipitation were encountered and dealt with. Guided by the outcomes of the Chapter 1 literature review, the experimental design subsequently focussed on the use of high-performance liquid chromatography (HPLC) with photodiode array (PDA) detection. The results suggest that the validated HPLC method can quantitatively measure the quality of the antimalarial by assessing active pharmaceutical

[1] The systematic review is published. Grech, J., et. al. (2017). "An Empirical Review of Antimalarial Quality Field surveys: The Importance of Characterising Outcomes." Journal of Pharmaceutical and Biomedical Analysis. **147**: pp 612 – 623.

ingredients (API) contents and detect their possible degradation products. The validated method was utilised to assess degradation observed in harsh degradation studies. Furthermore, the method by which to quantitatively assess the degradation products was proposed.

Chapter 3 investigates the degradation of the ATM/LUM co-formulated tablets using laboratory-based stability tests. The currently accepted standard for an accelerated stability test was compared against a novel application of a cyclic stability test. The cyclic stability test recreates the day/night fluctuation of temperature and relative humidity that medicines would be exposed to in an uncontrolled environment in the target country of the field survey, e.g. transport and storage in a non-airconditioned supply chain. These stability tests represent a situation of controlled and monitored conditions. The antimalarials were then analysed using physical measurements and the HPLC-PDA method validated in Chapter 2 for API content and degradation profiles, in addition to disintegration testing. The results suggest that medicines that are exposed to the cyclic fluctuation degrade quicker than when stored in static accelerated conditions even when the average temperature and humidity conditions are comparable. The degradation of the samples in the laboratory resulted in the observation of three degradant products. All samples passed the disintegration testing based on the British Pharmacopeia guidelines.

Chapter 4 presents atypical stability tests, in which antimalarials were stored in a shipping container in Darwin, Northern Territory, and outside in Perth, Western Australia. The two locations represent two different climate zones with different temperature and humidity conditions. These stability tests represent a situation of uncontrolled but monitored conditions. After storage for six months, the antimalarials were then analysed using physical measurements and the HPLC-PDA method validated in Chapter 2 for API content and degradant profiles, in addition to disintegration testing. The results were like those of Chapter 3, in which medicines

experience increased degradation when exposed to real-world cyclic conditions greater than the recommended storage conditions. The samples stored in Darwin experienced higher levels of degradation, with conditions that were similar to the the cyclic conditions of Chapter 3. All samples passed the disintegration testing based on the British Pharmacopeia guidelines.

Chapter 5 describes the implementation of the COPA protocol in a field survey of five administrative districts of Uganda, Africa. The field survey was an overt stratified random sampling method directed at collecting co-formulated ATM/LUM treatments and distributing written surveys regarding pharmacy practices of the visited points of distribution (PODs). The surveys identified that while most PODs do not have an effective form of climate regulation (i.e. an air-conditioning system), the medicines are generally stored for short period before sale (< 3 months). The collected medicines were then analysed using the HPLC-PDA method validated in Chapter 2. The results support the observations of the written survey, as only one location was found to possess a degraded medicine when the degradation profile was assessed. Furthermore, based on the packaging analysis of the collected samples another POD was found to have distributed a falsified medicine. All samples passed the disintegration testing based on the British Pharmacopeia guidelines.

Chapter 6 revisits the analysis technique of LC-MS, utilising an instrument with greater sensitivity that is capable of high-resolution mass spectrometry (HRMS) to confirm the presence of the two API and identify the three degradant products observed. Accurate mass information for the two API, ATM and LUM, was obtained and two of the three degradant products were identified as diketo-aldehyde, an ATM-related degradant, and desbenzylketo, a LUM-related degradant. The identity confirms the use of the two degradant products as indicators for degradation. However, the successful HRMS application still requires further development before HRMS can be replace of photo diode array detection.

Chapter 7 summarises the research output, detailing the successful development and implementation of the COPA protocol. The real-world applicability of novel cyclic stability testing (Chapter 3) is addressed, detailing how the results are reflective of those observed after storing antimalarials in Darwin (Chapter 4). With the proven successes of the COPA protocol in earlier chapters, Chapter 7 addresses the public health implications of the COPA protocol, and how it can be used to inform effective countermeasures, moving forward. Furthermore, the direction of future recommended research is also included. The recommendations include increasing engagement with pharmaceutical companies for field surveys and identifies improved analytical capabilities that would enhance the outcomes of the COPA protocol.

Certificate of Authorship

Except where clearly acknowledged in footnotes, quotations and the bibliography, I certify that I am the sole author of the thesis submitted today entitled –

The Development and Implementation of a Protocol for the Characterisation of Poor quality Antimalarials (COPA).

I further certify that to the best of my knowledge the thesis contains no material previously published or written by another person except where due reference is made in the text of the thesis.

The material in the thesis has not been the basis of an award of any other degree or diploma except where due reference is made in the text of the thesis.

The thesis complies with University requirements for a thesis as set out in the *Examination of Higher Degree by Research Theses Policy.* Refer to http://www.canberra.edu.au/current-students/canberra-students/current-research-students/hdr-policy-and-procedures

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Candidate's Signature Date

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Primary Supervisor's Signature Date

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This thesis is dedicated to the memory of Ms Ainsley Grech, my first support in the pursuit of forensics.

List of Publications

Papers published in refereed journals:

1. **Grech, J.**, Robertson, J., Thomas, J., Cooper, G., Naunton, M. and Kelly, T. (2017). "An Empirical Review of Antimalarial Quality Field surveys: The Importance of Characterising Outcomes." Journal of Pharmaceutical and Biomedical Analysis **147**: pp 612 - 623.
2. Walker, E. J., Peterson, G. M., **Grech, J.**, Paragalli, E. and Thomas, J. (2018). "Are we doing enough to prevent poor-quality antimalarial medicines in the developing world?" BMC Public Health **18**(1): p 630.

Additional publications detailing the research output from Chapter 2 (analytical method development and validation), Chapter 3 (novel Cyclic stability testing), Chapter 4 (atypical stability testing), and Chapter 5 (Ugandan field survey) are under development for publication in the near future. Identified journals are the Journal of Pharmaceutical and Biomedical Analysis, Malaria Journal, BMC Public Health or the Australian Journal of Forensic Sciences.

Poster presentations at international meetings:

1. **Grech, J.**, Robertson, J., Thomas, J., Cooper, G., Naunton, M., Mutambuze, I., and Kelly, T. (2017) “Rethinking the Approach of Medicine Stability Testing.” European Congress on Tropical Medicine and International Health, Antwerp, Belgium.

List of Abbreviations and Symbols

% CV	Coefficient of variance
AC	Accelerated
ACN	Acetonitrile
ACT	Artemisinin-based combination therapy
AM	Antimalarial medicine
AMF-m	Affordable Medicines Facility-malaria
AMO	Amodiaquine dihydrochloride dihydrate
ANOVA	Analysis of variance
API	Active pharmaceutical ingredient
AR	Artrin
ART	Artemisinin
ATM	Artemether
ATS	Artesunate
AUC	Area under the curve
Br. Ph.	British pharmacopeia
CO	Coartem
DBK	Desbenzylketo-lumefantrine
DDA	Dodecylamine
DE	Degraded
DESI-MS	Desorption electrospray ionisation-mass spectrometry
DHA	Dihydroartemisinin
DKA	Diketo-aldehyde
DPR	Degradant peak ratio
FA	Falsified
GMP	Good manufacturing practices
HPLC	High-performance liquid chromatography
HRMS	High-resolution mass spectrometry
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IP	Intellectual property
IS	Internal standard
L-ATM	labelled [^{13}C, D_3]-beta-artemether
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantitation

LOS	Lumefantrine analogue oxalate salt
LSD	Least significant difference
LU	Lumiter
LUM	Lumefantrine
MEDQUARG	Medicine quality assessment reporting guidelines
MKT	Mean kinetic temperature
MRM	Multiple reaction monitoring
NC	No characterisation
NGOs	Non-government organisations
NMRA	National medicine regulatory authority
PCA	Principal component analysis
PDA	Photodiode array
POD	Point of Distribution
PQM	Poor-quality medicine
PYR	Pyrimethamine
QC	Quality control
QCH	High-concentration quality control
QCL	Low-concentration quality control
QCM	Medium-concentration quality control
RH	Relative humidity
RT	Retention time
S/N	Signal-to-noise
SB	Substandard
SRS	Stratified random sampling
SUL	Sulfadoxine
TEA	Triethylamine
TLC	Thin-layer chromatography
USD	US dollar
UV	Ultraviolet
WHPA	World Health Professions Alliance
WS	Within quality standards
WWARN	Worldwide Antimalarial Resistance Network
XIC	Extracted ion chromatogram
XRD	X-ray diffraction

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Chapter 1. Introduction.

1.1. Malaria.

Malaria is one of the world's most dangerous infections, while approximately 2.9 billion US dollars (USD) was spent in 2015 to control and eliminate malaria, an estimated 212 million cases worldwide led to 429,000 deaths (World Health Organisation 2016). The deadly parasitic infection affects 3.2 billion at-risk people in 97 endemic countries, worldwide (World Health Organisation 2014b). For humans, there are four species of the *Plasmodium* genus that cause a malaria infection; *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*, (World Health Organisation 2015). However, it is *P. falciparum* that is the greatest health concern with the largest worldwide distribution and the highest risk of death. The lethality of a *P. falciparum* infection is caused by cerebral occlusions cause blockages in the blood vessels of the brain (Rasti *et al.* 2004). The distribution of malaria caused by all species of the parasite is presented in Figure 1.1.

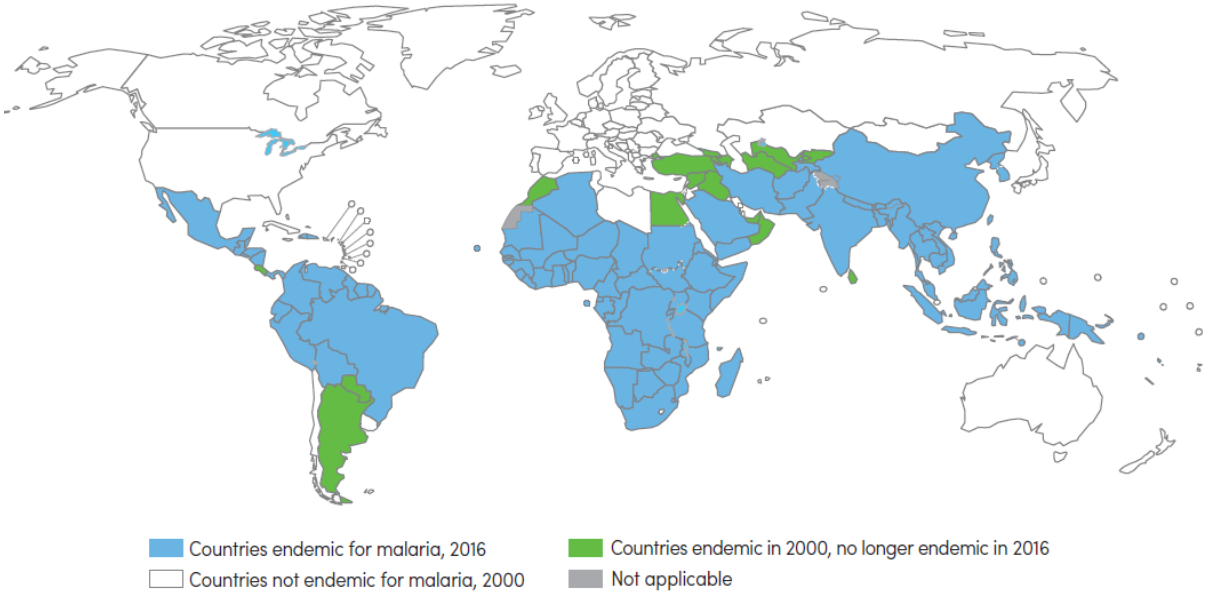

Figure 1.1. The countries endemic for malaria in 2000 and 2016 (World Health Organisation 2016).

It is clear in Figure 1.1 that the distribution of malaria is geographically aligned with poor and vulnerable societies that have limited access to treatment options and healthcare facilities (Sachs and Malaney 2002). Children under the age of five that live in sub-Saharan Africa fall victim to 90 % of the total global number of deaths (The United Nations Children's Fund 2013). In many cases, the only means of survival is treatment with high-quality antimalarial medicine (AM).

1.2. Treatment of Malaria.

The numerous antimalarial chemotherapeutic mono- and combination therapies can be categorised into five groups (Table 1.1): 1) quinoline derivatives, 2) antifolate, 3) artemisinin-derivative, 4) antibiotic and 5) artemisinin-based combination therapy (ACT). The history of antimalarial treatments has seen treatment options change in response to resistance. As a result of decades switching between antimalarial monotherapies due to the development of resistance, the common current recommended treatment is ACTs (World Health Organisation 2010a). Each ACT combines a highly effective, short half-life, artemisinin-derivative with a less effective, longer half-life, antimalarial medicine (Haynes *et al.* 2007). The premise of the combination is that the longer half-life antimalarial will limit the chance of recrudescence through ongoing protection after an effective reduction of the parasite by the artemisinin-derivative.

The number of ACTs distributed worldwide has increased from 98 million in 2009 to 181 million in 2013 (World Health Organisation 2014b). Of the 181 million treatments, approximately 95 % of the treatments were distributed in Africa alone. In determining which antimalarial agents to investigate in this research, all the treatment options included in the five groups listed below in Table 1.1 were considered. The selection incorporated the recommended treatment for malaria in the target areas (World Health Organisation 2010a; World Health Organisation 2014b) and procurement trends of world leaders in antimalarial procurement and

distribution the ACTs. Unfortunately, the medicines used by patients can be ineffective for many individuals; the reasons for which must be identifiable to counter this issue.

Table 1.1. Grouping of Antimalarial Medicine

Group 1: Quinoline Derivative			
i	Chloroquine	vi	Halofantrine
ii	Amodiaquine	vii	Lumefantrine
iii	Quinine	viii	Piperaquine
iv	Mefloquine	ix	Quinidine
v	Primaquine		
Group 2: Antifolate			
i	Sulfadoxine-pyrimethamine	v	Sulfamethoxypyrazine
ii	Proguanil	vi	Pyrimethamine
iii	Sulfalene + Pyrimethamine	vii	Sulfadoxine
iv	Atovaquone-proguanil		
Group 3: Artemisinin Derivative			
i	Artesunate	iv	Artemesinin
ii	Artemether	v	Arteether
iii	Dihydroartemisinin	vi	Artemotil
Group 4: Antibiotic			
i	Amoxicillin	iii	Tetracycline
ii	Doxycycline	iv	Clindamycin
Group 5: Artemisinin-based Combination Therapy (ACT)			
i	Artemether + Lumefantrine	iv	Artesunate + Sulfadoxine-pyrimethamine
ii	Artesunate + Amodiaquine	v	Artesunate + Pyronaridine
iii	Artesunate + Mefloquine	vi	Dihydroartemisinin + Piperaquine

1.3. What are Poor-quality Medicines?

Poor-quality medicines (PQMs) have been identified as a major health problem for over a quarter of a century (Sesay 1988). Formally addressing PQMs began in 1992 when the WHO agreed upon an official definition of a counterfeit medicine and implemented the definition two years later (World Health Organisation 1992; World Health Organisation 1999). The WHO definition has since changed to include substandard, spurious, falsely-labelled, falsified, counterfeit medicine categories until recent progress to simplify the definition to substandard and falsified (World Health Organisation 2011b; World Health Organisation 2017). Despite the progress to define PQMs, there generally remains no clear agreement on the definition describing each category (Almuzaini *et al.* 2013). The outcome of attempting to place a structured definition on a situation affected by many factors has led to disagreement and opposition. This difference is partially attributed to the conflicting interests of pharmaceutical companies and public health organisations (Attaran *et al.* 2012). The lack of clear distinctions between categories of PQMs has led to confusion, with companies protecting their patents from generic versions using the definition meant to protect against criminally counterfeited reproductions and distracting from countering other types of PQMs (Caudron *et al.* 2008; Mullard 2010). This limitation led to some countries' regulatory authorities interpreting model definitions to view counterfeit products as an intellectual property (IP) issue, resulting in an adverse effect on the international trade of quality generic medicine (Brant and Malpani 2011; Newton *et al.* 2011b). Disagreement over the exact definitions of PQMs and the requirements that limit each category has also caused confusion over the terminology and led to improper reporting in medicine quality field surveys (Shakoor *et al.* 1997).

Affirmative action is required to accurately characterise the results of medicine quality surveys to provide an informed course of action to combat the diverse problem of PQMs. Characterising poor-quality AMs will help initiate effective countermeasures through

cooperative action, providing similarly structured conclusions of field surveys, and steering future direction.

Figure 1.2 presents five categories of medicine quality including those within quality standards (legitimate) and of poor quality (Schweitzer 2007; Bate 2012). Two forms of legitimate medicines are innovator and generic medicines. While there are many differences between the two, e.g. price, source, and licenses required, both are manufactured in accordance with current good manufacturing practices (GMP) and are pharmaceutically equivalent (Schweitzer 2007). Generic products may be dispensed as an alternate to innovator (branded) products if the products are registered with the appropriate national medicine regulatory authority (NMRA) and do not impede on IP restrictions (Brant and Malpani 2011).

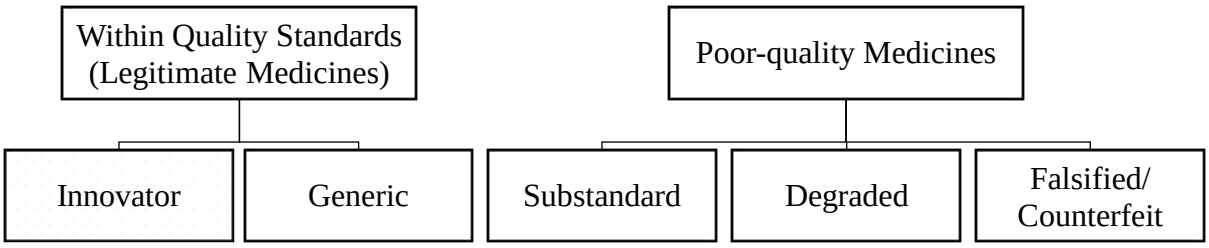

Figure 1.2. A flowchart presenting accepted categories used to describe medicine within quality standards (legitimate) (Schweitzer 2007) and of poor quality (Bate 2012).

Throughout the literature, four terms are widely used to describe PQMs; these are substandard, degraded, falsified, and counterfeit. In 2010, WHO redefined the substandard medicines as- "pharmaceutical products that do not meet their quality standards and specifications" (World Health Organisation 2010b). Substandard products that lack adequate active pharmaceutical ingredients (API) can have major, and potentially disastrous, implications including, but not exclusive to, the development of disease resistance, impacting the entire population (Taylor *et al.* 1995; Bloland 2001; Dondorp *et al.* 2010). Substandard products are not only limited to those with API content outside the accepted ranges. They also include those which contain adequate API content but without adequate bioavailability (Minzi

et al. 2003). Substandard products can be a result of deliberate cost-cutting mechanisms or through inadvertent poor manufacturing procedures (Wolinsky 1983; Shakoor *et al.* 1997; Tipke *et al.* 2008). Differentiating intent has proven difficult.

Degraded medicines refer to medicines sourced from a legitimate manufacturer, made with GMP, and within NMRA standards, which at a point throughout supply-chain or storage at the point of distribution (POD) degrade to outside NMRA quality standards (Newton *et al.* 2011b). An acceptable level of degradation is expected, hence the use of expiry dates and shelf-life indications as a demonstration of medicine quality (Capen *et al.* 2012). Therefore, all medicines that have passed their expiry date are considered degraded (Bate 2012). However, exposure to extreme environmental factors, such as temperature, relative humidity or direct exposure to sunlight/UV can lead to premature degradation before the stated expiry date (Amin and Kokwaro 2007). Medicines that have passed their expiry date and have been repackaged to present a later expiry date may be considered both degraded and falsified/counterfeit as the product presents with a fraudulent label. Fraudulent labelling of medicine products was the essence of the original WHO definition of counterfeit in 1992, declaring a counterfeit medicine to be "one which is deliberately and fraudulently mislabelled with respect to identity and/or source" (World Health Organisation 1992). The definition applies to "branded and generic products and counterfeit products may include products with correct ingredients, wrong ingredients, without active ingredients, with the incorrect quantity of active ingredient or with fake packaging" (World Health Organisation 1992). Initially intended to recognise counterfeiting as a criminal activity, with associated health implications, the second umbrella-statement has subsequently lead to various interpretations (Wondemagegnehu 1999). Associating the term 'counterfeit' with IP violations has detracted from its application towards public health, and as such, the term 'falsified' has been suggested as a replacement when concerning public health matters (Yong *et al.* 2015).

A consequence of associating counterfeit medicine with other counterfeit products (e.g. commercial products such as DVDs or clothing) is the implication that the victims are the manufacturing companies and not the patients (Newton *et al.* 2011b). Until patients are adequately addressed as the primary victims of counterfeit medicines the term falsified should be used to avoid confusion. Falsified medicines pose a significant danger to the consumer as the contents may be uncontrolled, unregulated in dose or content, and of unknown origin or adverse reactions (Wertheimer and Santella 2005).

The European Union's Falsified Medicines Directive is a noteworthy program that clearly states the discrepancies between counterfeit, falsified and substandard medicine products to ensure that there are no implications on IP-related infringements (Council of the European Union 2011). This standpoint has allowed the European Union to maintain steady progress addressing criminally-derived falsified medicines. Unfortunately, with the current standpoint of the WHO, ambiguous blanket terms have seemingly inhibited productive intelligence gathering and effective countermeasures. As a consequence, at least 19 shipments of generic medicines have been seized under the guise of 'counterfeit' products in transit through the European Union (Brant and Malpani 2011).

In the current literature, two leading perspectives in poor-quality medicines have presented alternate views on the characterisation of PQMs (Figure 1.3, (Newton *et al.* 2011b; Bate 2012)). View A is akin to the European Union directive; there is a distinction between falsified and counterfeit medicines reserving the term 'counterfeit' for trademark violations (Council of the European Union 2011; Bate 2012). View A also indicates that both falsified and counterfeit medicine products fall within the 'grey area' of substandard, addressing the issue of intent when classifying medicines as substandard. View B presents a view similar to the definitions of the WHO with counterfeit (alternatively referred to as spurious, falsely-labelled, falsified and counterfeit) products as an independent entity, with a minimal

crossover with substandard and degraded (Newton *et al.* 2011b). This view is beneficial regarding its simplicity but may lack the ability to transition into pragmatic countermeasures. Either view is acceptable if the definitions and their inclusion criteria are stipulated.

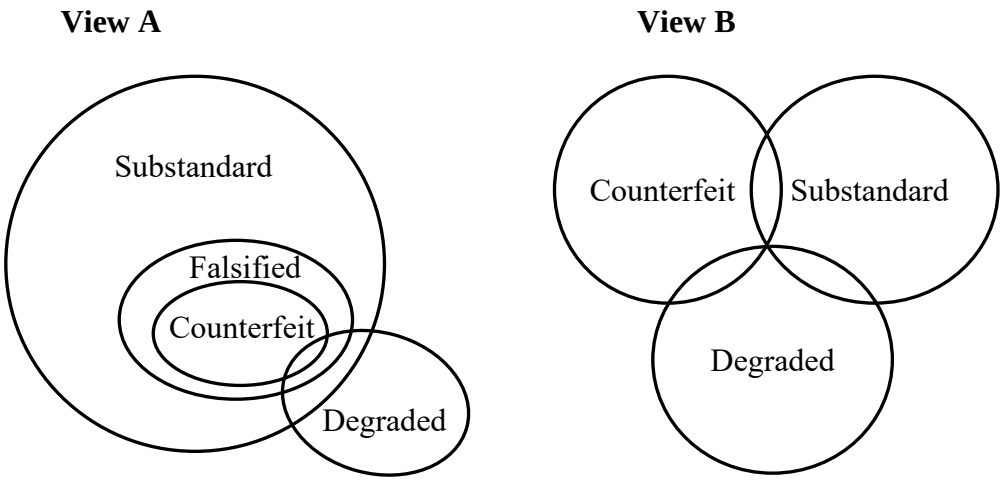

Figure 1.3. Two leading perspectives on PQMs. (View A (Bate 2012) and View B (Newton et al. 2011b)).

A systematic review of the Worldwide Antimalarial Resistance Network (WWARN) reported that only 32.3 % of the medicine quality reports explicitly described their applied definitions of medicine quality (Tabernero *et al.* 2014). A subsequent effect of excluding the applied definitions is that it may limit the understanding and applicability of the reports' conclusions. It is important to state the definitions as it develops the foundation to correctly characterise the conclusions, each of which has individual countermeasures (Attaran *et al.* 2012).

1.4. Consequences of Poor-quality Medicines.

The consequences of PQMs are severe and multi-faceted. Foremost, patients who receive ineffective treatment are vulnerable to a range of adverse effects. Falsified medicines may contain allergens (Sengaloundeth *et al.* 2009), or hazardous, incorrect ingredients (Kelesidis *et al.* 2007). Substandard medicines may contain too much or too little API leading

to adverse reactions in medicines with low therapeutic ranges (e.g. quinine) or provide no cure at all (Newton *et al.* 2006). Degraded medicines may produce potentially toxic degradation products, which risks further poisoning the patient (Verbeken *et al.* 2011). For example, degraded tetracycline, an antibiotic used to prevent malaria, has been known to cause kidney dysfunction (i.e. Fanconi syndrome) since the 1960s (Frimpter *et al.* 1963).

Ineffective treatments not only affect the patient but also have considerable effects on the community. Consumer confidence and perceptions of medicine quality are based on observed responses to treatment and therefore can influence the chances of others seeking treatment when infected (McCombie 1996; Wertheimer and Norris 2009; Björkman-Nyqvist *et al.* 2013). Loss of confidence in the genuine product can place health care professionals or health care services at risk if it is seen that treatment is unsuccessful (Alghannam *et al.* 2014).

Moreover, PQMs have been shown to promote the spread of antimalarial-resistant strains through underdosing the patient, due to low API content or limited bioavailability (Maponga and Ondari 2003; Tipke *et al.* 2008; Karunamoorthi 2014). Typically, the correct dose kills both sensitive and resistant parasites. However, low doses only kill the sensitive parasites, allowing the survival of the 'fitter' resistant strains leading to their uninhibited replication and spread between hosts (White *et al.* 2009). The process of resistance development is termed 'selection pressure' (Amin and Kokwaro 2007; Wertheimer and Norris 2009). It occurs as the treatment selectively kills particularly susceptible parasites, while not effecting resistant parasites, allowing uninhibited growth (White *et al.* 2009). This has been found to be increasingly apparent in drugs with a prolonged half-life, such as sulfadoxine-pyrimethamine (SUL-PYR) (Amin and Kokwaro 2007). Another apparent trend demonstrates that resistance tends to develop in areas of low transmission, such as South-East Asia, before transmission to areas of high transmission, e.g. Sub-Saharan Africa (White *et al.* 2009).

The probability of resistance to develop has been found to be very small, even in the occurrence of treatment failure. White *et al.* (2009) explored the processes which need to occur for resistant parasites to thrive inside the host before transmission through the population. The development of resistance is only significant at a population level if it is transmitted to other people through vector re-uptake of a large number of resistant parasites. However, resistance should not be considered as binary, a resistant or non-resistant parasite, but as a continuous degree which develops from a 'low-grade' to a 'high-grade' resistance level. High-grade resistance is detailed as the survival of a standard, therapeutic dose of the treatment and typically results from multiple mutations transitioning from low-grade resistance (White *et al.* 2009). Subtherapeutic doses allow the survival of low-grade resistant parasites which may have been eradicated through a standard dose. The progressive transition to high-grade resistance is what has rendered antimalarials such as chloroquine, SUL-PYR, mefloquine, and artemisinin (ART) ineffective as monotherapies.

There is a significant monetary repercussion of the development of resistance as relatively cheap drugs will become ineffective and require newer drugs to take their place (Wertheimer and Norris 2009). This situation became apparent as a Kenyan retail audit in 2002 demonstrated that when compared with the previously effective treatment, chloroquine, newer treatments such as artemisinin, mefloquine, and halofantrine were 16, 17, and 22 times more expensive, respectively (Amin and Snow 2005).

The financial consequences of consuming PQMs are two-fold; the patient is at a loss after purchasing an ineffective treatment, and following the development of resistance, patients are forced to buy the newer, more expensive treatments (Kelesidis and Falagas 2015). For example, in 2006, the comparative price between the then first-line treatment, SUL-PYR, and the new ACT treatments were tenfold (White *et al.* 2011). The series of consequences all stem

from the provision of PQMs with each point of the cycle leading to increasingly significant follow-on effects. Unless active countermeasures are taken, the cycle will likely keep repeating.

1.5. Countermeasures to Poor-quality Medicines.

The basis for why patients seek a particular treatment for malaria include price, availability, and efficacy (McCombie 1996). Therefore, if high-quality medicines are supplied at affordable prices, patients may find less reason to purchase cheaper, lower quality alternates (World Health Organisation 1999; Bate *et al.* 2011). Since 90 % of patients in developing countries pay for treatment themselves, it is important to keep prices affordable (Brant and Malpani 2011). In May 2001, the manufacturer of the AM, Coartem® (combination treatment of artemether and lumefantrine), Novartis, affected a memorandum of understanding with the WHO for the large-scale provision of Coartem® at cost price to the governments of malaria-endemic developing countries (World Health Organisation 2011a). This ten-year agreement saw the provision of 400 million treatments and a price reduction of up to 60 %. These types of agreements are vital as a hypothesis for the demand of inferior medicines is that the consumers will opt for affordable medicine options, even at the sacrifice of quality (Bate *et al.* 2011). Similarly, scarcity or erratic supply of essential medicines has been correlated to the facilitation of falsified medicines (World Health Organisation 1992). However, when PQMs are present, the effective countermeasures for each category are different.

Falsified medicines target the genuine medicine supply chains to infiltrate legitimate markets. Therefore effective countermeasures are based on strengthening the supply chain through traceable authentic medicines (Agbaraji *et al.* 2012). The push for countermeasures is supported through technological advancement. A simple technology that has seen relative success recently is an SMS-based verification process, for example, the Sproxil-Defender or m-Pedigree system (Agbaraji *et al.* 2012). Both systems work on the premise of a ‘scratch-off’ feature that reveals a unique code that can be verified via an SMS by the end-point consumer

with the pharmaceutical manufacturer (Spink *et al.* 2016). The FDA has supported the implementation of two technologies to counter falsified medicines; radio-frequency identification tags and CD-3 (Mackey and Liang 2011; Batson *et al.* 2016).

The countermeasures for falsified medicines are derived from the criminalisation of the practice, that can be supported through actions including, but not limited to; strengthening policing and customs, investigating cross-border crimes, seizing proceeds of crime, and prosecuting offenders (Attaran *et al.* 2012). These actions are directed towards the criminal syndicates and do not match the requirements to stop substandard or degraded medicines, and punishments associated with PQMs are relatively lenient and highly variable between countries. Progressive legislation addressing medicine quality has been noted in Nigeria, which now has 12 laws directly relating to drugs, poisons, food control, product registration, and adulteration (Spink *et al.* 2016).

Alternately, practices such as the effective procurement strategies detailed above can ensure that quality medicines establish and maintain the largest market share (Gostin and Buckley 2013). When improper procurement strategies are employed, manufacturers can be pressured to produce medicines at a lower cost. In an attempt to maintain low prices while still preserving profits, the manufacturer may sacrifice quality leading to substandard products (Wolinsky 1983). However, substandard products do not always derive from criminal intent. Substandard medicines can be remedied through the actions of NMRA and quality control services (Newton *et al.* 2011b), through actions such as forming manufacturing and quality controls and strengthening logistics and stock management processes (Attaran *et al.* 2012).

Since degraded medicines result from the breakdown of quality medicines, supply-chain management is particularly important as medicines are at risk of premature degradation not only during transport but also during their storage at the POD (Hogerzeil and De Goeje 1991; Hall *et al.* 2016). In Brazil, medicine storerooms have been assessed for the presence of

preventative measure to stop degradation, such as; air-conditioning, protection from sunlight, and periodic temperature and humidity records (Nogueira *et al.* 2011). Alternately, the SMS-based programme that previously tested for stock-outs can also be employed for replacing expired stock, but first, the process needs to be developed (Barrington *et al.* 2010).

1.6. Review of Previous Medicine Quality Field Surveys.

The systematic literature review presented below delivered key insights into the paradigm of medicine quality field surveys that helped direct the scope of the Characterisation of Poor-quality Antimalarials (COPA) research. Since publishing the review in May 2017 (Grech *et al.* 2017), the further release of field survey data has been monitored and collected. Therefore, the information stated below will describe the processes that generated the publication and an update of the data released since.

1.6.1. Data collection process.

Screening of article abstracts was completed using the following inclusion criteria: a) related to antimalarial medication, b) was a primary source, c) published in a peer-reviewed journal, d) described *in-vivo* or *in-vitro* testing, e) was not repeated testing, and f) outlined pharmacopoeia/quality requirements.

A systematic review of scientific reports of the quality of AM was undertaken, searching the databases of SCOPUS, SciFinder, ScienceDirect, Web of Knowledge, PubMed, and EMBASE. There was no restriction placed on the earliest search date and journals were included up to the search date, June 17th, 2016.

All searches were performed in combination with the term “antimalarial”, either using multiple search panels or combined in the same search field using “AND”. The search included the additional terms; “counterfeit”, “substandard”, “falsified”, OR “poor-quality” AND “medicine” OR “drug”, to include all categories of PQMs. Only articles written in English were included.

Further relevant studies were identified through searches of reference lists of selected studies and previously published review articles (n = 47). Figure 1.4 illustrates the search strategy and the flow chart for the data selection process used in this systematic review. From this list of abstracts, full-text versions were sourced for 142 articles deemed eligible for further investigation. Full-text articles were excluded from quantitative synthesis based upon; non-reporting the country of origin for each sample, non-reporting the exact number of samples analysed, the sampling method was biased towards finding either authentic or PQM, or the pharmacopoeia requirements for adequate quality were not reported.

From January 2017 to November 2018, newly released field survey information has been collected through monitoring, the Google Scholar alerts for the search terms, " "poor quality" antimalarials" and " "degraded" antimalarials". The journal articles identified through monitoring the alerts were scrutinised with the same data inclusion and exclusion criteria as described above. Through this method, seven new field surveys were identified and included in the updated assessment.

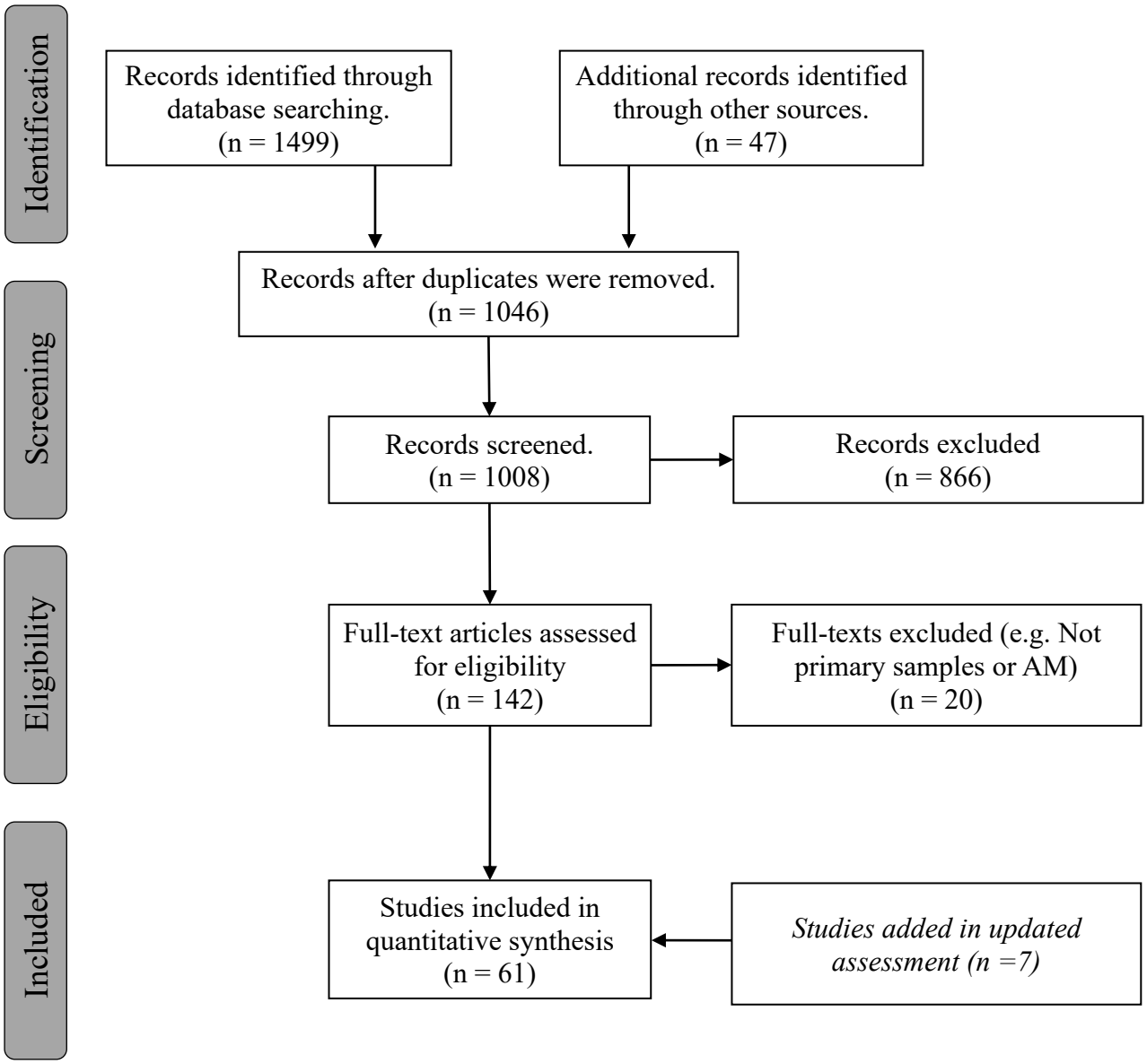

Figure 1.4. Flowchart of the search strategy and data selection process for original systematic review and updated assessment.

1.6.2. Categories of Quantitative Data.

For the final quantitative synthesis, all of the selected full-text articles were examined for many important criteria: the country where the samples were purchased, whether the region is rural or urban, types of AM, analytical techniques applied, sampling techniques, sampling date/range, the total number of samples collected, the number of samples which failed analysis, and analysis of the packaging. For criteria that were not required for quantitative syntheses, such as rural/urban region or date of sampling, 'not specified' was an acceptable result.

1.6.2.1. *Risk of bias.*

Sampling and sub-sampling technique/s and repetition of samples from previous work in addition to including new samples (e.g. longitudinal surveys) have been identified as potential confounding factors, which can bias the survey results. The potential bias associated with the sampling technique is multi-faceted. Executing a convenience method of sampling can result in bias, as the researchers do not follow a regulated process when deciding the sites from which to sample. As no complete list of all sampling sites in the area is required to undertake this sampling method, it is a regular method applied in assessing AM quality, though it is not representative of the area as a whole. This convenience sampling bias can be extended when the researchers sought out places known to possess poor-quality AM. This sampling can occur due to word-of-mouth indications or through the previous testing in the area. Therefore, it is important to note that for the majority of the literature gathered; the results only represent the dispensaries visited and does not represent the entire population.

The heterogeneity of the included studies restricts potential data synthesis. The significant variance in sample collection methods and sample size means that it is difficult to interpret the accumulated data. It is important to understand that due to the lack of statistical significance based on sample collection, the cumulative data are only representative of the samples collected and not of the population as a whole.

1.6.3. Antimalarial Medicine Quality Survey Results.

The results presented below have been updated and reflect the field surveys published in the period before 2017 and afterwards. The benefit of presenting the data in this manner is that insight can be gained into the recent surge in public statements and publications calling on involvement into improved field survey practices, such as Bassat *et al.* (2016) and Walker *et al.* (2018). Summary information of the assessed field surveys can be found in Appendix 1.A.

To simplify the analysis of results, AMs were classified into five groups; 1) quinolone derivatives, 2) antifolates, 3) artemisinin-based monotherapies, 4) antibiotics and 5) ACTs. Details of the AMs in each group can be found in Table 1.1. A summary table of the collated data from the 68 field surveys is available in Appendix 1.A. The summary table presents key information of the field survey and comments on whether the field survey can characterise the results, based on the analytical methods.

Overall, 1865 of the 10198 samples tested (18 %) were reported to be of poor quality. The group with the highest percentage of failed AMs were antifolates (27 %), followed by artemisinin-based monotherapies (24 %), quinolone derivatives (21 %), ACTs (17 %), and antibiotics (8 %). Figure 1.5 indicates that after a dramatic percent increase in failed AMs surveys in 2001 – 2005 across the five groups, there has been a steady decline in the % reported failed samples over time.

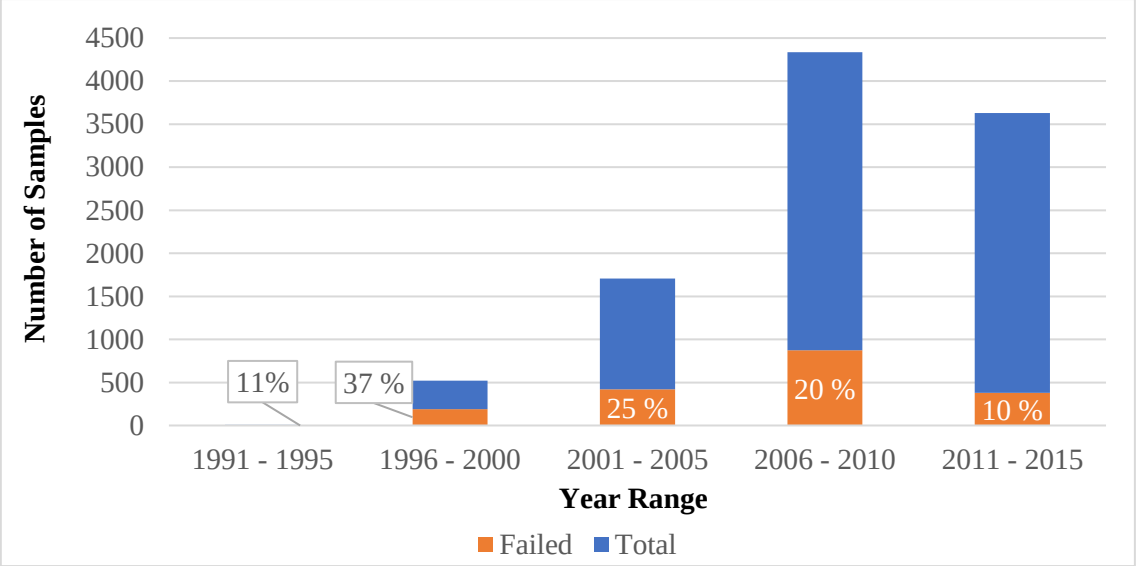

Figure 1.5. The number of AM samples (total analysed and failed) across the five groups as part of the included field surveys from 1991 - 2015.

However, the percentage of failed ACTs has increased from 2006 – 2010 (15 %) to 2011 – 2016 (21 %). This trend may be due to ACTs being the recommended first-line treatment for a *P. falciparum* malaria infection in many malaria-endemic countries (World Health Organisation 2010a; World Health Organisation 2014b).

The seven updated field surveys collected 2377 samples, from which 7 % (n = 167) were found to be of poor quality, continuing the decreasing trend in the percentage of failed samples observed since 2006 – 2010. For these seven field surveys, the group with the highest percentage of failed samples was antibiotics (11 %), and the group with the lowest percent was antifolates (5 %). ACTs saw a significant reduction with 7 % of the tested samples failing analysis, down from 21 % in the recent period.

1.6.4. Critique of Survey Features.

1.6.4.1. *Sample Size.*

The number of samples that were investigated varied greatly between surveys, ranging from a single sample (Gaudiano *et al.* 2006; Keoluangkhot *et al.* 2008) to 1633 included samples (Pribluda *et al.* 2012). Figure 1.6 demonstrates the varying sample sizes of the

reviewed surveys; the majority analysed 50 samples or less (56 %). Data have been updated since publication (Grech *et al.* 2017) to include seven newly reported field surveys post-2017. The distribution of sample sizes did not deviate greatly in the updated surveys. With samples sizes ranging from 10 to 1,444 samples tested in these 7 additional field surveys (Figure 1.6).

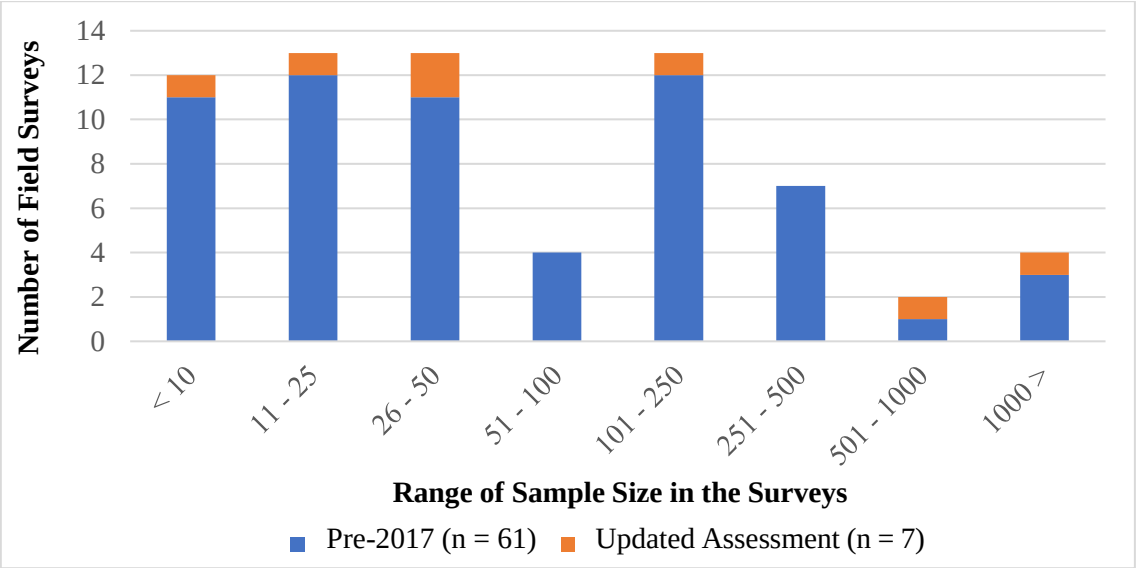

Figure 1.6. Distribution of sample sizes across the included field surveys.

Low sample size can lead to an overestimation of the effect of the survey and misrepresent the population being investigated (Button *et al.* 2013). It is important to address low sample size as a limitation of field surveys because the misreporting of the findings of a survey may be detrimental to addressing the key issue (World Health Organisation 2003; World Health Organisation 2006a).

1.6.4.2. *Sampling Method.*

Field surveys were assessed to determine the mode by which the samples were collected. Samples collected through police or customs seizures (Yong *et al.* 2015) are referred to as seizures. Convenience sampling may occur when the samples or sampling sites are selected based on convenience, e.g. ease of access. Directed sampling methods possessed a specific reason for why the sampling site was chosen. For example, selecting medicines based

on usage and price (Shakoor *et al.* 1997) or based on the results of prior survey responses (Klein *et al.* 2012). A large number of field surveys did not specify the applied sampling method. Many field surveys listed the location (e.g. district) that the field surveys sampled but did not specify how those sites were chosen. In the event the sampling method is not specified, one could assume that samples were collected via a convenience method, or a similar, non-representative sampling method.

Figure 1.7 summarises the mode of sampling method employed in the field surveys. While 13 field surveys (21 %) did not specify the sample collection method; convenience and directed sampling methods were described a total of nine (15 %) and ten (16 %) times, respectively. An additional 12 (20 %) and 16 (26 %) field surveys utilised simple random and stratified sampling methods, respectively, either in isolation or combination with other sampling methods (i.e. directed or convenience). The popularity of convenience sampling is expected given the low-cost of sampling, and it does not require stringent allocation of sampling sites (Newton *et al.* 2009). The field surveys that utilised seizures, convenience, and directed sampling methods, or did not specify the method are examples of non-probability sampling and lack the statistical power of the other methods, such as simple random and stratified (Stehman 1999).

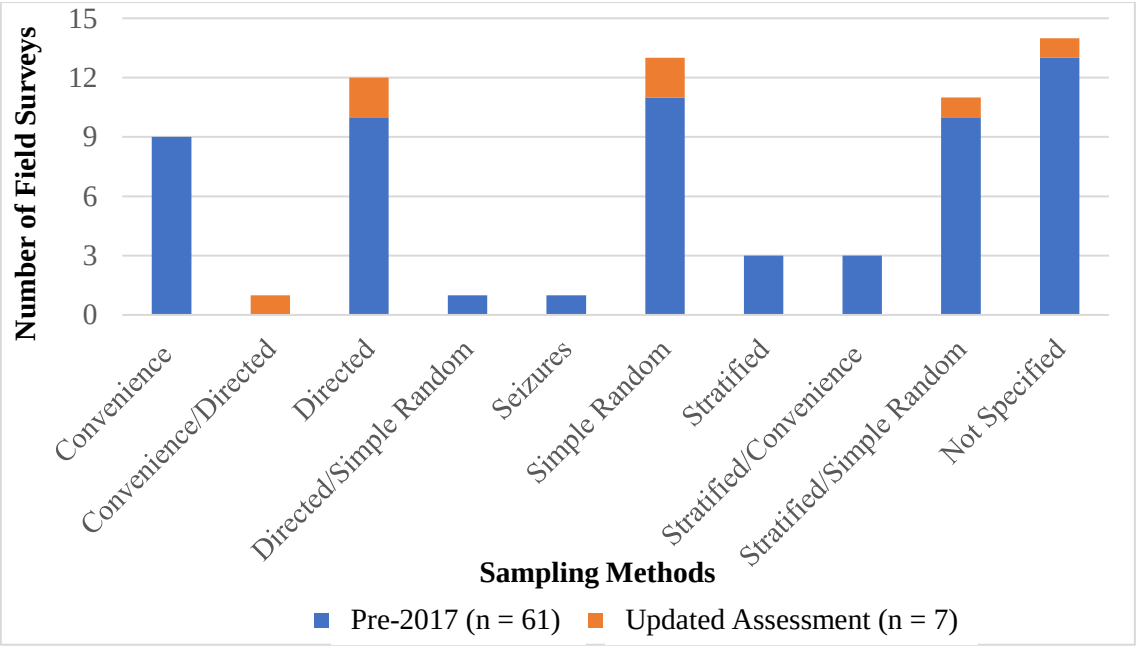

Figure 1.7. Distribution of sampling methods across the included field surveys. Updated to include newly reported field survey results.

In Figure 1.7, random sampling has been used individually and paired with stratified sampling. Random sampling allows the results to be representative of the population and stratification of an area allows for the reduction of sites that are required to be visited. Stratification can be based upon geographical administrative districts or other variables (e.g. climate zones) depending on the potential outcomes of the field surveys (Sengaloundeth *et al.* 2009). The potential negatives aspects of random sampling are the requirement for a complete list of possible sampling sites and the usually higher associated costs (Newton *et al.* 2009).

A positive identified in the seven new field surveys is that only a single article did not include the sampling method that was used in selecting the sampling sites. Moreover, three (43 %) articles stated using a random sampling method.

1.6.5. Geographical Regions.

Reported countries were grouped into one of 22 geographical regions of the United Nations Statistics Division (2013). The included field surveys were conducted in 38 countries in 12 geographic areas, with five surveys reporting on multiple regions. The three

predominantly reported geographical regions were East Africa (20 %), West Africa (28 %), and South-East Asia (18 %).

The United Nations geographical regions were also applied to the reported number of confirmed cases of malaria by the WHO (Global Health Observatory 2013). Since the year 2000, 14 of the 22 geographical regions have reported confirmed cases of malaria. However, these regions do not match those that were investigated for PQMs. Table 1.2 compares relevant components of the two data sets; demonstrating that not all regions with confirmed cases have been adequately investigated and conversely, not all investigated regions contain confirmed malaria cases.

Table 1.2. The United Nations Geographical regions that have been studied in all the included field surveys or have had WHO confirmed malaria cases.

UN Statistics Division I.D. Number	Region	No. of Surveys (%)	WHO Confirmed Malaria Cases
1	Eastern Africa	18 (20.2)	+
2	Middle Africa	13 (14.6)	+
3	Northern Africa	1 (1.1)	+
4	Southern Africa		+
5	Western Africa	25 (27.8)	+
6	Caribbean		+
7	Central America		+
8	South America	8 (8.9)	+
10	Central Asia		+
11	Eastern Asia		+
12	Southern Asia	3 (3.3)	+
13	South-East Asia	16 (17.8)	+
14	Western Asia		+
15	Eastern Europe	1 (1.1)	
16	Northern Europe	2 (2.2)	
20	Melanesia	3 (3.3)	+

The two countries investigated with no WHO confirmed malaria cases were Estonia and the Russian Federation. Further investigation through the WHO Regional Office for Europe website revealed that their centralised information system for the infectious disease had

reported 456 new cases of malaria between the year 2000 – 2013 (World Health Organisation 2014a). However, none were reported in Estonia in that period.

The comparison between the WHO confirmed malaria cases data set and the list of investigated regions was to assess whether the two corresponded; do a greater number of cases generate greater interest and vice versa? This correlation appears true as East and West Africa are the two most investigated regions, which coincides with the two regions with the largest number of confirmed malaria cases at approximately 35 % and 21 %, respectively (World Health Organisation 2012). However, South-East Asia is ranked fifth in confirmed malaria cases while being third with respect to the number of investigations. The emergence of disease resistance in the Thai-Cambodian border area of South-East Asia may provide a reason for increased interest in the region (Fairhurst *et al.* 2012).

Another commonly reported geographical discriminator in field surveys is the description of the sampling region as rural or urban. Field surveys included in the review were assessed as to whether the sampled regions were rural, urban, both rural and urban, or not specified. With no common applied definition of rural and urban, field surveys were measured by the term they presented or by the description of the region, e.g. metropolis areas were considered urban. Figure 1.8 demonstrates that most field surveys did not specify the type of population area that was surveyed (n = 28, 46 %), followed by only urban areas (n = 17, 28 %), both urban and rural (n = 11, 18 %), and lastly only rural areas (n = 5, 8 %). Moreover, Figure 1.8 demonstrates that neither rural nor urban regions possess a significantly higher percentage of failed samples.

Even in the new field surveys, there was a lacking declaration of geographical region. In many cases, the allocation had to be assigned based on the population and location. There is an obvious need to clearly define the requirements for each allocation so that implementation may be improved.

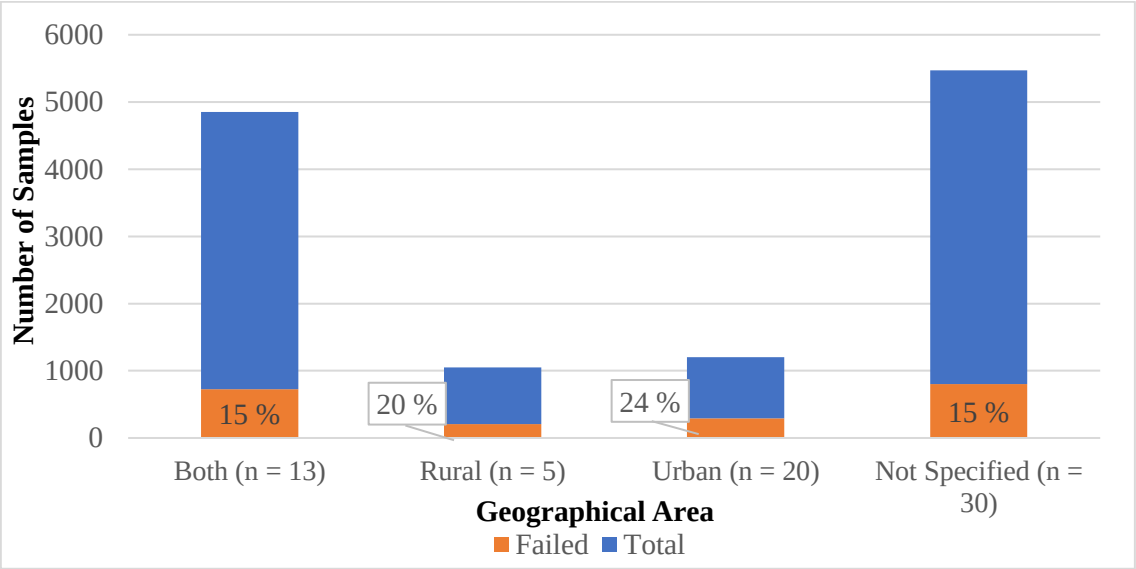

Figure 1.8. Distribution of all the included field survey results (total number of samples analysed and the number of failed samples) across the reported geographical sampling region.

The type of sampling region has implications on medicine quality as it is typically understood that rural areas possess less structured medical supply chains that their urban counterparts (Barrington *et al.* 2010). However, the results of this review indicate that while the percentage is still high (20 %), rural areas did not contain a large percentage of PQMs compared with other population areas.

1.6.6. Analytical Capabilities.

All the analytical methods reported in the literature review are allocated to one or more groups representing the three categories of PQMs (Figure 1.9). These groups represent what characterisation can be achieved by using an analytical method or combination thereof. Packaging analysis should be the first point of establishing the quality of AM as fraudulent packaging is an indication of falsified medicines. Packaging analysis does not require any specialised equipment, costly analysis or lengthy training. What is necessary is the participation of pharmaceutical companies through the provision of reference packaging or as a minimum, images of the packaging for comparison (Newton *et al.* 2011a; Yong *et al.* 2015). Only 28

surveys (46 %) reported assessing the packaging of the medicines in any form. The implications of overlooking packaging analysis are that PQMs cannot be determined to be falsified and furthermore cannot be confirmed to be substandard. Of these 28 surveys, 12 surveys (20 %) characterised their results as containing falsified samples while four (7 %) did not characterise their results despite performing packaging analysis, which would have allowed them to comment upon falsified status.

Only packaging analysis is required for characterisation of a falsified medicine when applying the WHO-accepted definition. However, an indication of a falsified medicine adapted in some reports was if the medicine contained zero API (Stenson *et al.* 1998; Syhakhang *et al.* 2004; Phanouvong *et al.* 2013). In these circumstances, presumptive testing such as colour reaction tests and thin layer chromatography (TLC) are included as they are both capable of reporting the presence of an API. TLC can serve as a qualitative or quantitative method when combined with other techniques; the eluted spot can be removed and analysed by a more sensitive method, or the spot intensity is measured by a densitometer or spectrometer (World Health Organisation 2006b). At sufficient concentration, TLC can reveal impurities or degradant products, though no such reports have been produced with AMs (Kaur *et al.* 2010). However, TLC can indicate an acceptable content range of 80 – 100 % when compared reference calibrators; allowing for ± 10 % error for visual inspection (Basco 2004; Vijaykadga *et al.* 2006). In many cases, the lower limit, 80 % (± 10 %), would be outside acceptable quality standards in recognised pharmacopoeia (e.g. British Pharmacopeia, International Pharmacopeia, and USP) (Osei-Safo *et al.* 2014). The inconsistent thresholds seemingly restrict TLC as a semi-quantitative analytical technique.

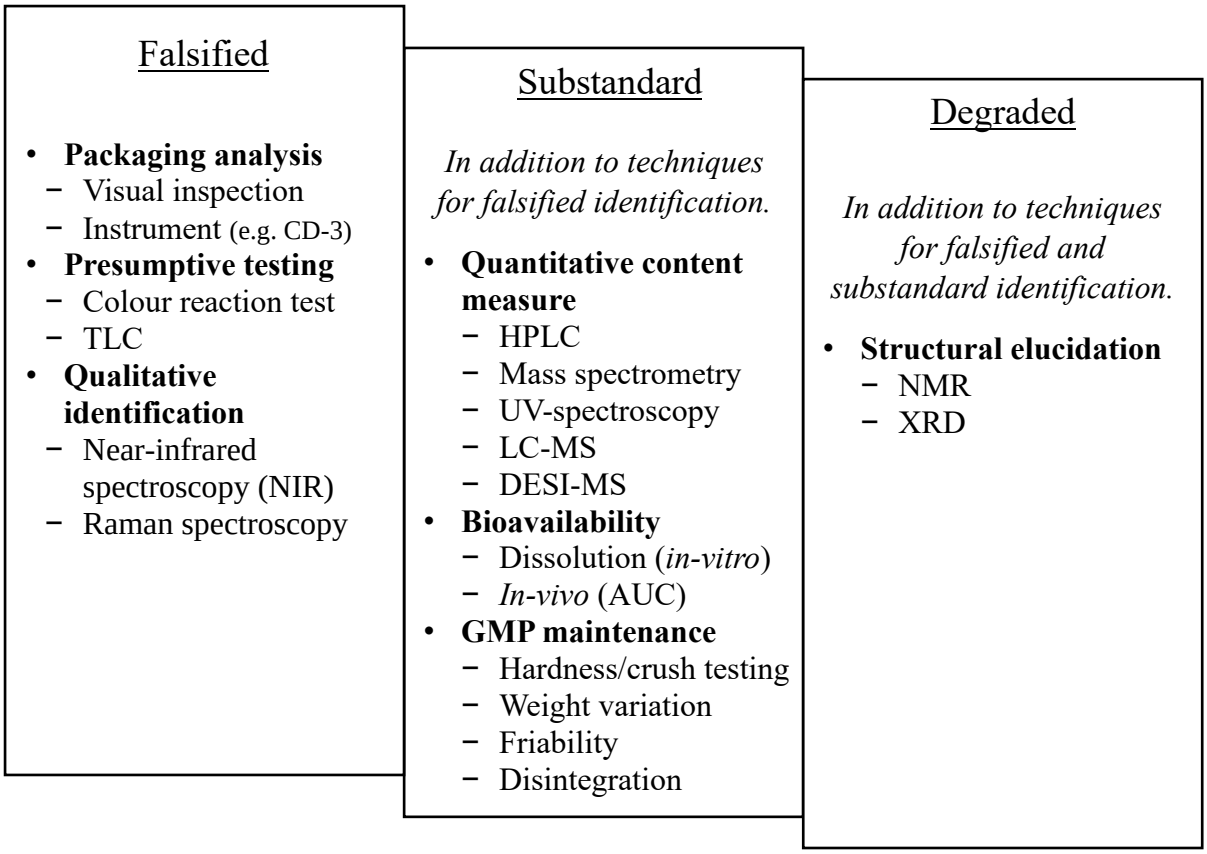

Figure 1.9. Distribution of applied analytical techniques required to characterise PQMs as falsified, substandard or degraded. Abbreviations: Area under the curve (AUC), Desorption electrospray ionisation-mass spectrometry (DESI-MS), X-Ray Diffraction (XRD), nuclear magnetic resonance (NMR).

The assessment to classify a medicine as substandard should include both content and bioavailability analysis. Even when the medicine is found to contain the required amount of API, inadequate release of the API can result in an ineffective, suboptimal treatment (Kelesidis and Falagas 2015). Dissolution is a valuable assessment of the theoretical basis of bioavailability (Amidon *et al.* 1995), though not giving a definitive indication of API content. Dissolution was utilised to give an *in-vitro* indication of bioavailability in 23 surveys (38 %) and was included in combination with content analysis in 20 occurrences (34 %). Indications of following current GMP such as hardness/crush testing, weight variation, friability, and disintegration can provide evidence of a quality product. However, indications of less than GMP cannot provide reasoning to consider a product to be substandard if the product is still

capable of possessing and delivering the correct amount of API to the patient upon consumption. Once the medicine has been determined to be from a legitimate source and substandard, analysis progresses to ascertain any alterations in chemical structure or to identify degradation markers.

To conclusively determine if a medicine has degraded, more scrutiny is required through elucidating any changes to the chemical structure of the medicine. The changes may take the form of degradation of API and subsequent formation of degradation products or alterations to the crystalline structure of the mixture (Agrahari *et al.* 2015). As stated in ICH guidelines, medicine manufacturers are required to report manufacturing impurities and the anticipated degradation products before expiry, including their anticipated levels (International Conference on Harmonisation 2006). Similar techniques are utilised in the reviewed literature to ascertain evidence of degradation through loss of API, e.g. high-performance liquid chromatography (HPLC) (Taylor *et al.* 2001; Gaudiano *et al.* 2006; Osei-Safo *et al.* 2014), changes to the API chemical structure (e.g. NMR (Maggio *et al.* 2014)) or changes to the crystalline pattern of the compound (e.g. XRD (Agrahari *et al.* 2015)). Three reports characterised their findings as degraded; however, only one conclusively characterised that degradation occurred through identifying degradation products/impurities (Gaudiano *et al.* 2006; Newton *et al.* 2011a; Seear *et al.* 2011). Multiple stability-indicating HPLC/UV spectroscopy methods have been developed for detecting degradation products of artemether (Shrivastava *et al.* 2010; De Spiegeleer *et al.* 2012; Mondal *et al.* 2014). While a UV detector does not give specific mass information about the degradant, it can be considered a fit-for-purpose technique to indicate the medicine is degraded through identifying separate degradant peaks. Of the 61 included reports, 23 (38 %) had no characterisation presented, as poor-quality tablets were only said to ‘fail’ analysis or be ‘outside quality standards’. The

reporting of findings with vague descriptions restricts the ability to comprehend and effectively address the problem of poor-quality AMs.

All 61 studies were assessed to determine whether their characterisation aligned with the analysis techniques applied, and in situations where no characterisation occurred, were evaluated as to whether an accurate characterisation was possible. Some of those who did not characterise had the capabilities to do so based upon the analytical techniques applied. In four situations (7 %), no characterisation was reported for failed samples despite performing adequate analysis (e.g. packaging inspection and HPLC) to determine if either falsified or substandard products were present (Meos *et al.* 2008; Evans *et al.* 2012; Pribluda *et al.* 2012; Fadeyi *et al.* 2015). Five surveys (8 %) based their characterisation as substandard upon non-compliance with GMP indicators and not upon content measurement or bioavailability indicators (Lon *et al.* 2006; Ofori-Kwakye *et al.* 2008; El-Duah and Ofori-Kwakye 2012; Phanouvong *et al.* 2013; Samali *et al.* 2014). Ten surveys (16 %) sought to identify falsified medicines based upon the presence of the API (e.g. colour testing or TLC) without assessing the packaging. While two (4 %) surveys solely based their characterisation as falsified on these outcomes rather than packaging analysis (Stenson *et al.* 1998; Basco 2004; Syhakhang *et al.* 2004; Phanouvong *et al.* 2013).

The obvious improvement in the field surveys identified 2017 and 2018 was the uniform adoption of packaging analysis as a routine assessment of medicine quality, with 100 % of the new field surveys reporting using the technique. Moreover, all but one reported using HPLC, a method capable of detecting substandard/degraded medicines. The improvement in the analytical capability of the field surveys is encouraging for the accurate characterisation of the results in the future.

1.6.7. Characterisation of Results.

In 2009, Newton *et al.* (2009) published a checklist to follow when attempting a medicine quality field survey, referred to as the Medicine Quality Assessment Reporting Guidelines (MEDQUARG). The intention of the MEDQUARG checklist is to improve the overall quality of field surveys so that generalised observations can be made using the outcomes of multiple field surveys in an area. Of the items on the checklist, the accurate characterisation of results is an important step. While all types of PQMs are potentially harmful, the required countermeasures are different (Attaran *et al.* 2012). To effectively combat PQMs, it is important to distinguish between each type where possible. The MEDQUARG checklist was further endorsed in a 2014 review by Tabernero *et al.*, which indicated the merits of the checklist on understanding the epidemiology of PQMs (Tabernero *et al.* 2014).

Included articles were assessed based on the conclusions of the field surveys. Outcomes were recorded as falsified (FA), substandard (SB), degraded (DE), within quality specifications (WS), no characterisation (NC), or a combination of these (Figure 1.10). The criteria by which the characterisations were judged are based on the above definitions (Section 1.3). Surveys were recorded as NC if the results were indecisive or ambiguous, e.g. reporting as ‘outside pharmacopoeia specifications’ or ‘failed’ without justification or explanation. The most common characterisations were seen to be either substandard (n = 23, 38 %), followed by none at all (n = 22, 36 %).

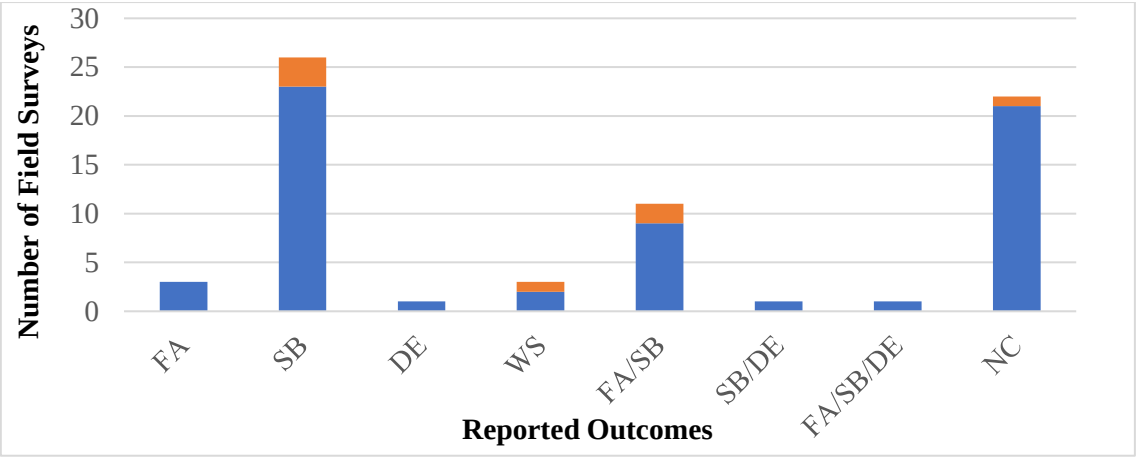

Figure 1.10. Characterised reported outcomes of the included field surveys of AM quality. Abbreviations: falsified (FA), substandard (SB), degraded (DE), within quality standards (WS), and no characterisation (NC).

The updated field survey data presented very positive indications of transitioning to accurate characterisation (Figure 1.10). Only a single field survey did not specifically characterise the results further than poor-quality (Nakalembe 2018). Furthermore, the characterisations that were concluded in the remaining six field surveys aligned with the analytical capabilities described in the research. Kaur *et al.* (2017) presented a high-quality field survey, addressing the possible characterisation of medicines as falsified, substandard, or degraded. While not reporting degraded medicines, the paper states that no degradation products were detected and therefore none were characterised as such.

The major finding derived from these data is how common results were seen not to conform to current definitions or were characterised based upon incomplete assessment. In total, 13 (21 %) field surveys identified some samples as falsified, five of these (38 %) did so without performing packaging analysis. Moreover, of the 34 surveys to conclude the presence of substandard medicines, only 23 (67 %) first ruled out the chance of being falsified medicines through assessing the packaging. Conversely, only two of the 34 substandard findings proceeded to assess degradation empirically. Three field surveys reported identifying degraded

medicines; however, only one based the characterisation upon the presence of degradation markers (Gaudiano *et al.* 2006; Newton *et al.* 2011a; Seear *et al.* 2011).

Three concerns were identified while assessing accurate characterisation of field surveys; 1) presenting no final characterisation, 2) incomplete analysis procedures, and 3) characterisation that does not conform with current definitions. While incomplete characterisations are undesirable, circumstances such as minimal funding, restricted access to analytical instruments, and limited availability of reference material (e.g. comparative packaging) are often unavoidable (Nayyar *et al.* 2012). As such, the characterisation should address the limitations and present whether alternates are possible (Keoluangkhot *et al.* 2008; Newton *et al.* 2011a). However, incomplete or absent characterisations may be preventable. The benefits of characterisation are immeasurable, with outcomes assisting in creating effective countermeasures against PQMs.

1.7. Outcomes of Effective Characterisation of Poor-Quality Medicines.

Accurate characterisation initiates a unified action through providing proceeding countermeasures or through supporting currently established methods. There are many government, non-government organisations (NGOs), and inter-governmental organisations assisting in the provision of high-quality AMs through involvement in aspects including; prequalification, procurement services, dissemination of information and awareness, and education and training. The President's Malaria Initiative is one such organisation, implementing strategies to mitigate PQMs, including; educating shopkeepers, informing consumers on how to recognise falsified medicines, helping strengthen local technical capacities for medicine quality testing and maintenance of quality control strategies (Ziemer 2015). The mere presence of NGOs in the local medicine supply market can reduce the likelihood of incumbent drug stores selling counterfeit medicines by 20 – 21 % (Björkman-Nyqvist *et al.* 2013). It is, therefore, paramount that the presence of an influential NGO is taken

into consideration when determining the factors associated with the occurrence of poor-quality AMs. The following sections detail a few examples of the NGOs and IGOs that are actively working against PQMs.

1.7.1. Prequalification.

The WHO prequalification is a six-stage process and is detailed in Annex 10 of WHO Technical Report No. 961 (World Health Organisation 2011c). The six stages are; 1) submission of an expression of interest, 2) receipt and processing of expressions of interest, simultaneously 3a) assessment of dossiers and 3b) inspection of manufacturing and research sites, 4) decision on prequalification, 5) listing as prequalified products, and 6) maintenance of the list. The transaction summary reported by the Global Fund indicates that 2004 - 2015 over $ 381 million USD were spent in 2376 AM transactions across 73 countries. Of the transactions logged by the Global Fund between 2004 and 2015, it was found that 1,907 (80 %) were for a product which had been prequalified by the WHO and produced by a prequalified manufacturer (The Global Fund 2015). This statistic demonstrates the influence that the prequalification system possesses in influencing procurement trends. Prequalified AMs correlate with greater stability compared with those not prequalified, lending support to better quality in tropical climates (Hall *et al.* 2016). Accurate characterisation can assist in stage six of the prequalification process (maintenance of the list). The WHO Technical Report No. 961 references a standard operating procedure for investigating issues concerning prequalified pharmaceuticals (World Health Organisation 2011c). Results of investigations are publically posted via Medical Product Alerts. Survey results of characterised PQMs not only makes the WHO aware of the issue but may also assist in directing an expedited response.

1.7.2. Procurement Services.

A highly influential method employed by NGOs to ensure the distribution of high-quality medicines is the provision of procurement services. The exact procurement information

by many NGOs is not widely available (Mackintosh *et al.* 2011). However, one of the leading procurers of AM treatments is The Global Fund (2015) who have made their procurement information openly available online. This information has provided a useful insight into procurement services. Other significant procurement services are provided by; International Dispensary Association, *Médicins san Frontières*, United States Agency for International Development, The Bill and Melinda Gates Foundation, Clinton Health Access Initiative, and the WHO (Gostin and Buckley 2013). The procurement of products by the Global Fund in Cambodia is steered by procurement guidelines (Kingdom of Cambodia 2006). These guidelines stipulate that pharmaceuticals for procurement must appear in the standard treatment guidelines of the WHO, comply with quality standards, and be at competitively low prices. An accepted method of ensuring product quality is the acceptance of the product under the WHO Prequalification Program. A recent report presented the effect of the Affordable Medicines Facility-malaria (AMF-m) on availability, price, and market share of ACTs in eight African regions (Tougher *et al.* 2012). In all but one country, the presence of the AMF-m improved the AM market through an increase in AM availability, reduction in the price of AMs, or an increase in market share of quality-assured ACTs. The decrease in price was able to be achieved through increased negotiating power of the organisation and through a co-payment fund in which the AMF-m supported importers by subsidising the cost from the manufacturers (Tougher *et al.* 2012).

Procurement services are not simply restricted to medicine purchases but also extend into supply and distribution. The typical distribution chain of NGOs is relatively simpler and more streamlined when compared with both the public and private sector (Yadav *et al.* 2011). Previously published data have witnessed that without proper regulation of temperature and humidity, medicines during transit are exposed to conditions 12 - 15 °C higher than ambient temperature in the area (Hogerzeil and De Goeje 1991; Hogerzeil *et al.* 1992). Accurate

characterisation of degraded medicines can give an indication of potential issues in the distribution supply chain and therefore help direct an effective countermeasure to reduce future PQMs.

1.7.3. Information and awareness.

An unfortunate occurrence in disseminating information regarding PQM to the public is that some pharmaceutical companies are reluctant to report circumstances of counterfeiting as it may damage their legitimate business (Cockburn *et al.* 2005). Therefore it is important for organisations without financial motivation to raise awareness of the issue and inform the public. The WWARN provides such a service through the collection and presentation of information associated with AM resistance. With the support of other NGOs such as the Bill and Melinda Gates Foundation, WWARN provides a service directed at raising awareness of poor-quality AMs. As PQM is associated with disease resistance (Nayyar *et al.* 2012), an aspect of the service provided by WWARN is the Antimalarial Quality Surveyor (AQS). The WWARN AQS is part of the Mahidol Oxford Tropical Medicine Research Unit through the support of the Wellcome Trust. Critical information gathered through the WWARN AQS has been systematically reviewed and presented in an academic journal (Tabernero *et al.* 2014). Although access to peer-reviewed journal articles by the general population in low- and middle-income countries is highly unlikely, access to an internet website is a more viable method of information gathering.

Moreover, without simplified and concise provision of the information within the technical articles, it is possible that the reader may not fully understand the conclusions. Through this service, 43 non-peer reviewed articles have been collated, reporting from over ten countries. In addition to the hundreds of available peer-reviewed articles, the non-peer reviewed articles are gathered from local and international press services, representing what

one may encounter in low- and middle-income countries. This information demonstrates the importance of services such as WWARN in raising awareness.

1.7.4. Education and Training.

Education is important, as patients that are better informed are more likely to seek treatment at a health centre and then source medicines from licensed dispensaries (Klein *et al.* 2012). Misdiagnosis (including self-diagnosis) can lead to inaccurate community understanding of the treatment's effectiveness, e.g. self-limiting bacterial infections can portray effective treatment of malaria despite the patient possessing no malarial infection at all (Björkman-Nyqvist *et al.* 2013). The Global Fund supports the Family Health International 360 initiative which has been active in Pailin along the Thai-Cambodian border since 2007 (FHI360 2015). Their presence in each region is directed towards the education and training of locals on malaria prevention, diagnosis, and treatment services. Information regarding the correct transmission pathway of malaria has been linked with correct consumer practices once an infection has occurred (Björkman-Nyqvist *et al.* 2013). Foreigners travelling to malaria-endemic regions may seek health-related information from travel agents or insurers (Leggat 2000).

1.8. Conclusions.

The data extracted from the literature review demonstrates the capabilities of generalising the outcomes field surveys but also highlights the shortcomings that restrict them. Insight into the type and number of failed samples of a particular region can help direct an informed response. However, the current data set is incomplete. The ability to generalise the results of field surveys will be strengthened through uniformity in reporting, addressing key criteria such as; the applied sampling method, the utilised analysis techniques, explicitly stating the number of samples analysed and failed, referencing the applied pharmacopoeia criteria, and acknowledging any survey limitations. Through rigorously addressing these criteria the

researchers gain the ability to characterise their results, giving an exact description of the categories of medicines that were found in the field survey. From this detailed information, effective countermeasures can be formed considering the precise type of PQMs in the region. Key organisations must follow the suggestions of the MEDQUARG checklist and generate a uniform procedure collecting, analysing, reporting and characterising the results of field surveys.

1.9. Aims of the COPA Research.

The primary aim of the COPA research is to provide practical guidance into medicine quality field surveys through the development of a protocol that will characterise poor-quality antimalarials. The secondary aim of the COPA research is the successful implementation of the COPA protocol in an environment in which PQMs have previously been reported, Uganda, Africa.

Additional outcomes accomplished in completing the above aims will be to gain a better understanding of the applicability of the current stability testing regimes. This aspect is critical to the practical application of the COPA protocol. Stability testing is imperative in developing a process to detect degraded medicines. However, without fully investigating the conditions to which the medicines are exposed and the resulting medicine degradation, the laboratory research may lack the intended real-world application. This investigation includes the implementation of a novel stability test, diurnal cyclic variation, that replicates day/night cycles and the effect that has on the medicines.

The successful completion of the above aims would deliver a robust protocol for presenting medicine quality in field surveys as falsified, substandard or degraded. The accurate characterisation can better inform effective countermeasures to best fight the widespread issue of poor-quality medicines.

1.10. Overview of the COPA Research.

Figure 1.11 presents the structure of the COPA project; with the major aims of the project on the left-side, the tasks required to achieve the aims on the right, and the chapters in which each task is performed under each title.

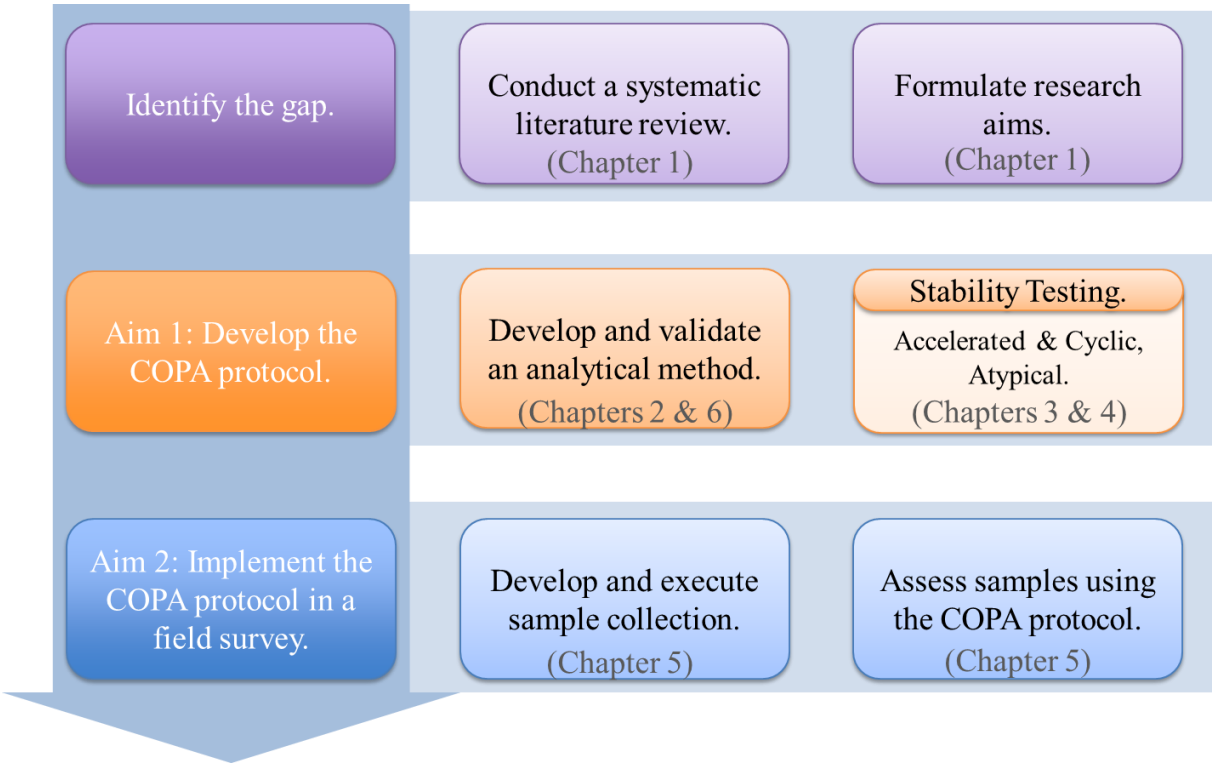

Figure 1.11. Structure of the COPA project. Including references to the chapters in which each component is addressed.

Medicine quality advocates have initiated the procedures and structures in which to assess and report medicine quality surveys through the MEDQUARG checklist proposed in 2009. While recent medicine quality field surveys have seemingly espoused more components of the checklist, there is still lack in the practical guidance that can completely address the outcomes of the MEDQUARG checklist. The principles of the checklist rely on the accurate characterisation of PQMs as falsified, substandard, and degraded. Therefore, the practical guidance provided by the COPA protocol will specifically address all three categories.

Given there are many types of AMs and a larger number of manufacturers, selecting those to target was achieved by analysing the transaction history of The Global Fund (2015). The Global Fund is one of the largest procurers of antimalarial agents and over the last ten years had procured over $ 380 million USD worth of antimalarials (The Global Fund 2015). Therefore, their procurement trends provide invaluable insight into current market tendencies. For this research, the two antimalarial compounds selected were artemether (ATM) and lumefantrine (LUM), which are often administered as a mixed (co-formulated) tablet commonly referred to as an ACT. Co-formulated ATM and LUM treatments were selected as the outcome from the research would have the largest impact, by incorporating the medicines that have been more widely distributed recently.

The first element of the research was the creation of a fit-for-purpose laboratory method. The analytical method had to be quantifiable across a wide concentration range to account for the changing concentrations typical of substandard medicines, and adequately broad to allow the detection of other unidentified falsified compounds. The method was validated in accordance with international standards to assist international engagement and implementation. The development and validation of the analytical method are described in Chapter 2.

Chapters 3 and 4 bridge the gap between laboratory-based stability testing and the real-world conditions to which medicines are exposed. Newly developed medicines are routinely assessed for their stability during the supply chain and further distribution. However, the assessment is performed in a laboratory setting. Chapter 3 repeats the current standard convention and introduces a novel cyclic stability test to move closer to the uncontrolled conditions of supply chains lacking climate control. Chapter 4 monitors medicines as they are exposed to uncontrolled conditions in an atypical stability test and measures the similarities in

the degradation to those of laboratory-based stability tests. The results of Chapters 3 and 4 inform the COPA protocol in detecting and assessing degraded medicines.

The next element of the research is the implementation of the COPA protocol on samples collected during a field survey throughout representative administrative districts in Uganda, Africa. The administrative districts were selected based on climate information to observe whether the varied conditions resulted in different degradation profiles, and ultimately demonstrate the COPA protocols capability to account for ranging climate conditions. The complete field survey, including information concerning the pharmacy practices of the sampled pharmacies, is described in Chapter 5.

Appendix 1.A. Summary table of the assessed field surveys.

Table 1.3 presents key information of the field survey and comments on whether the field survey can characterise the results, based on the analytical methods.

Abbreviations:

Accidental (AC), Bioavailability (BIO), British Pharmacopeia (Br. Ph.), Convenient (CO), Content (CONT), Degraded (DE), Directed (DI), Falsified (FA), Good manufacturing processes (GMP), International Pharmacopeia (Int. Ph.), Not specified (NS), Packaging (PACK), Simple Random (SR), Stratified (ST), Substandard (SU).

Table 1.3. Summary table of the collected data from 68 field surveys (incorporating the original literature review [1 – 61] and the updated assessment [62 – 67]).

Ref	Author	Geography		Sample Method	Date	Sample #	% Fail	Analysis Technique						Standards	Charact-erisation
		Region	Class					PACK	FAL	SUB			DEG		
										CONT	BIO	GMP			
[1]	Abdi, Y. *et. al.*	1	Urban	CO	NS	9	**11%**				✓			- *	NC
[2]	Abdo-Rabbo, A. *et. al.*	14	Both	ST/CO	NS	50	**28%**			✓	✓			USP	SU
[3]	Affum, A.O. *et. al.*	5	NS	SR	2011	16	**6%**	✓	✓	✓				Int. Ph.	SU
[4]	Aina, B. *et. al.*	5	Urban	ST	NS	32	**59%**			✓	✓	✓		BP	NC
[5]	Amin, A.A. *et. al.*	1	NS	DI	2002	116	**41%**			✓				USP	SU
[6]	Antony, P. & Temu-Justin, M.	1	NS	DI	1999	2	**50%**		✓	✓	✓	✓		USP	NC
[7]	Atemnkeng, M.A. *et. al. (a)*	2	NS	NS	NS	19	**32%**		✓	✓				USP & Ph. Eur.	NC
[8]	Atemnkeng, M.A. *et. al. (b)*	1, 2	NS	SR	2004	24	**38%**	✓		✓				Ph. Eur.	SU
[9]	Awemu, G. *et. al.*	5	Urban	NS	NS	15	**33%**			✓	✓	✓		Br. Ph	NC
[10]	Basco, L.K.	2	Rural	SR	2001-‘02	284	**39%**		✓					TLC Test	FA
[11]	Bate, R. *et. al. (a)*	12	Urban	SR	2008-‘09	119	**7%**		✓			✓		MiniLab	NC
[12]	Bate, R. *et. al. (b)*	5	NS	CO	2011	150	**17%**	✓		✓				Int. Ph.	SU
[13]	Chikowe, I. *et. al.*	1	NS	ST/CO	2014-‘15	112	**88%**	✓	✓	✓				Int. Ph.	SU
[14]	Ehianeta, T. *et. al.*	5	Urban	NS	NS	13	**85%**			✓				USP	NC
[15]	El-Duah, M. & Ofori-Kwakye, K.	5	Urban	DI	2010	14	**100%**	✓	✓			✓		Int. Ph.	SU
[16]	Esimone, C. *et. al.*	5	NS	NS	NS	9	**11%**		✓			✓		USP & Br. Ph. *	NC

Table 1.3. Summary table of the collected data from 68 field surveys (incorporating the original literature review [1 – 61] and the updated assessment [62 – 67]).

Ref	Author	Geography		Sample Method	Date	Sample #	% Fail	Analysis Technique						Standards	Charact-erisation
		Region	Class					PACK	FAL	SUB			DEG		
										CONT	BIO	GMP			
[17]	Evans, L. *et. al.*	8	Both	DI	2009	77	**58%**	✓		✓	✓	✓		USP & Br. Ph & Int. Ph.	NC
[18]	Fadeyi, I. *et. al.*	5, 16	Rural	CO	NS	20	**15%**	✓	✓	✓	✓			USP	NC
[19]	Gaudiano, M.C. *et. al. (a)*	2	Urban	NS	NS	1	**100%**			✓				No PC	DE
[20]	Gaudiano, M.C. *et. al. (b)*	1, 2	Urban	SR	2005	30	**47%**	✓		✓	✓	✓		USP	FA/SU
[21]	Hebron, Y. *et. al.*	1	NS	SR	NS	15	**27%**			✓	✓	✓		USP	SU
[22]	Hehonah, N. *et. al.*	20	Urban	NS	NS	7	**71%**			✓				USP	NC
[23]	Hetzel, M.W. *et. al.*	20	NS	SR	2011	360	**10%**			✓				Br. Ph., Int. Ph. & USP	SU
[24]	Jande, M. *et. al.*	1	Urban	NS	NS	9	**56%**			✓	✓			USP	NC
[25]	Kamau, F. *et. al.*	1	NS	NS	1992 - 2000	45	**22%**			✓				Br. Ph.	NC
[26]	Kaur, H. *et. al. (a)*	1	Rural	ST	2005	301	**13%**			✓	✓			USP	SU
[27]	Kaur, H. *et. al. (b)*	1	Both	ST/SR	2010	1281	**12%**			✓		✓		No PC	NC
[28]	Keoluangkhot, V. *et. al.*	13	NS	DI	2006	1	**100%**	✓		✓				No PC	SU
[29]	Khuluza, F .*et. al.*	1	NS	SR	NS	28	**11%**	✓	✓	✓	✓	✓		USP	FA
[30]	Kibwage, I. & Ngugi, J.	1	NS	CO	NS	33	**70%**			✓	✓			USP	NC
[31]	Klein E., *et. al.*	5	Urban	DI	2010	33	**39%**	✓					✓	No PC	SU
[32]	Krech, L., *et. al.*	13	NS	ST	2003-‘12	1309	**2%**	✓	✓	✓	✓	✓		USP	FA/SU

Table 1.3. Summary table of the collected data from 68 field surveys (incorporating the original literature review [1 – 61] and the updated assessment [62 – 67]).

Ref	Author	Geography		Sample Method	Date	Sample #	% Fail	Analysis Technique						Standards	Charact-erisation
		Region	Class					PACK	FAL	SUB			DEG		
										CONT	BIO	GMP			
[33]	Lalani, M., *et. al.*	12	Both	ST/SR	2009	134	**25%**	✓	✓	✓	✓	✓		USP	SU
[34]	Lon C., *et. al.*	13	Both	CO	2003	427	**29%**	✓	✓			✓		Int. Ph. & USP	FA/SU
[35]	Mbinze, J., *et. al.*	2	NS	NS	NS	6	**0%**		✓					USP	WS
[36]	Meos, A, *et. al.*	15, 16	Urban	CO	NS	135	**8%**	✓		✓	✓	✓		Ph. Eur.	NC
[37]	Minzi O., *et. al.*	1	Urban	DI	NS	33	**36%**		✓	✓	✓			USP	SU
[38]	Nair A., *et. al.*	20	Urban	ST/SR	2009	14	**100%**	✓	✓	✓	✓			USP	FA/SU
[39]	Newton P., *et. al.*	1, 2, 5	NS	NS	2007	163	**48%**	✓		✓			✓	No PC	FA/SU/DE
[40]	Odeniyi, M., *et. al.*	5	NS	NS	NS	8	**63%**			✓	✓	✓		Br. Ph.	NC
[41]	Odunfa O., *et. al.*	5	Both	SR	NS	20	**55%**	✓	✓		✓	✓		Int. Ph. & USP	SU
[42]	Ofori-Kwakye, K., et. al.	5	Urban	NS	2006	17	**59%**	✓	✓			✓		Int. Ph.	SU
[43]	Ogwal-Okeng J., et. al.	1	NS	DI	2001	92	45%			✓				USP	NC
[44]	Onwujekwe O., *et. al.*	5	Both	ST/SR	NS	225	**27%**			✓	✓			USP	NC
[45]	Osei-Safo, D., *et. al.*	5	Both	CO	2010-‘11	16	**50%**	✓	✓	✓				Int. Ph.	SU
[46]	Phanouvong, S., *et. al.*	13	NS	ST/SR	NS	709	**1%**		✓			✓		USP & Int. Ph.	FA/SU
[47]	Pribluda V., *et. al.*	8	NS	CO	2002-‘09	1633	**12%**	✓	✓			✓		Int. Ph., USP, Br. Ph	NC
[48]	Rimoy, G., *et. al.*	1	NS	NS	NS	9	**0%**				✓			- *	WS

Table 1.3. Summary table of the collected data from 68 field surveys (incorporating the original literature review [1 – 61] and the updated assessment [62 – 67]).

Ref	Author	Geography		Sample Method	Date	Sample #	% Fail	Analysis Technique						Standards	Charact-erisation
		Region	Class					PACK	FAL	SUB			DEG		
										CONT	BIO	GMP			
[49]	Samali, A., *et. al.*	5	NS	SR	NS	8	**63%**	✓				✓		Br. Ph. *	FA/SU
[50]	Sawadogo C., *et. al.*	1, 2, 3, 5	NS	DI/SR	NS	26	**62%**			✓	✓	✓		USP	NC
[51]	Seear, M., *et. al.*	12	Urban	ST/CO	NS	100	**80%**	✓		✓				Br. Ph.	SU/DE
[52]	Shakoor O., *et. al.*	5, 13	NS	DI	NS	43	**33%**			✓				Br. Ph.	SU
[53]	Stenson, B., *et. al.*	13	Both	ST/SR	1997	193	**44%**		✓	✓				USP	FA
[54]	Syhakhang L., *et. al.*	13	Both	ST/SR	1999	163	**27%**		✓	✓				Br. Ph. & USP *	FA/SU
[55]	Tabernero, P., *et. al.*	13	Both	ST/SR	2012	146	**35%**	✓	✓	✓				USP, Ph. Eur. & Int. Ph.	SU
[56]	Taylor R., *et. al.*	5	NS	SR	NS	31	**32%**			✓				Br. Ph.	FA/SU
[57]	Tipke M., *et. al.*	5	Rural	CO	2006	77	**42%**		✓	✓		✓		Ph. Eur. *	SU
[58]	Vijaykadga S., *et. al.*	13	Rural	DI	2003-‘04	369	**7%**	✓	✓	✓		✓		TLC Test	SU
[59]	Visser, B., *et. al.*	2	Urban	ST/SR	2014	432	**1%**	✓	✓	✓		✓		Ph. Eur.	FA/SU
[60]	Yeung, S., *et. al.*	13	NS	ST/SR	2010-‘11	291	**27%**	✓		✓				USP	SU
[61]	Yong, Y., *et. al.*	13	NS	AC	2008	144	**26%**	✓	✓	✓				USP, Br. Ph. & INT. PH.	SU
[62]	Kaur, H., *et al.*	2	Both	CO/DI	2013	677	**9%**	✓	✓	✓			✓	BP	FA/SU
[63]	Nakalembe, L.	1	NS	ST/SR	2017	30	**13%**	✓	✓	✓				USP	NC
[64]	Adeyemi, D., *et al.*	5	Urban	NS	2016	20	**30%**	✓	✓	✓	✓	✓		USP	SU
[65]	Mziray, S., *et al.*	1	Both	DI	2012-‘15	1444	**3%**	✓	✓			✓		-	FA/SU

Table 1.3. Summary table of the collected data from 68 field surveys (incorporating the original literature review [1 – 61] and the updated assessment [62 – 67]).

Ref	Author	Geography		Sample Method	Date	Sample #	**% Fail**	Analysis Technique						Standards	Charact-erisation
		Region	Class					PACK	FAL	SUB			DEG		
										CONT	BIO	GMP			
[66]	Ndwigah, S., et al.	1	NS	DI	2014	37	**0%**	✓	✓	✓				USP	WS
[67]	Prah, J., *et al.*	5	Urban	SR	2014	10	**10%**	✓	✓	✓		✓		USP	SU
[68]	Schiavetti, B., *et al.*	2	Urban	SR	2014	159	**36%**	✓	✓	✓				IP	SU

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Chapter 2. Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) and High Performance Liquid Chromatography- Photodiode Array (HPLC-PDA) Method Development and Validation.

2.1. Background.

For the successful development of the COPA protocol, the protocol must include an analytical capability to measure the quality of the antimalarial API and provide insight into any potential degradation of the product. In doing so, the COPA protocol will be able to successfully characterise the product as substandard or degraded, two of the three categories of a PQMs.

The target API for this study were ATM and LUM. The structures for the two analytes are illustrated in Figure 2.1.

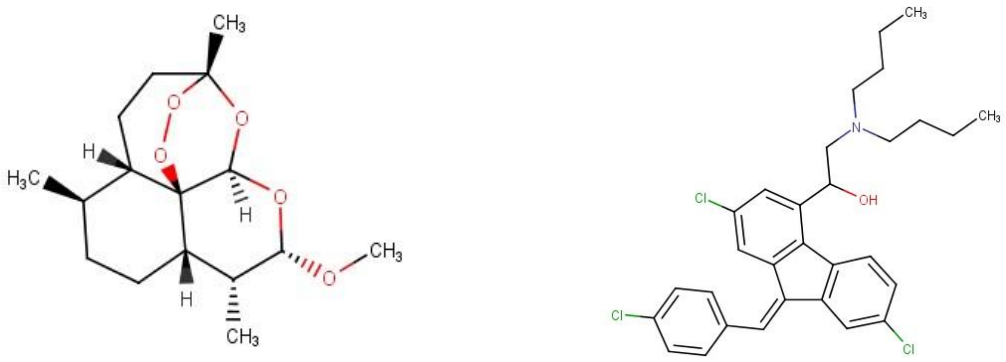


Figure 2.1. The molecular structure of ATM (left) and LUM (right).

Therefore, the aim of this chapter was to develop and validate an analytical method that was capable of quantitively measuring the percent content of the APIs (ATM and LUM) and any degradant products that may occur. The method was required to be validated in accordance

with ISO 17025 standards. Guidance for selecting the analytical method was based on the results of the literature review presented in Chapter 1.

2.2. The Development of a Liquid Chromatography-Tandem Mass Spectrometry Method for the Detection of Artemether and Lumefantrine.

As reported in Chapter 1, liquid chromatography-mass spectrometry had been implemented in many antimalarial field surveys but applied different analytical details depending on the specific antimalarial being investigated. Earlier research by Hodel *et al.* (2009) reported a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method that was capable of simultaneously determining 14 antimalarials in a single analysis. The antimalarial analytes in this research included the four predominant artemisinin-derived combination therapies; ATM, artesunate (ATS), amodiaquine, and LUM. For this reason, the method described in the Hodel *et al.* (2009) paper was initiated in the LC-MS/MS method development. For the Hodel *et al.* (2009) method, the mobile phase consisted of 20 mM ammonium formate in ultrapure water (solvent A) and acetonitrile (ACN, Solvent B), both with 0.5 % formic acid. The LC-MS/MS was used in electrospray ionisation (ESI) positive ion mode, the nitrogen drying gas was at 350 °C, and the capillary voltage was set 4000 volts.

The aim of the LC-MS/MS method was to quantify the API content of ATM/LUM combination therapies and to detect potential degradant products. The collection of detailed mass information of the degradation products was intended to provide insight into the degradation pathways.

2.2.1. Materials and Chemicals.

The reference standards for the target APIs, ATM (product no. A2190) and LUM (product no. L0256), were manufactured by Tokyo Chemical Industries ATS (batch no. A2150610, Guilin Pharmaceuticals Co., Ltd, Guangxi, China). Labelled [^{13}C, D_3]-beta-artemether (L-ATM, > 99 % pure, ALSACHIM, Strasbourg, France), and

lumefantrine-analogue oxalate salt (LOS, 97.1 % pure, ALSACHIM) were evaluated as internal standards (IS'). Figure 2.2 illustrates the molecular structure of the two internal standards, L-ATM and LOS. The L-ATM and LOS reference standards were kindly provided by the WWARN Reference Standards Programme.

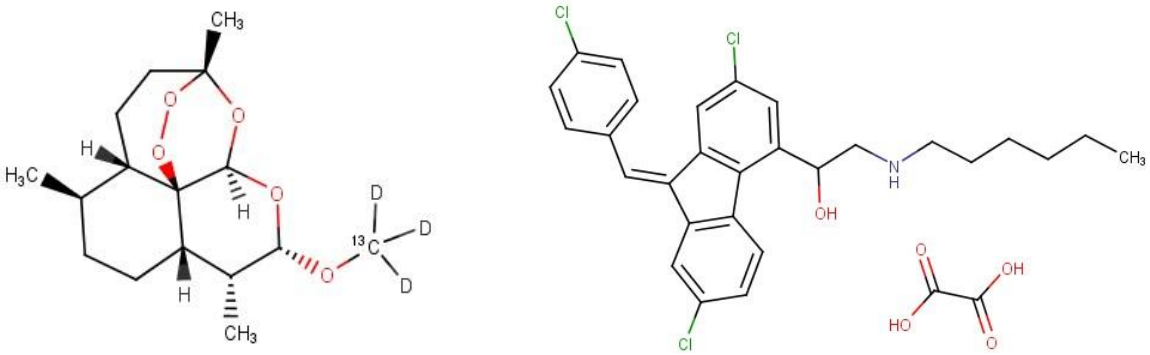


Figure 2.2. The molecular structures of L-ATM (left) and LOS (right).

Mobile phase components including formic acid (HCOOH, 98 %, product no. 33015-2.5L), ammonium acetate ($NHCH_3COO$, 98 %, product no. A1542-250G), and ammonium formate (NH_4COOH, 97 %, product no. 15264100G) were procured through Sigma-Aldrich (St Louis, Missouri, USA). Glacial acetic acid (CH_3COOH, > 99.8 %, product no. AC03461000) was sourced through Scharlau (Sentimenat, Spain). Chromatography-grade Methanol (CH_3OH, 99.9 %, product no. 100233) was supplied by Honeywell Burdick and Jackson (Ulsan, Korea). LiChrosolv® ACN (99.99 %, product no. 1.00030.4000) was produced by Merck KGaA (Darmstadt, Germany). All mobile phase solutions were filtered through 0.22 µm Nylon discs under vacuum (Grace Davison Discovery Sciences nylon discs, Rowville, Victoria) prior to use. Millipore water was obtained from Sartotrius Arium 611VF water purification system.

2.2.2. Instrumentation.

The LC-MS/MS was the Agilent Technologies 1260 Infinity series comprised of the 1260 µ-degasser (G1379B), 1260 binary pump (G1312B), 1260 auto-sampler (G1329B), a

1290 thermostat (G1330B) and a 6410 triple quadrupole mass spectrometer (6410B). The analytical column used was an Agilent Technologies Zorbax SB-C18 Rapid Resolution HT 2.1 x 50 mm (1.8-micron, 82770-902). During LC-MS/MS assessment an Agilent LC/MS API ElectroSpray ionisation (ESI) source (G1948B) was used for ionisation.

The data acquisition software was Agilent MassHunter Workstation Acquisition (version B.04.01, © Agilent Technologies, Inc. 2003 – 2011). Optimisation was performed using the MassHunter Optimizer (version B.04.01, © Agilent Technologies, Inc. 2008 – 2010).

Preparative equipment included; the pH 510 meter produced by Eutech Instruments PTE LTD (Blk 5, Singapore), the AUX320 electronic balance (readability > 0.1 mg) from Shimadzu (Rydalmere, Australia), the FXP10M ultrasonicator manufactured by Unisonics Australia, Brookvale, Australia.

2.2.3. Initial Mobile Phase Composition.

The initial mobile phase composition explored conditions based on the method presented in (Hodel *et al.* 2009) for the determination of 14 antimalarial drugs that included ATM, ATS, and LUM. The aqueous solutions in Millipore water that were considered during the development phase were: 0.5 % *v/v* formic acid (pH 2.30), 1.0 % *v/v* formic acid (pH 2.21), and 20 mM ammonium formate with 1.0 % *v/v* formic acid (pH 2.58). However, the optimal aqueous mobile phase was 20 mM ammonium formate with 0.5 % *v/v* formic acid in Millipore water (solvent A), measured pH 2.84. The organic phase (solvent B) solely consisted of ACN.

2.2.4. MS Optimisation of ATM, L-ATM, LUM, and LOS.

Deuterated IS' are the gold standard for analytical analysis. In the absence of a deuterated standard of LUM, an oxalate salt analogue, LOS, was an appropriate substitute as an IS. The purpose of this experiment was to optimise the MS parameters and obtain mass information for ATM, L-ATM, LUM, and LOS certified reference material.

Certified reference material stated the expected mass of ATM (298.4 g/mol), L-ATM (302.38 g/mol), LUM (528.4 g/mol), and LOS (590.92 g/mol). Analytical standards of ATM, L-ATM, and LOS were created at 10 µg/mL concentration and 1 µg/mL for LUM.

The mobile phase compositions comprised of 20 mM ammonium formate in 0.5 % *v/v* formic acid (A) and ACN (B). In the absence of an analytical column (i.e. direct infusion), the initial mobile phase program was set at 50 % B at 0.2 mL/min. The MS/MS was set to ESI positive (+) ion mode full-scan (range: 50 – 600 *m/z*). For each analytical standard, the parent ion *m/z* was confirmed through full scan mode in the MassHunter Workstation Acquisition, and the multiple reaction monitoring (MRM) quantifier and qualifier ions were then identified using the MassHunter Optimizer.

Once the mass information had been collected, the analytical standards were then separated using the Zorbax SB-C18 analytical column. A gradient elution program was employed using 20 mM ammonium formate in 0.5 % *v/v* formic acid (A) and ACN (B): 60 % B for one and a half minutes before increasing to 85 % B over one minute, holding for five minutes, and returning to 60 % B for five minutes to equilibrate before commencing the next analysis.

Table 2.1 demonstrates the acquired MRM information for ATM, LUM, and their IS. The masses seen for LUM and LOS in the full-scan mode are consistent with $[M+H]^+$ ionisation. However, for ATM and L-ATM, the *m/z* 221 ions indicate potential insource fragmentation, as the expected $[M+H]^+$ values are 299 and 303, respectively. Furthermore, the quantifier and qualifier ion information gathered for ATM and L-ATM are very similar and cannot be confidently differentiated based on the mass information.

Table 2.1. MRM Information for the target API and their internal standard (IS).

Analyte	Parent ion *m/z*	Quantifier ion *m/z*	Qualifier ion 1 *m/z*	Qualifier ion 2 *m/z*
ATM	221.1	163.2	43.0	77.1
L-ATM (IS)	221.0	163.1	43.1	77.0
LUM	528.2	510.2	346.1	276.2
LOS (IS)	500.0	484.1	43.1	276.1

The fragmentation of ART derivatives during the ionisation process have been reported numerous time in the literature (Souppart *et al.* 2002; Peys *et al.* 2005; Keoluangkhot *et al.* 2008; Hodel *et al.* 2009; Huang *et al.* 2009; Vandercruyssen *et al.* 2014). Figure 2.1 demonstrates the chemical structures of the two ART derivatives of interest and the proposed structures of the in-source fragmented parent and quantifier ions.

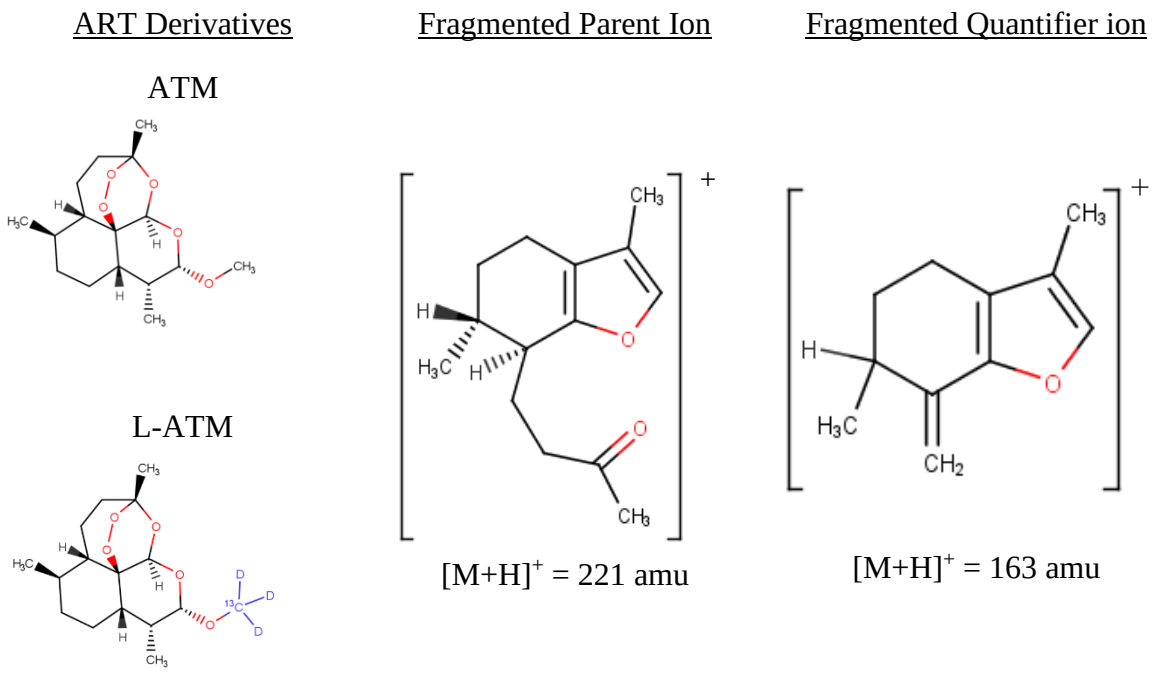


Figure 2.3. Proposed Chemical structure of the target ART derivatives and the proposed structures of the detected fragments following in-source fragmentation.

The in-source fragmentation of ART derivatives resulted in the non-deuterated and deuterated forms of ATM no longer having a mass difference for their parent ions, and therefore

a loss in mass selectivity. Chromatographic separation of these compounds would not be expected to occur as demonstrated in Figure 2.4.

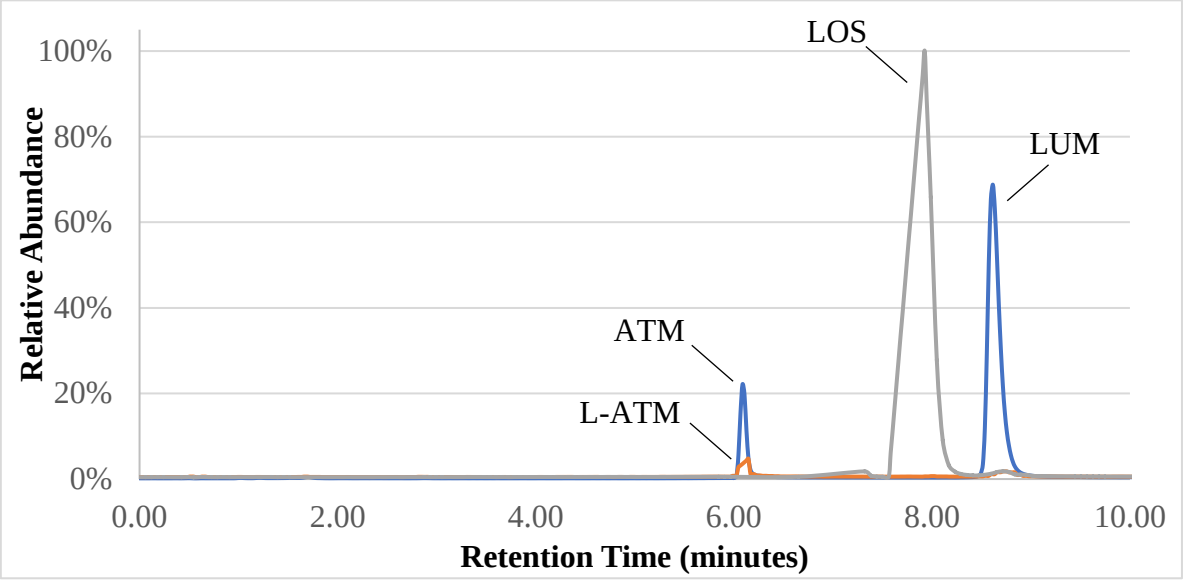


Figure 2.4. Total ion chromatogram of ATM, L-ATM, LUM, and LOS. LC-MS/MS parameters: Solvent A consisted of 20 mM ammonium formate in 0.5 % formic acid and Solvent B was ACN. Gradient elution program: 45 % B for 3 mins, increasing to 85 % B over 0.5 mins and hold for 6.5 mins before re-equilibration. Monitored MS MRM conditions detailed in Table 2.1.

ESI was selected for method development as it is considered to be a 'soft' ionisation technique (Kujawinski *et al.* 2002). However, the in-source fragmentation of ART derivatives restricts the use of deuterated IS, which possesses identical retention times (RTs) and similar fragmented mass spectral profiles. Therefore, when using LC-MS/MS, ATM and LATM cannot be confidently distinguished based on those criteria.

2.2.5. Impact of in-source fragmentation on detection of ATM degradants using LC-MS/MS.

The purpose of this experiment was to probe the impact of in-source fragmentation on degradation products; therefore, only ATM was assessed as LUM had not previously displayed in-source fragmentation. Contrary to obtaining the MS information of analytes with reference

standards gathering MS information of degradation products first requires the mixtures be chromatographically separated first.

ATM standards underwent ‘harsh degradation’ to create degradation products for preliminary identification purposes. A harsh degradation study exposes the medicine products to conditions far greater than is those expected to be encountered during supply or distribution. The purpose of the harsh degradation study is to degrade the medicine to an extreme degree in a short period to observe the degradation products created.

Powdered standards of approximately 20 mg of ATM for the harsh degradation study were placed in an oven at 150 °C and removed at 30 and 60 minutes. The powder was then dissolved in ACN to create standard concentrations of 1 mg/mL. The mobile phase composition comprised of 0.1 % *v/v* formic acid in Millipore water: ACN (55:45) at 0.6 mL/min, using the Agilent Zorbax SB-C18 column maintained at 35 °C. LC-MS/MS reflected the conditions in Section 2.2.4 (range: *m/z* 50 – 600 amu).

As seen in Figure 2.3, four degradant peaks at the RTs approximately 2.5, 4.3, 4.8, and 5.3 minutes (labelled Harsh Peak 1 – 4, respectively) were generated during the harsh degradation process. These peaks were analysed for both the qualitative presence of the fragments and a quantitative measure of the fragments’ MS ratios of major quantifier and qualifier ions.

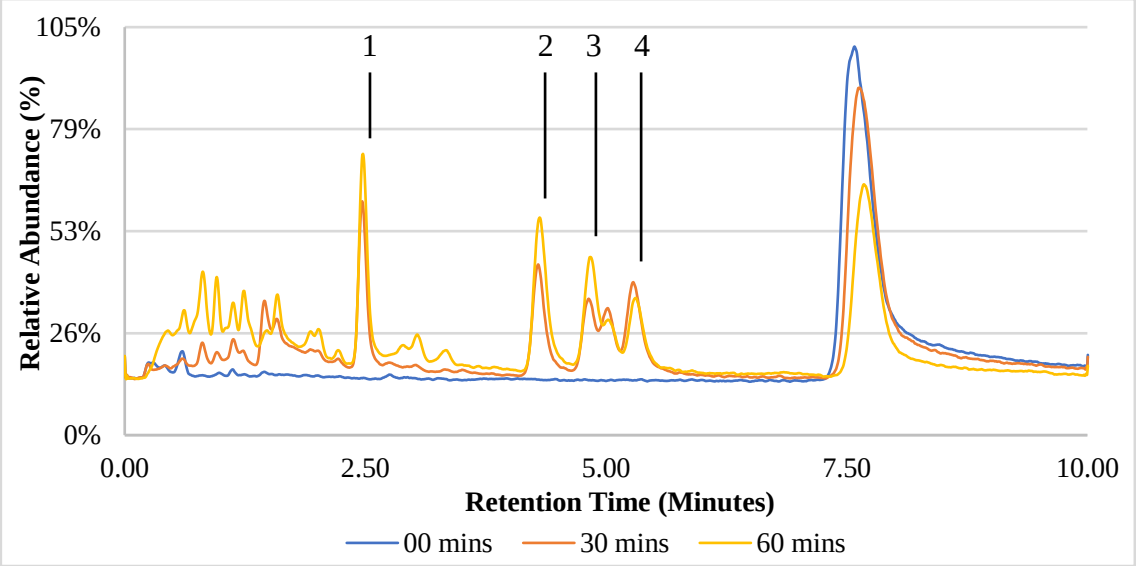


Figure 2.5. Chromatograms of ATM that has undergone harsh degradation (heated at 150 °C for either 30 or 60 minutes). Harsh Peak 1 (2.48 min), Harsh Peak 2 (4.33 min), Harsh Peak 3 (4.87 min), and Harsh Peak 4 (5.28 min).LC-MS/MS parameters: Isocratic elution program at 0.6 mL/min of 55 %/45 % *v/v* 0.1 % acetic acetic acid and ACN. Separated using an Agilent Technologies Zorbax SB-C18 Rapid Resolution HT 2.1 x 50 mm x 1.8-micron held at 35 °C.

The mass spectrum for all four major peaks presented a very similar spectral profile, with the highest intensity seen at *m/z* 253, followed by *m/z* 221 and 275. This spectral profile was seen across all time increments that contained degradant peaks. The two ions, *m/z* 253 and 275, not observed when observing ATM. Table 2.2 presents the average ratio of the highest peak (*m/z* 253; quantifier ion) and the two lesser peaks (*m/z* 221 & 275; qualifier ions). The latter three degradants all possessed similar ratios and cannot be confidently differentiated.

Table 2.2. Average *m/z* intensity ratios of predominant *m/z* for the four major harsh degradant peaks of ATM.

RT (mins)	2.5	4.3	4.8	5.3
m/z ratio		Mean (SD)		
253/221	5.6 (0.2)	3.5 (0.1)	3.2 (0.1)	3.8 (0.2)
253/275	12.6 (0.5)	9.9 (0.2)	8.7 (0.2)	9.0 (0.5)

The analysis of the degradant products using LC-MS/MS is hindered if the MS profiles of the degradant peaks are identical. Therefore, measures were pursued to improve the stability of the degradant products during analysis.

2.2.6. Stabilisation of ART derivatives.

Two method adaptations were considered to stabilise the ART derivatives during the ionisation process; the use of a stabilising agent and altering the aqueous mobile phase component. (Keoluangkhot *et al.* 2008) reported success in the analysis of ART derivatives in acetic acid containing dodecylamine (DDA) as a stabilising adduct. However, the paper reports that the mass spectrometry occurred independently of the HPLC separation. Therefore, further steps were taken to adopt the direct MS method into a working LC-MS/MS method with a mobile phase containing acetic acid.

Direct infusion into the MS was performed using an SDR Scientific syringe pump (model no. 55-4150EU) at the rate of 1 µL/min. The MS conditions were adjusted in the MassHunter Workstation to provide the 'softest' conditions to avoid any in-source fragmentation. The capillary voltage of the ionisation source was + 4000 V, the fragmentor voltage was optimised at 75 V (range from 5 – 225 V), and the cell voltage was set to 1 (range from 0 – 8, no unit). The nebuliser pressure was 10 psi, and the nitrogen drying gas temperature was 300 °C. The MS was set to a full-scan ESI+ mode with a *m/z* range between 100 and 800 amu.

Two ATM-based preparations were created: A) 0.01 % ATM *w/v* in 50:50 methanol/0.1 % acetic acid *v/v*, B) Solution A with 100 µM DDA. The peak area for the major ions in both Solutions A and B were recorded and the analyte identified from the *m/z* ratio (Table 2.3).

The initial mobile phase composition was derived from the direct infusion conditions; using 0.1 % *v/v* acetic acid in Millipore water (A) and 0.1 % *v/v* acetic acid in methanol (B)

(50:50) at a flow of 0.4 mL/min (Souppart *et al.* 2002; César *et al.* 2011). The Zorbax SB-C18 analytical column temperature was set at 35 °C.

Table 2.3 presents the five most abundant ions detected in the direct injection of Solutions A (0.1 % ATM) and B (0.1 % ATM + 100 µM DDA). None of the five ions represents the expected ion $[M+H]^+$ for intact ATM. Instead, the top three ions were adducts of ATM and either DDA or sodium (Na). The most abundant *m/z* ratio was 484, indicating an adduct of ATM (298 amu) with DDA (185 amu) and hydrogen (1 amu), demonstrating the possibility of stabilising ATM with DDA.

Table 2.3. The five most abundant ions of Solutions A (0.1 % ATM) and B (0.1 % ATM + 100 µM DDA).

m/z	Peak Area (x 10^6)		Formulation
	Solution A	Solution B	
484	-	1.56	$[ATM+DDA+H]^+$
619	1.49	1.49	$[2ATM+Na]^+$
321	1.27	1.05	$[ATM+Na]^+$
267	1.00	0.97	$[ATM\ Fragment]^+$
221	1.01	0.81	$[ATM\ Fragment]^+$

With the success of creating an ATM+DDA adduct through direct injection, the components of the injection solution were adapted into a mobile phase program. The initial conditions were an isocratic program of 0.1 % *v/v* acetic acid in Millipore water (A) and 0.1 % acetic acid in methanol (B) at a 50:50 ratio. However, LUM did not elute within an acceptable time (< 60 minutes). Therefore, the organic modifier of mobile phase B was changed to ACN. The ratio of A to B were adjusted numerous times to achieve adequate chromatography. With 75 % B, both ATM and LUM eluted at an acceptable RT of fewer than 10 minutes). However, ATM was predominantly observed as the fragment *m/z* 267. Moreover, a high abundance of

$[DDA+H]^+$ (*m/z* 186) suggested that the conditions were not as conducive for forming the $[ATM+DDA+H]^+$ adduct.

2.2.7. Conclusions.

Reproducible identification of unfragmented ions of ART derivatives and their degradants using LC-MS/MS was not possible. The results collected did not provide reliable mass information. Consequently, the identification was based solely on the RT of the analytes and not the mass information. As identified in the literature search detailed in Chapter 1, 61 % (n = 37) of the included surveys related to antimalarials reported having access to an HPLC while only 11 % (n = 7) reported using a combination of HPLC and mass spectrometry (i.e. LC-MS/MS). Therefore, adapting the method for HPLC application grants the ability to increase the reach and application of the method, while avoiding the challenges associated with the in-source fragmentation, particularly with respect to the degradant peak identification. Therefore, the focus turned to the development of a HPLC separation using photo diode array (PDA) detection of ART and LUM.

2.3. The Development of a High-Performance Liquid Chromatography-Photo Diode Array (PDA) Method for the Detection of Artemether and Lumefantrine.

2.3.1. Materials and Chemicals.

The reference material of the target APIs was ATM and LUM, and the IS' were ATS and LOS. The organic mobile phase was LiChrosolv® ACN (Merck KGaA, Darmstadt, Germany) and the aqueous mobile phase comprised of Millipore grade water obtained from Sartorius Arium 611VF water purification system with an inorganic modifier. Malonic acid ($CH_2(COOH)_2$, > 99 %, product no. M1296-100G), ammonium hydroxide solution (NH_4OH, 28 - 30 % *w/v*, product no. 221228500MLA), phosphoric acid (H_3PO_4, 85 % *w/v*, product no. 695017-2.5L), formic acid, ammonium acetate, and ammonium formate were purchased from

Sigma Aldrich (St Louis, Missouri, USA). Triethylamine (TEA, $(C_2H_5)_3N$, 98 %, product no. 2766710) was manufactured by The British Drug House Limited (Poole, England). All solutions were filtered through 0.22 μm nylon discs under vacuum prior to use.

2.3.2. Instrumentation.

Separation was achieved using an Agilent 1200 series HPLC system. The chromatography column was again an Agilent Technologies Zorbax SB-C18 Rapid Resolution HT 2.1 x 50 mm (1.8-Micron, 82770-902) analytical column. Detection was performed with a photodiode array (PDA) detector with a target range from 200 to 400 nm for peak identification. The wavelengths specifically used for API detection were: 200 nm for ATM and ATS and 250 nm for LUM and LOS.

2.3.3. Optimisation of Mobile Phase Conditions.

Initial HPLC-PDA parameters were based on the previous LC-MS/MS method development (see Section 2.2). The mobile phase composition was 0.1 % *v/v* acetic acid as Solvent A and ACN as Solvent B. The mobile phase conditions were further developed through adaptation of a buffer to achieve a constant pH. For all buffer solutions, an isocratic elution program was set at a solvent ratio of 60:40 A/B at 0.6 mL/min and the column temperature was maintained at 35 °C.

Multiple aqueous mobile phases (A) were made to the pH levels of 2.5, 3.0, and 4.0, and were used in the separation of ATM, LUM, and their IS. Buffers of formic acid and ammonium formate in Millipore water at different ratios created solutions with the pH of 2.5 and 3.0. A buffer of acetic acid and ammonium acetate in Millipore water created the pH 4.0 solution. The three aqueous mobile phase solutions were interchanged as Solvent A while Solvent B remained ACN throughout.

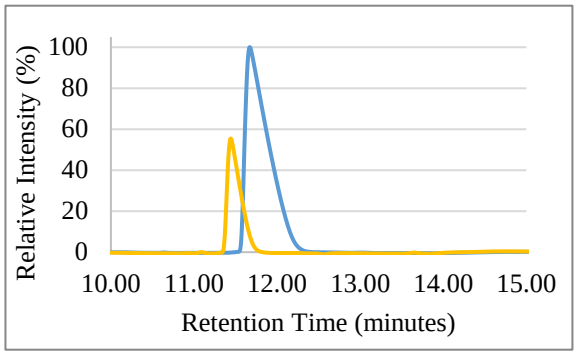
During the method development three aqueous mobile phase (solvent A) solutions were evaluated in combination with ACN as solvent B (26.5 mM formic acid/ammonium formate

pH 2.5, 26.5 mM formic acid/ammonium formate pH 3.0, and 17.5 mM acetic acid/ammonium acetate pH 4.0).

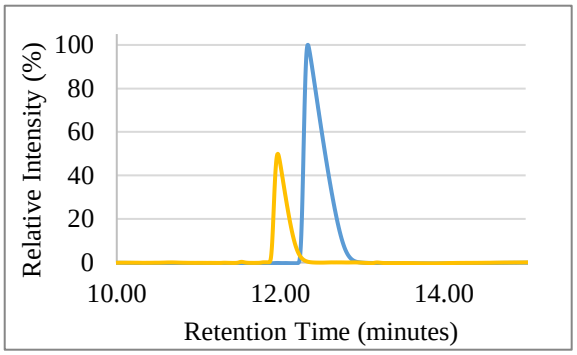
The two ATM and ATS peaks were easily resolved in all three pH conditions. In addition, there were no significant changes observed in peak shape for the two analytes. However, being a structural analogue of LUM, LOS demonstrated very similar RTs to LUM, and a pH buffer was necessary to achieve constant separation between the two analytes.

The change in RT and peak shape for 50 µg/mL LUM and LOS because of changing the mobile phase pH can be seen in Figure 2.6. At pH 2.5, poor peak resolution between LUM and LOS was observed. While pH 3.0 did not achieve complete baseline separation between the two analytes, there was adequate separation while still maintaining an acceptable peak shape with some peak tailing occurring. Baseline separation between LUM and LOS was achieved at pH 4.0; however, there was significant tailing observed in the LUM peak and a reduction in the relative intensity as a result. Furthermore, known impurities in the LOS standard elute immediately before LOS and could, therefore, contribute to the peak area of LUM under pH 4.0 conditions. Therefore, pH 3.0 was implemented for on-going experimentation.

a)



b)



c)

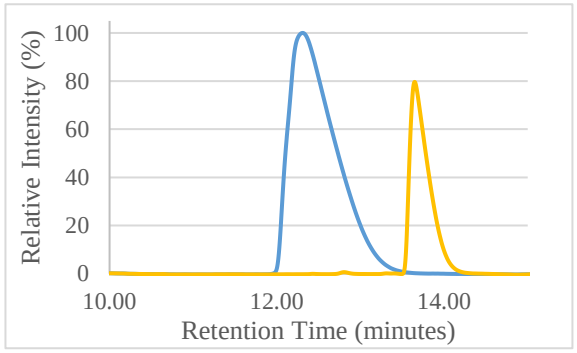


Figure 2.6. Chromatograms of LUM and LOS in changing pH of the Solvent A: a) pH 2.5, b) pH 3.0, c) pH –4.0. Legend: LUM = blue, LOS (IS) = yellow. Chromatographic conditions: gradient elution at 40 % B held for 8 mins, increasing to 70 % B over 2 mins, held for 4 mins before re-equilibrating to 40 % B over 3 mins and holding for 4 mins. Separated using an Agilent Technologies Zorbax SB-C18 Rapid Resolution HT 2.1 x 50 mm x 1.8-micron held at 30 °C. Flow rate constant at 0.6 mL/min.

2.3.3.1. *Precipitation of LUM in the HPLC-PDA Mobile Phase.*

During the validation process, unpredictable HPLC system pressure increases were observed during analysis. After troubleshooting the issue, it was concluded that the pressure increases were due to precipitation of LUM in the mobile phase after injection into the HPLC system. ATM has previously demonstrated that it is readily soluble in ACN; however, LUM required acid to ionise the compound before dissolving (Osei-Safo *et al.* 2014). LUM and LOS had been initially dissolved in 0.1 % *v/v* phosphoric acid in a Millipore water solution (Sridhar *et al.* 2010; Kalyankar and Kakde 2011). However, after injection, the analytes are suspended

in the mobile phase; therefore, the mobile phase must be able to maintain LUM and LOS in solution and be compatible with HPLC and/or LC-MS/MS analysis. Unfortunately, the phosphoric acid in the first dissolving solution is not compatible with LC-MS/MS (Dolan 2016). With intentions for the COPA protocol be adapted for LC-MS/MS in the future, phosphoric acid could not be used and therefore, alternate acids were assessed as the dissolving solvents.

Different acid-containing solutions were assessed for two criteria: a) ability to dissolve LUM at 500 µg/mL concentration as the extraction solution and b) compatibility with analysis using HPLC-PDA and LC-MS/MS. The acids assessed were: a) 0.1 % *v/v* formic acid, b) 0.5 % *v/v* formic acid, and c) 0.1 % *w/v* malonic acid. All solutions were adjusted to pH 3.0; formic acid solutions were buffered with ammonium formate, and malonic acid was pH adjusted with 10 % *w/v* ammonium hydroxide. The extraction solution consisted of a combination of 60 % acidic solution and 40 % ACN (*v/v*).

Firstly, solid LUM powder was weighed, dissolved in solution and sonicated for a minimum of 15 minutes. The extraction solution was visually inspected for precipitated LUM. The extraction solution containing 0.1 % *v/v* formic acid was not capable of completely dissolving LUM at a concentration of 500 µg/mL after sonication based on visual appearance. Both the 0.5 % *v/v* formic acid and 0.1 % *w/v* malonic acid solutions were capable of dissolving LUM. Therefore, these two solutions were progressed to assess compatibility with HPLC-PDA analysis.

Secondly, the buffer solutions were made as solvent A and ACN as solvent B. The combination of the two solvents was required to present a limited effect on the HPLC system pressure (indicating no blockages). The analysis of blank samples indicated that 0.5 % *v/v* formic acid overloaded the PDA-detector, given ultraviolet (UV) absorbance of formic acid is highest in the low- to mid-200 nm range (Singleton *et al.* 1987). As this range is used for the

detection of ATM, the interference from formic acid was not acceptable. The malonic acid-based mobile phase did not cause such interference and therefore, was included in further chromatographic method development.

2.3.4. Extraction of ATM and LUM Co-Formulated Tablets.

Tablets were firstly weighed for total mass and then crushed into a fine powder using a Silent Knight® Pill Crusher (product no. SK0500, Links Medical Products, Inc.) inside low-density polyethylene pouches (product no. PC1000, Links Medical Products, Inc.). The powdered tablets portions analysed for ATM were dissolved in ACN (and then filtered through 0.22 µm nylon filter). The powdered tablet portions analysed for LUM were dissolved in a mixture of mobile phase solvents A and B (60:40 *v/v*). The concentration of the extract was altered depending on the experiment. All solutions were then sonicated for 30 minutes, including vortexing three times, before filtration through a 0.45 µm nylon filter to remove any insoluble material. The filtered extract was then diluted to the required concentration for analysis.

Due to the partial solubility of LUM in ACN, a consequence of the above extraction method was that varying amounts of LUM would be present in an ATM extract. However, the relatively low concentration of LUM in the extract, and the above chromatographic conditions, there is no negative repercussion in its presence as any ATM present in the LUM extract is below the LOD of the instrumentation.

2.3.5. Development of the Chromatographic Conditions.

2.3.5.1. *Optimisation of Resolution of ATM and Harsh Degradant Peaks.*

The starting conditions were based on the previous mobile phase composition of 40 % B. To further refine ATM's peak shape and to provide adequate separation of degradant peaks the starting mobile phase ratio was slightly altered to assess 40 % B, 43 % B, and 45 % B. The multiple degradant peaks generated after the harsh degradation of ATM that were

presented above in Figure 2.5 (see Section 2.2.5) eluted in the minutes preceding ATM. Therefore, the potential presence of these peaks was considered when assessing the optimal mobile phase ratio.

The increase in the organic phase to 45 % B saw a dramatic increase in the resolution of the ATM peak. However, the RT decreased to such an extent that there would not be adequate time for the satisfactory separation of the degradant peaks. A starting ratio of 43 % B provided an adequate compromise between ATM peak shape and RT. Therefore, 43 % B was used at the starting point for future method development.

2.3.5.2. *Improving LUM Peak Shape with TEA and a Gradient Program.*

Two approaches were taken to improve the baseline separation between LUM and LOS in addition to improving the peak shape; adding TEA to the mobile phase and applying a gradient elution program.

TEA is a common counter to the tailing of basic compounds as it competes for uncapped silanol sites in the column, as demonstrated in (Kalyankar and Kakde 2011) when 0.05 % *v/v* TEA was added to improve LUM peak shape. Three concentrations were assessed for the impact on reducing LUM tailing and effect on the mobile phase buffer pH; 0.01 %, 0.05 %, and 0.10 % *v/v* TEA. Adequate peak improvements were witnessed with 0.01 % *v/v* TEA with minimal pH effects on the mobile phase. To further minimise the pH effect, TEA was added to the mobile phase before the final pH adjustment with ammonium hydroxide.

While determining the optimum conditions for LUM and LOS peak shape and separation, a gradient profile with an immediate increase in % B instead of a progressive ramp was investigated. Adjustments in % B during the gradient ranging between 65 – 75 % (before selecting 75 %), changed the RT for the analytes but proportionally increased tailing, which resulted in no baseline separation. The gradient was slowly applied over two-minutes after the

elution of ATM and before LOS. There was significant tailing seen for both LUM and LOS inhibiting baseline separation between the two analytes using these gradient conditions.

2.3.6. Final Chromatographic Conditions.

The aqueous phase consisted of 1 % *w/v* malonic acid and 0.01 % *v/v* TEA in Millipore water (solvent A) adjusted to 3.0 (± 0.03 pH) with 10 % *w/v* ammonium hydroxide in Millipore water. The organic phase consisted of LiChrosolv® ACN (solvent B). All solutions were then filtered through 0.22 µm nylon discs under vacuum (Grace Davison Discovery Sciences, Rowville, Victoria).

The gradient elution program was initiated at 43 % B, increasing to 75 % B at eight minutes over two minutes, holding for four minutes before re-equilibrating to 43 % B over three minutes and holding for four minutes (Figure 2.7). ATM and ATS were best observed on the 200 nm UV-wavelength. LUM and LOS were best observed on the 250 nm UV-wavelength. The RT (± 10 %) for ATS (IS) was 2.07 minutes, ATM was 6.72 minutes, LOS (IS) was 11.78 minutes, and LUM was 12.07 minutes (Figure 2.7). RT shift was adjusted by using the IS' RT as references. Each peak was confirmed by observing the UV spectrum (200 – 400 nm) at the peak apex.

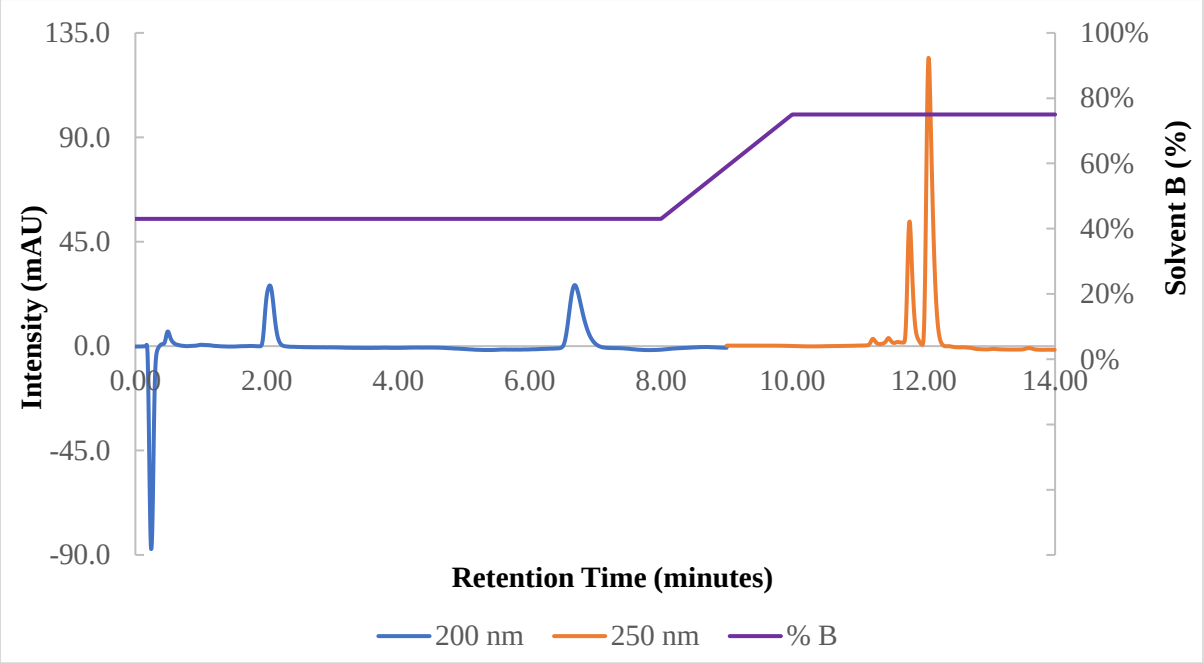


Figure 2.7. Final chromatogram conditions observed at 200 nm (0 - 8 minutes) and 250 nm (8.00 - 14.00 minutes). Left to right, Peak 1 = ATS, Peak 2 = ATM, Peak 3 = LOS, and Peak 4 = LUM. Chromatographic conditions: gradient elution (shown in purple) at 43 % B held for 8 mins, increasing to 75 % B over 2 mins, held for 4 mins before re-equilibrating to 43 % B over 3 mins and holding for 4 mins. Separated using an Agilent Technologies Zorbax SB-C18 Rapid Resolution HT 2.1 x 50 mm x 1.8-micron held at 30 °C. Flow rate constant at 0.6 mL/min.

2.4. Validation of the HPLC-PDA Method.

The following validation was performed in accordance with International Organisation of Standardisation 17025 standards, following the guidelines of the National Association of Testing Authorities Technical Note 17 (National Association of Testing Authorities 2013). Guidelines for the validation components not covered thoroughly under the Technical Note (e.g. stability) were sought in other literature (Peters *et al.* 2007).

2.4.1. Materials and Chemicals.

The target APIs were ATM and LUM, and the IS' were ATS and LOS. Dihydroartemisinin (DHA, > 98 %, product no. D3793) and ART (> 97 %, product no. A2118)

were procured through Tokyo Chemical Industries (Tokyo, Japan). Sulfadoxine (SUL, > 95 %, product no. S7821-10G), amodiaquine dihydrochloride dihydrate (AMO, analytical grade, product no. A2799-5G), and pyrimethamine (PYR, analytical grade, 46706-250MG) were procured through Sigma-Aldrich (St Louis, Missouri, USA).

Macleods Pharmaceuticals Limited (Mumbai, India) donated branded Lumiter tablets (batch no. 15TAI061, expiry date 11/2018) and the powder API used to manufacture that batch; ATM (batch no. AM2/20150805) and LUM (batch no. RMG-1120/15).

2.4.2. Selectivity.

Selectivity was tested through analysing five common antimalarial API. Standards of the antimalarials; ART, DHA, and AMO were created at a concentration of 1 mg/mL, and SUL and PYR were created at a concentration of 0.5 mg/mL. No interference was demonstrated if the potential interferent API was observed with a different RT and spectrum shape when compared with the target analytes.

AMO (0.247 mins), ART (2.513 mins), and SUL (0.380 mins) presented as single peaks while DHA and PYR portrayed as two peaks at 1.100 then 1.733 minutes and 0.273 then 0.560 minutes, respectively. All the RTs are outside the acceptable limits (± 2.5 %) target API's RT. AMO and PYR are seemingly unretained as both APIs elute at the same RT solvent front, observed as the dip in the baseline intensity at approximately 0.253 minutes. SUL appears to have slight retention, eluting shortly after the solvent front. However, there is adequate differentiation between all four API.

2.4.3. Linearity

For the measurement of linearity, calibration curves were investigated using a linear regression model and required a minimum of four replicates at five concentration levels. Calibrator concentrations for ATM were 100, 200, 500, 1250, 2500 µg/mL and 5.0, 10, 25, 50,

125 µg/mL for LUM, with an injection volume of 5 µL. Linearity is expressed as a coefficient of determination (r^2) and was calculated using Microsoft Excel.

Based on a residual analysis, all calibration models possess a linear regression model with no weighting. The dependent variable was generated as a peak area ratio of the analyte of interest divided by its accompanying IS. As seen in Figure 2.8, the r^2 value for ATM was 0.9996 and LUM was 0.9949, indicating a high linear correlation.

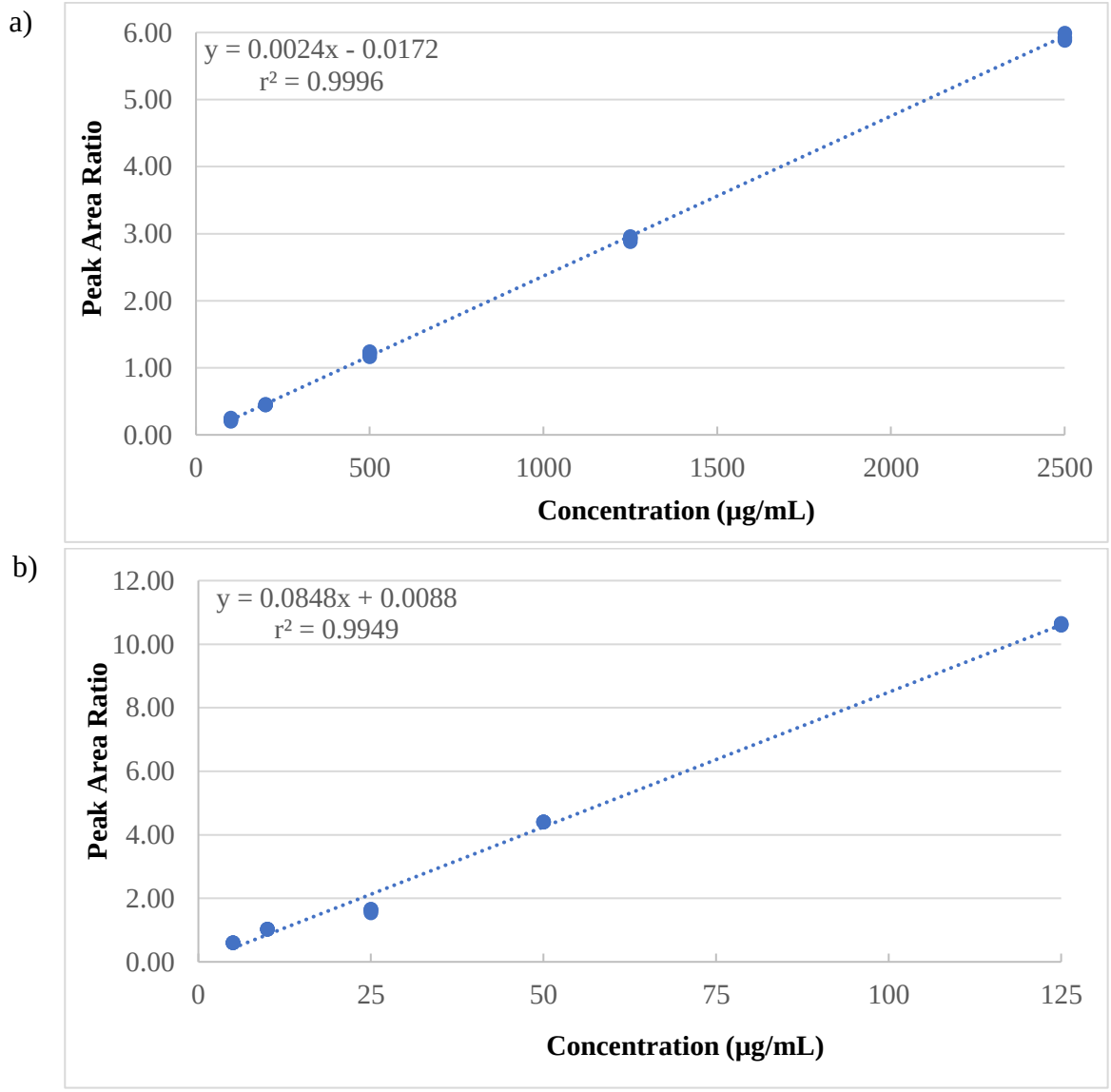


Figure 2.8. Five-point calibration curve for a) ATM (r^2 = 0.999) and b) LUM (r^2 = 0.995).

2.4.3.1. *Limits of Quantitation and Detection.*

The lower limit of quantitation (LOQ) was determined to be the lowest concentration on the calibration curve that met the following criteria; produced a Gaussian-shaped peak, demonstrated a RT of ± 0.5 minutes of the calibrator mean, had a minimum signal to noise (*S/N*) ratio of 10:1, and demonstrated adequate precision and accuracy (≤ 20 %). The lower limit of detection (LOD) is the lowest concentration that meets the same criteria as the LOQ except only requires a *S/N* ratio of 3:1.

The upper limit of quantitation for LUM was determined to be the highest calibrator that presented a Gaussian-shaped peak with baseline peak separation. The upper and lower limits of quantitation form the measuring interval for each API. For the tested concentrations of ATM, the lower LOD and LOQ were demonstrated at 100 µg/mL. For LUM, the lower LOD and LOQ were determined to be 5 µg/mL.

2.4.4. Accuracy and Precision.

To assess the accuracy and precision, three quality control (QC) replicates at a low (QCL), medium (QCM), and high (QCH) concentrations for both ATM and LUM were evaluated.

The three QCs for were 120, 600, and 1800 µg/mL for ATM and 6, 30, 80 µg/mL for LUM, corresponding to QCL, QCM and QCH, respectively.

Repeatability was assessed with five replicates of three QC samples in a single day (degrees of freedom = 4) while intermediate reproducibility was evaluated using three QC standards in five replicates per day initiated on four different days (degrees of freedom = 19). Accuracy is expressed as a percentage of the expected concentration (%). Precision was measured as the percent coefficient of variance (% CV).

For valid accuracy and precision results, the outcome of analysis needed to be within ± 20 % for QCL and ± 15 % for QCM and QCH (Peters *et al.* 2007). As seen in Table 2.4, the

required acceptance levels were met in all occurrences except for the QCM of LUM on a single day, which recorded an intra-assay accuracy of 81 %. However, the inter-day accuracy of the QCM of LUM was within the validation criteria.

Table 2.4. Assessment of the accuracy and precision of the method.

	ATM			**LUM**		
QC Level	QCL	QCM	QCH	QCL	QCM	QCH
Nominal conc. (µg/mL)	120	600	1800	6	30	80
Intra-day accuracy (%) Range	91 - 114	111 - 112	108 - 114	92 - 113	81 - 107	89 - 108
Intra-day precision (%) Range	3 - 11	2 - 6	0 - 2	0 - 1	0 - 1	0 - 1
No. of determinations	5	5	5	5	5	5
Inter-day accuracy (%)	105	111	112	99	97	96
Inter-day precision (%)	11	3	3	9	11	8
No. of determinations	20	20	20	20	20	20

2.4.5. Ruggedness.

The method was measured for its susceptibility to changes in mobile phase pH, temperature control of the column, and flow rate to assess the ruggedness of the HPLC-PDA method. The regular conditions for these conditions were pH of 3.0, a column temperature of 30 °C, and a flow rate of 0.6 mL/min. The HPLC method was considered resistant to pH and temperature changes if the RT for all four analytes of interest were less than 2.5 % and there was still acceptable baseline separation.

2.4.5.1. *Adjusting the Mobile Phase pH.*

The pH of the mobile phase was assessed at 2.9 and 3.1, representing ± 0.1 pH of the target pH. The initial solutions were created by dissolving 0.1 % *w/v* malonic acid in Millipore water and adding 0.01 % *v/v* TEA; however, different volumes of 10 % *w/v* ammonium hydroxide were used to achieve the target pH.

There were no observed changes in RT for ATM or ATS. At pH 3.1, there were minor fluctuations in the baseline that did not affect the detection of ATS or ATM. There were changes in the RT for LUM and LOS. However, both analytes were affected equally, which did not impact the baseline separation, and the RT changes were less than 2.5 %. Therefore, the method was proven to be resistant to pH changes between the range of 2.9 to 3.1 pH.

2.4.5.2. *Adjusting the Column Temperature.*

Two column temperature settings were assessed at 30 °C and 40 °C, representing ± 5 °C of the normal column temperature, 35 °C. All three temperatures are within the limitations of the column of 60 °C max.

There were no observed changes for ATS at either temperature setting. For ATM, 30 °C and 35 °C produced identical RTs; however, at 40 °C there was an RT shift of 4.2 % that is outside the acceptable RT change. Again, there were changes in RT for LUM and LOS. However, both analytes were affected equally, which did not impact the baseline separation, and the RT changes were less than 2.5 %. Therefore, the method was only found to be unaffected when the temperature fluctuated between 30 °C and 35 °C.

2.4.6. Recovery.

Sample preparation involves separating ATM and LUM based upon different solubility in ACN and subsequent filtration using 0.45 µm nylon filtration discs. To assess the impact of the filtration step, three sample preparation series were performed: a) Triplicate QCs for the three concentration levels (QCL/QCM/QCH) without filtration, b) triplicate QCs for the three levels filtered through the 0.45 µm nylon disc, and c) five replicates of dissolved ATM/LUM tablets (QCM) filtered through the 0.45 µm nylon disc. Recovery was quantitatively measured as a percentage of the expected concentration using a five-point calibration curve.

Using laboratory standards, the recovery for ATM without the extraction step returned on average recovery of 88 % for QCL, 103 % for QCM, and 101 % for QCH. After the inclusion

of an extraction step the recovered percentages were 86 % for QCL, 104 % for QCM, and 97 % for QCH. The five replicates generated from powdered tablets demonstrated an average of 98 % at an equivalent QCM concentration.

Comparing the process of extraction through filtration ($\bar{x}$ = 96 %, s = 8 %) and no filtration ($\bar{x}$ = 97 %, s = 8 %) for ATM presented no significant difference ($t(16)$ = -0.38, p 0.05). When comparing extracted laboratory standards and extracted powdered tablets ($\bar{x}$ = 98 %, s = 4 %) for ATM demonstrated no significant difference ($t(12)$ = -0.64, p 0.05).

Using laboratory standards, the recovery of LUM without the extraction step was on average 97 % for QCL, 92 % for QCM, and 95 % for QCH. The inclusion of an extraction step recovered 97 % for QCL, 92 % for QCM, and 98 % for QCH. The five replicates generated from powdered tablets demonstrated an average recovery of 97 % at an equivalent QCM concentration.

Comparing the process of extraction via filtration ($\bar{x}$ = 90 %, s = 5 %) and no extraction ($\bar{x}$ = 92 %, s = 4 %) presented no significant difference for ATM ($t(16)$ = -0.38, $p \leq 0.05$). Comparing the process of extraction via filtration ($\bar{x}$ = 96 %, s = 4 %) and no extraction ($\bar{x}$ = 85 %, s = 6 %) presented no significant difference for LUM ($t(16)$ = 0.64, $p \leq 0.05$). When comparing extracted laboratory standards ($\bar{x}$ = 90 %, s = 5 %) and extracted powdered tablets ($\bar{x}$ = 99 %, s = 4 %) for ATM demonstrated no significant difference $t(12)$ = -0.64, $p \leq 0.05$. When comparing extracted laboratory standards ($\bar{x}$ = 85, s = 6 %) and extracted powdered tablets ($\bar{x}$ = 103 %, s = 14 %) for LUM demonstrated no significant difference $t(12)$ = -0.28, $p \leq 0.05$. Therefore, the use of a filtration step as part of the extraction and analysis process did not significantly affect the overall recovery of the target analytes.

2.4.7. Matrix Effects.

Matrix effects were considered to come from potential influences of tablet excipients on the analysis of the two API. To assess this influence, two peak area calibration curves of

three concentration levels, in duplicate, were analysed; one made from extracted tablet samples and another from the raw API used to make the tablet batch. The three calibration levels were Level 1, 3, and 5, for both API (see Section 2.4.2). Matrix effects, either suppression or enhancement, were observed if the slope of the three-point calibration curve presented a difference of more than 10 %.

For ATM and LUM, the slope of the tablet trendline was 4 % and 7 %, different to the trendline of the raw API, respectively; therefore, indicating acceptable matrix effects.

2.4.8. Stability.

Stability was quantitatively measured as a percentage of the expected concentration using a five-point calibration curve. Stability during each process was achieved when the concentration of the QC samples did not deviate outside the accepted limits of ± 20 % for QCL and ± 15 % for QCM and QCH.

2.4.8.1. *Short-Term Stability.*

Short-term stability was assessed with triplicate QC samples at two concentration levels (QCL/QCH) for both API. The QCs were stored in the refrigerator at 4 °C for a maximum of seven days. The concentrations of the QCs for initial reading (t = 0) were compared to those on day seven (t = 7), using the corresponding five-point calibration curve. The analytes were reported to be stable in the refrigerator (4 °C) over a short-term if the average % change in concentration for each QC level was less than 10 %.

The percent change for QCL and QCH for ATM were 6 % and 1 %, respectively. The absolute percent change for QCL and QCH for LUM were 3 % and 1 %, respectively. These values indicate that the standards are stable in the refrigerator for seven days.

2.4.8.2. *Freeze/Thaw Stability.*

Freeze/that stability was assessed with triplicate QC samples at two concentration levels (QCL/QCH) for both API. After an initial analysis, the standards then underwent three

freeze/thaw cycles before subsequent analysis. Also, two sets of two tablet samples from an identical blister pack were analysed. One set was analysed to generate a baseline measurement, while the other set underwent three freeze/thaw cycles before subsequent analysis. The tablets underwent sample preparation, as detailed in Section 2.3.4, to create separate ATM and LUM extracts. The extracted solutions were diluted to the QCH concentrations prior to analysis.

For the ATM standards, two of the low QCs saw a 9 % gain in the calculated concentration and was therefore within the accepted content levels. The remaining standards were all within ± 3 % of the initial readings. For the two ATM tablet extracts, the percent change in calculated concentration was 2 % and 4 %.

For the LUM standards, in all situations except for one high QC, the difference between the initial and the final concentrations were less than ± 4 %, and therefore within acceptable limits at 15 %, except for the one QCH. The LUM tablet extracts were analysed at an average content of 92 %, remaining within the acceptable content requirement.

2.4.8.3. *Autosampler Stability.*

Triplicate QC standards at three concentration levels (QCL/QCM/QCH) for both API were analysed every four hours over 48 hours while located in the autosampler at room temperature (n = 12 for each QC level). Results are expressed as % CV of the calculated concentration over the total timeframe. The standards were stable in the autosampler over the analysis period if the % CV was less than 10 %.

The % CV for ATM over the 48-hour period was 8 %, 3 %, and 2 % for QCL, QCM, and QCH, respectively. The % CV for LUM over the 48-hour period was 1 %, 2 %, and 0 % for QCL, QCM, and QCH, respectively. These values indicate that the analytes are stable in the autosampler over a 48-hour period.

2.4.8.4. *Long-Term Stability.*

Duplicate tablet samples were stored in a – 20 °C freezer for 337 days. The tablets underwent sample preparation as detailed in Section 2.3.4, to create separate ATM and LUM samples. The extracted solutions were diluted to the QCH concentrations prior to analysis.

For LUM and ATM, the average recovery of the duplicate samples was 91 %, and 107 %, respectively, therefore remaining within the acceptable content requirements.

2.4.9. Carryover.

Samples derived from tablet extracts were made up to 150 % of the QCH concentrations for ATM and LUM. Three replicate injections of the extract standards for both ATM and LUM were followed by a zero standard, a blank containing the IS' of both target API, ATS and LOS, respectively. The concentration of the IS, ATS and LOS, were 450 and 15 µg/mL, respectively. Carryover was observed if the zero-standard following the high concentration sample injection presented a higher peak area ratio than that of the respective LODs for ATM and LUM. It was expected that due to the tablet extraction method, the ATM extract would contain LUM at a high concentration (see Section 2.4.3). Therefore, the following zero blank contained both ATS and LOS.

In all six replicates, there were no concentrations of ATM nor LUM that were above their LOD. Therefore, no carryover was demonstrated.

2.5. Application of the Validated HPLC-PDA for Assessing Degradant Profiles.

The above sections demonstrate a validated HPLC method for application in the COPA protocol to determine the quality of the API within ATM and LUM co-formulated antimalarials. However, for the HPLC method to fulfil the requirements to accurately characterise poor-quality antimalarials, the method must also measure any potential degradation. The following section details how the validated HPLC method can be applied in determining degraded ATM and LUM co-formulated antimalarials.

2.5.1. Harsh Degradation Study.

Five co-formulated tablets of ATM and LUM, branded as Lumiter (batch no. 15TAI061, expiry 11/2018, Macleods Pharmaceuticals), were first weighed and then crushed to a powder using the Silent Knight tablet crusher (as in Section 2.3.4). For each tablet, portions of the powder, equivalent to 10 mg of both API, were measured and placed into a 20 mL glass scintillation vial. As Lumiter tablets (strength 20/120) weighed approximately 240 mg total, the approximate portion size for ATM was 120 mg and 20 mg for LUM.

The degradation studies for the two API were performed separately. For LUM, the powder was placed in a Labec laboratory oven (model no. OHCH36SS, NSW, Australia) at 125 °C for 15-minute increments, up to 60 minutes (n = 5). The melting point for ATM is 125 °C, therefore, the powder was placed in the oven at 90 °C for 0, 15, 30, 60, and 120 minutes (n = 5). After removal from the oven, the plastic lids of the scintillation vials were placed on the vials, and the vials were placed in the - 20 °C freezer for approximately five minutes to cease any further degradation.

The heat-treated powders were then dissolved and filtered using the procedure in Section 2.3.4 and diluted to concentrations of 2000 µg/mL for ATM and 90 µg/mL for LUM, prior to HPLC analysis (Section 2.3.6).

The three common detected degradant peaks were detected, and peak area measured, at the 200 nm wavelength. Peak 1 and 3 were associated with ATM, and Peak 2 was associated with LUM. Figure 2.9 presents recorded chromatograms (t = 0, 30, 120 minutes) that include these three peaks (bordered in red), in addition to ATM and ATS (bordered in blue).

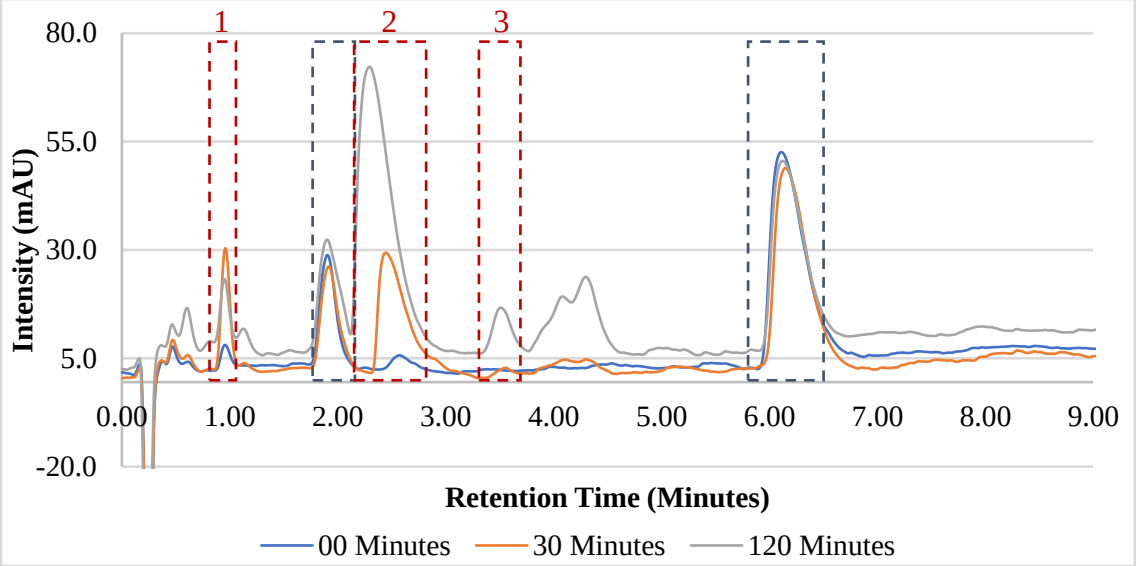


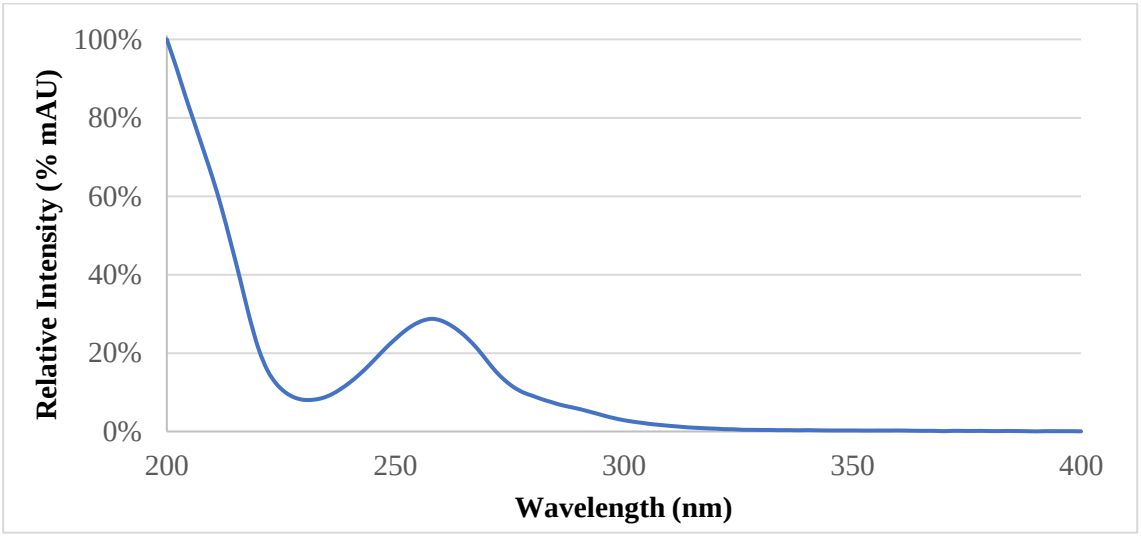
Figure 2.9. Resulting chromatogram (200 nm) of the ACN extract from ATM/LUM tablets that were kept in an oven at 90 °C for 0, 30 or 120 minutes. Degradant Peak 1 (0.95 mins), Degradant Peak 2 (2.35 mins) and Degradant Peak 3 (3.72 mins); all bordered in red. ATM (6.17 min) and ATS (1.95 min); both bordered in blue. For chromatographic conditions see Figure 2.6.

The degradant peaks were best observed at the 200 nm wavelength, which is the wavelength in which ATM was observed. However, this observation does not infer that the degradants are products of ATM. By consequence of generating a concentrated sample for ATM, the ATM extract also contains a higher concentration of the degradant products. Therefore, the peak area for the three degradants listed in Figure 2.10 were measured at 200 nm, (the same wavelength as ATM), as the degradants were more concentrated in the ATM extract.

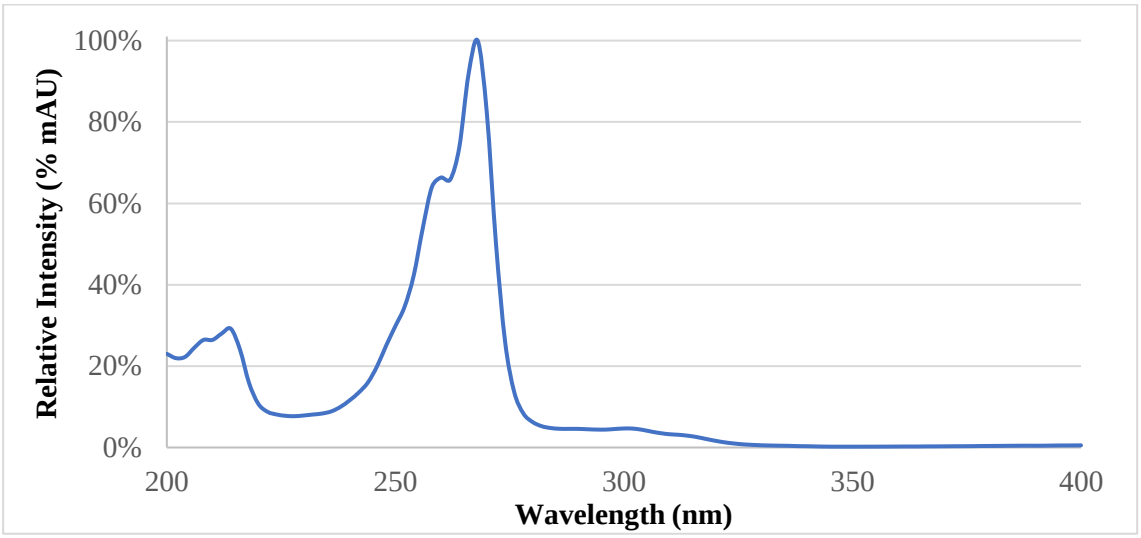
Degradant Peak 2 presents a uniquely shaped spectrum (Figure 2.10) with optimal absorbance at 268 nm and a characteristic shoulder at 262 nm, different to both Degradant Peaks 1 and 3. The characteristic structure of Degradant Peak 2 has been previously reported in Verbeken *et al.* (2011), which associated that spectrum shape with a desbenzylketo (DBK) derivative of LUM. The structure of DBK is a possible option as a degradant product of LUM;

however, confirmation of the degradant product would require mass confirmation and structural elucidation.

a) Degradant Peak 1 $\bar{x}$ RT = 0.95 minutes



b) Degradant Peak 2 $\bar{x}$ RT = 2.35 minutes



c) Degradant Peak 3 $\bar{x}$ RT = 3.72 minutes

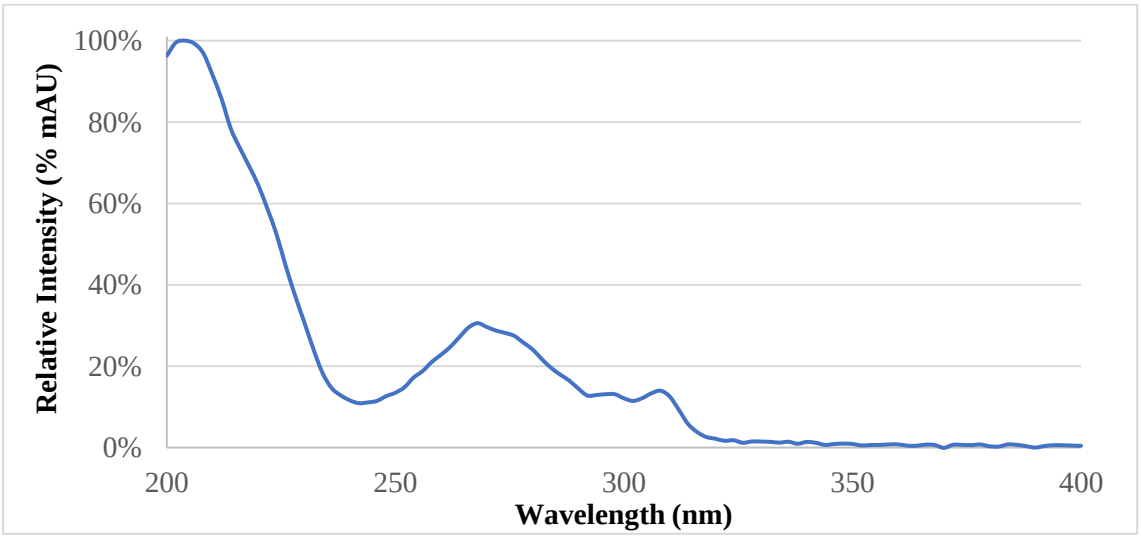


Figure 2.10. The spectrum of three degradant peaks (RT 0.95, 2.35 and 3.72 mins), spectrum range between 200 to 400 nm.

For Degradant Peak 1, the plot of the degradant peak ratio (DPR) over time indicates secondary degradation of the Peak around the 30-minute time-period (Figure 2.11). For the first three time-periods, the DPR increases, as is expected as the degradant is generated from the decomposition of the API. However, the slope from 30 minutes to the final time-period decreases to approximately 50 % of the recorded maximum. Two trendlines that explain the increase and decrease time-periods (n = 3) both demonstrate a strong r^2 value for the linear relationships; validating the secondary degradation hypothesis.

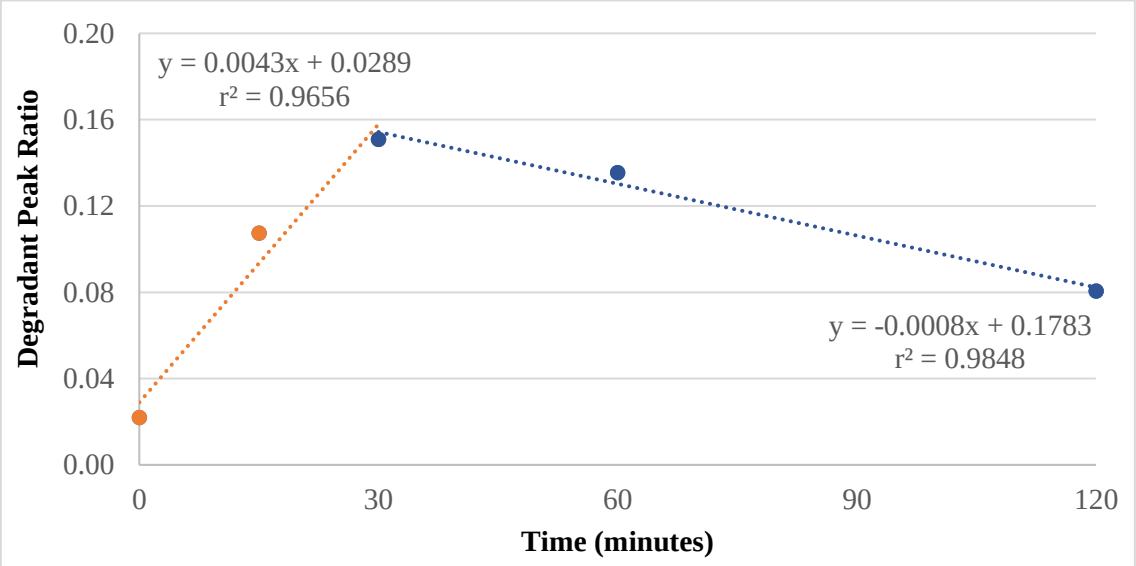


Figure 2.11. The DPR dependent on the time stored at 90 °C.

The secondary degradation that is observed for Degradant Peak 1 may negatively impact the assessment overall trends in later chapters. If the secondary degradation is not uniform, as is expected, then the quantitative assessment described in the following section may be affected.

Degradant Peak 2 was only detected in those powders that were stored in the oven; for T0, Peak 2 was below the LOD. The DPR was calculated for the four detected peaks and plotted against the time in which the samples were stored in the oven (Figure 2.10). An r^2 value of 0.9823 indicates that the increase in DPR is highly dependent on the time in which the powdered samples were stored at 125 °C.

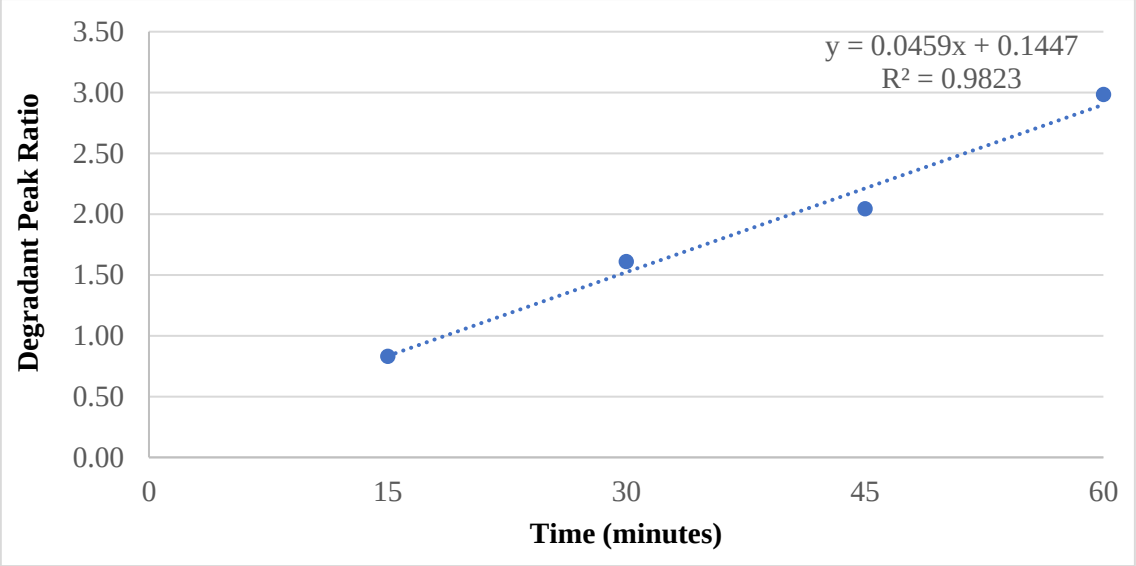


Figure 2.12. The DPR of Peak 2 dependent on the time stored at 125 °C (r^2 = 0.9823).

Further comparison of the DPR against the loss in LUM maintained the strong relationship (r^2 = 0.7323) presented in a negative slope. Therefore, there was a strong relationship between the loss in API and the generation of the degradant product.

2.5.2. Quantitative Assessment of the Degradant Profile.

Assessing the above degradant peaks in multiple tablets taken from the same conditions can generate a degradation profile for those conditions. For example, medicines that were accidentally left on the tarmac during the supply chain can be assessed as a group to provide strength to the assessment of the medicine's quality and insight into the conditions for which they were exposed. The following process is a recommended method by which to quantitatively assess the degradation profile of a storage condition.

The collected samples are assessed for degradation by observing the three degradant products identified in Figure 2.10. Degradation products are detected if the associated HPLC peak possessed a *S/N* ratio greater than 3. Degradant peaks with a *S/N* ratio greater than 10 were used to assess the degradation profile quantitatively. The noise calculation involves measuring the baseline height deviations of a corresponding blank standard. Quantitative

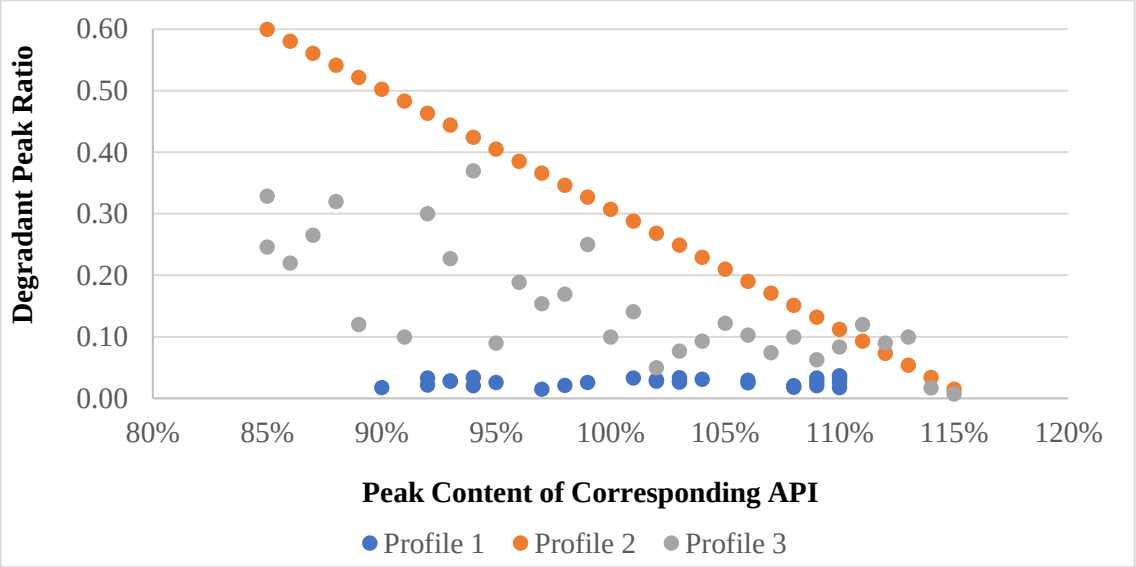
assessment involves calculating the DPR; the portion of degradant produced relative to the loss of the associated API (Equation 2.1).

$$Degradant\ Peak\ Ratio = \frac{Degradant\ Peak\ Area\ x\ Peak\ Purity\ (\%)}{Corresponding\ API\ Peak\ Area}$$

Equation 2.1. Calculation of degradant peak ratio (DPR).

The percent of the corresponding API is measured as a peak area ratio (PAR) of the API and its IS. Two measurements of interest when observing the plot are the DPR and the percent content of the corresponding API (Figure 2.13). Firstly, the coefficient of determination (r^2 value), calculated to measure the proportion of variance in the dependent variable that is related to changes in the independent variable. Secondly, the slope of the relationship, both the direction and the degree.

Figure 2.13 presents three simulated types of degradant profiles. Profile 1 represents a profile where very little degradant product was detected, indicated by a small slope value, and any product detected is not explained by the loss of API, indicated by the weak r^2 value. Profile 2 represents a profile where more degradant was detected, indicated by the high slope value, and the generation of the degradant product was the sole product of API loss, indicated by the strong r^2 value. Furthermore, an r^2 value of 1 also suggests that the degradant product behaved identically to the API in the instrument. Such an occurrence is not typically expected. Profile 3 represents an expected profile; that is there is a negative slope, indicating the more degradant product is created as more API is lost, and the r^2 value is lower, indicating that multiple variables explain the relationship, e.g. more than one degradant product. Therefore, when quantitatively assessing a degradant profile, the two quantitative measures, slope and r^2 value, were used to compare the profile to that of a baseline sample set.



Profile 1: r^2 = 0.030. Slope = 0.015. Profile 2: r^2 = 1.0. Slope = -1.95.
Profile 3: r^2 = 0.566. Slope = -0.771

Figure 2.13. Example assessment of three simulated degradant profiles.

2.6. Conclusions.

The purpose of Chapter 2 was to present an analytical method that was able to characterise ATM and LUM tablets as substandard or degraded. Implementing a LC-MS/MS method was not successful because in-source fragmentation of ATM and its derivatives negated the benefit of collecting mass information of the API and degradation products. However, the successful development of a validated HPLC method, presented the capacity to quantitatively assess substandard antimalarials and observe whether the medicines had degraded. Therefore, fulfilling the analytical requirement of the COPA protocol.

The harsh degradation studies executed during the method development were utilised to generate degradation products under extreme conditions. However, these conditions do not strictly reflect the conditions to which medicines would be exposed under real-world conditions. Therefore, the next progression of the COPA protocol is to implement that the HPLC method to analyse medicines that have undergone laboratory-based stability tests.

Chapter 3. Laboratory-based Stability Testing.

3.1. Background.

Stability studies are one mandatory requirement in the approval of new drug substances and products. The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) determines the conditions to which new medicines are exposed. The ICH guideline, Q1A(R2), recommends stability testing protocols including temperature, relative humidity (RH), and duration for the requirements of stability testing in four climate zones (International Conference on Harmonisation 2003).

The ICH Q1A(R2) guidelines recommend conditions for three types of stability studies; accelerated, intermediate, and long-term storage conditions. Each study has their storage conditions; accelerated at 40 ± 2 °C and 75 ± 5 %RH for six months, intermediate at 30 ± 2 °C and 65 ± 5 %RH for six months, and long-term at 25 ± 2 °C and 60 ± 5 %RH for 12 months. The long-term stability study may be performed using the intermediate temperature and humidity conditions for 12 months in lieu of doing both studies.

The variable that is common between all three ICH-recommended stability studies is the constant conditions. The temperature and humidity are set and remain constant, and the medicines are stored for the given timeframe.

The ICH guidelines propose that the storage conditions adequately account for temperature excursions based on a mean kinetic temperature (MKT) expression; which infers that “a single virtual temperature can be determined at which the loss rate is equivalent to that of this changing pattern of temperature” (Haynes 1971). The MKT was calculated for all storage conditions described below using the formula in Equation 3.1. The time segments (n) were recorded each 30-minute internal over the entire storage period.

$$T_K = \frac{\frac{-\Delta H}{R}}{\ln(\frac{e^{-\Delta H/RT_1} + e^{-\Delta H/RT_2} + \ldots + e^{-\Delta H/RT_n}}{n})}$$

Equation 3.1. The formula to calculate the mean kinetic temperature (Haynes 1971). Where ΔH = activation energy constant, R = universal gas constant, and T_n = absolute temperature.

While MKT aims to account for heightened temperature excursions, it is not clear whether MKT can also accommodate for repeated temperature variations that occur within acceptable climate conditions. It is generally observed that throughout a daily (diurnal) 24-hour period, the temperature and RH conditions vary: During the day the temperature is typically higher than at night, and RH is inversely proportional to temperature. Therefore, in PODs in tropical regions that do not possess air-conditioning systems, it is expected that the temperature and RH will fluctuate each diurnal cycle. Hence, this chapter explored the degradation of medicines when exposed to accelerated stability testing, in addition to cyclic temperature and RH storage conditions. A long-term stability test was also attempted; however, equipment failures led to this component of the research being discontinued.

The effects of the storage conditions were measured through four means; physical measurements, the content of the active pharmaceutical ingredient (API) using HPLC-PDA, assessing the degradant profile using HPLC-PDA, and disintegration testing. All measurements were compared against a baseline of medicines that were stored in controlled air-conditioning, under the recommended storage conditions listed on the packaging (under 30 °C and out of direct sunlight).

The aim of this chapter was to conduct laboratory-based stability testing with the aim to measure the effects of heightened storage conditions on the quality of three brands of antimalarials medicines. To measure the effects, the current stability testing status-quo is first

recreated; a baseline data set of non-degraded medicines and the ICH accelerated stability test. Furthermore, this chapter introduces a novel storage condition, the cyclic stability study.

3.2. Storage Information.

The recreation of a typical diurnal cycle was attempted using a Memmert Climate Control Chamber (model no. HCP153L, Schwabach, Germany). The climate chamber was controlled and monitored using the Memmert Celsius 10.0 software. The Celsius program recorded temperature and humidity conditions during the stability testing.

3.2.1. Baseline Medicines.

The baseline medicines were stored inside a safe in the exhibit store of Forensic Studies at the University of Canberra. The medicines were stored between 31/03/2016 and 26/04/2017 and temperature was measured in 30-minutes time intervals, equating to 18700 time-segments used to calculate MKT. The conditions within the exhibit store were monitored by a Lascar data logger. The average recordings were 20.4 °C and 42.1 %RH, and the MKT for the baseline storage was calculated to be 20.4 °C (Equation 3.1). The maximum recordings were 25.0 °C and 64.5 %RH. The minimum recordings were 14.5 °C and 25.0 %RH. On average throughout the day, the temperature changed 1.9 °C, and the humidity changed 4.2 %RH. The average temperature and RH recordings for the baseline samples are displayed in Figure 3.1; the range indicates the minimum and maximum recording for each day.

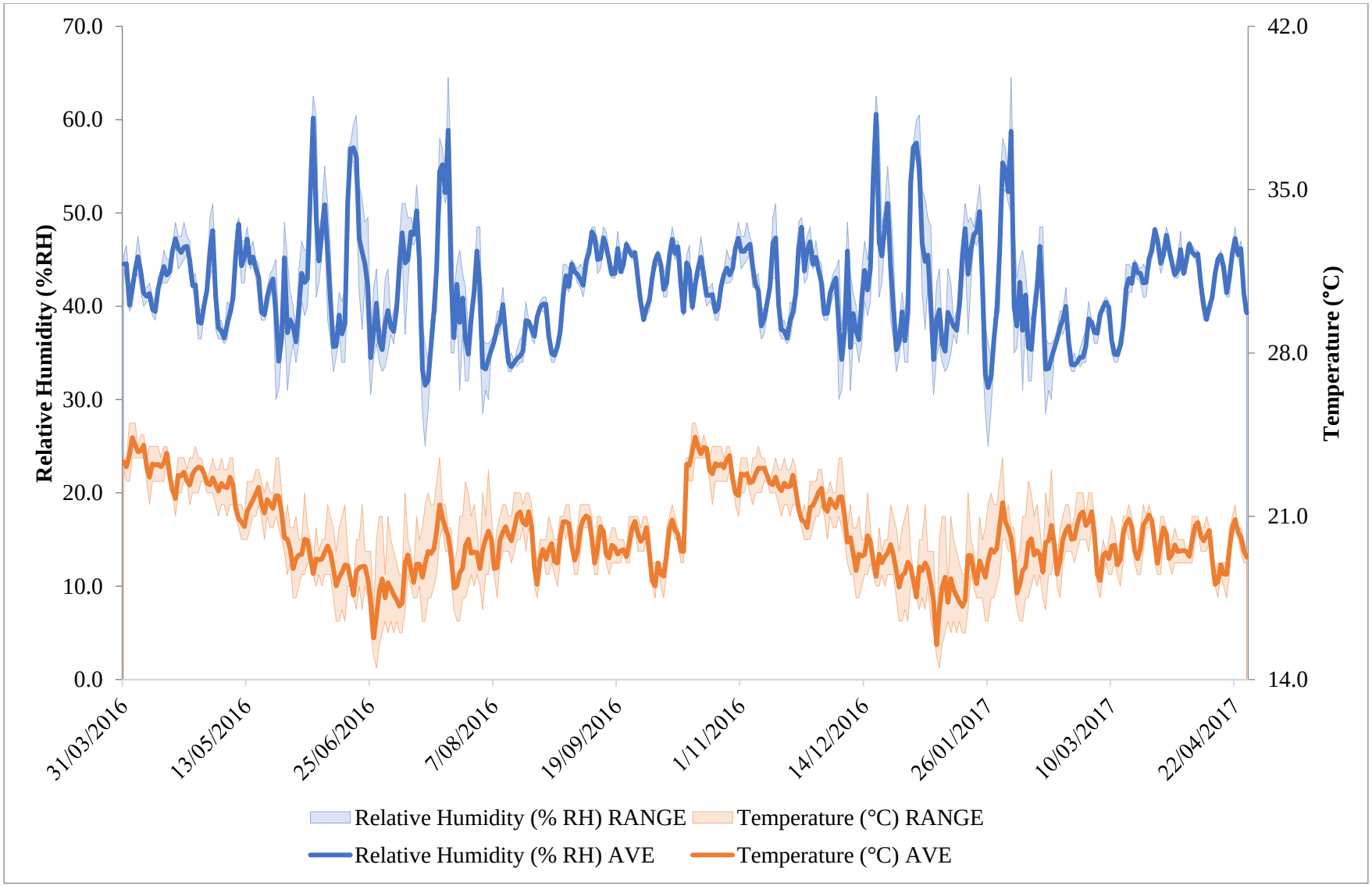


Figure 3.1. The average and range of temperature and RH conditions in which the baseline medicine samples were stored over 380 days.

3.2.2. Accelerated Stability Testing.

The accelerated stability testing occurred between 16/05/2016 and 22/11/2016 (190 days) and was measured in 30-minutes time intervals, equating to 9134 time-segments used to calculate MKT. Temperature and RH were configured to maintain 40 ± 1 °C and 75 ± 2.5 %RH in accordance with ICH guidance. The average temperature was 39.9 °C, and the humidity was 74.9 %RH, and the MKT for the accelerated study was calculated to be 39.9 °C. The minimum and maximum recordings were 39.0 °C and 24.0 %RH, and 40.0 °C and 77.0 %RH, respectively. On average throughout the day, the temperature changed 0.6 °C, and the humidity changed 2.6 %RH. Temperature and RH excursions outside the acceptable range were recorded three and 14 times, respectively. Excursions were the result of opening the chamber to remove medicines for experimentation or building-wide power outages. Moreover, an error in data collection of the climate chamber occurred for a 27-hour period. These excursions are unlikely to have significantly impacted the experiment.

3.2.3. Cyclic Stability Testing.

The cyclic stability testing conditions were set to replicate climate conditions that were typical of Uganda, the target country in Chapter 5. Local weather conditions in Kampala, Uganda (11/02/2015 – 18/02/2015) were collected from a weather reporting website (www.worldweatheronline) as an hourly report including temperature and RH. The maximum temperature and RH recording for each hour segment throughout the week was determined and was used to calculate the conditions for the diurnal cycle. The program contained 24 steps, equal to the temperature and RH changes seen for each hour segment during the day (Figure 3.2).

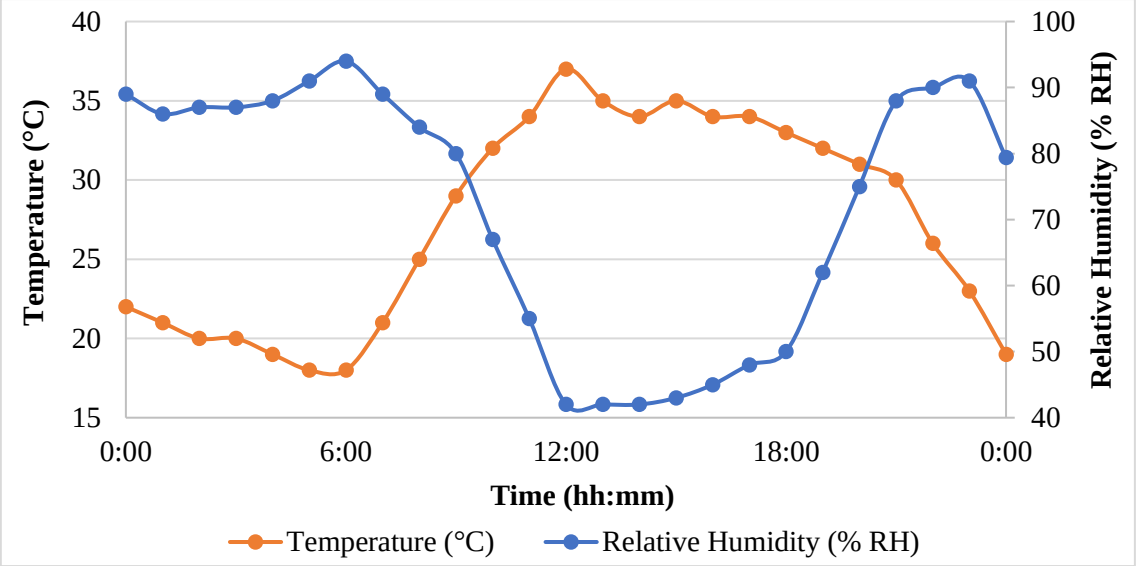


Figure 3.2. Observed average temperature and relative humidity fluctuations over a 24-hour period in Kampala, Uganda (11/02/2015 – 18/02/2015).

To align with the ICH accelerated stability testing guidelines, the settings for the diurnal cycle were set to possess an average temperature of 40 °C and a relative humidity of 65 %RH. The reason why the RH was not an average of 75 %RH, as per the accelerated stability testing conditions, was that the resulting maximum %RH was higher than the capacity of the Memmert climate chamber (> 90 %RH). For the final settings, the temperature range was 28.5 – 51.3 °C (average 40 °C) and relative humidity ranged between 38 – 85 %RH (average 65 %RH).

The cyclic stability testing occurred between 29/12/2016 and 27/06/2017 (180 days) and was measured in 30-minute time intervals, equating to 8625 time-segments used to calculate MKT. The average conditions throughout the testing period were 40.3 °C, and 64.6 %RH, and the MKT for the cyclic study was calculated to be 42.7 °C. The minimum and maximum recordings were 28.5 °C and 38.0 %RH, and 51.2 °C and 84.5 %RH, respectively. On average throughout the day, the temperature changed 21.7 °C, and the humidity changed 45.9 %RH.

The highest temperature variability between the set conditions and those recorded by the climate control chamber was witnessed during the temperature reduction phase during the cycle between 2230 and 0630 (Figure 3.3). The model of the Memmert climate chamber did

not possess an active temperature reduction method (i.e. an exhaust fan) and therefore struggled with the rapid decrease rate. For humidity, the variability between the set RH and the recorded RH was negligible, demonstrated in a deviation of less than 1 %RH. The Memmert Climate Chamber unexpectedly lost power for eight and a half hours during the experiment, resulting in a period of no recordings.

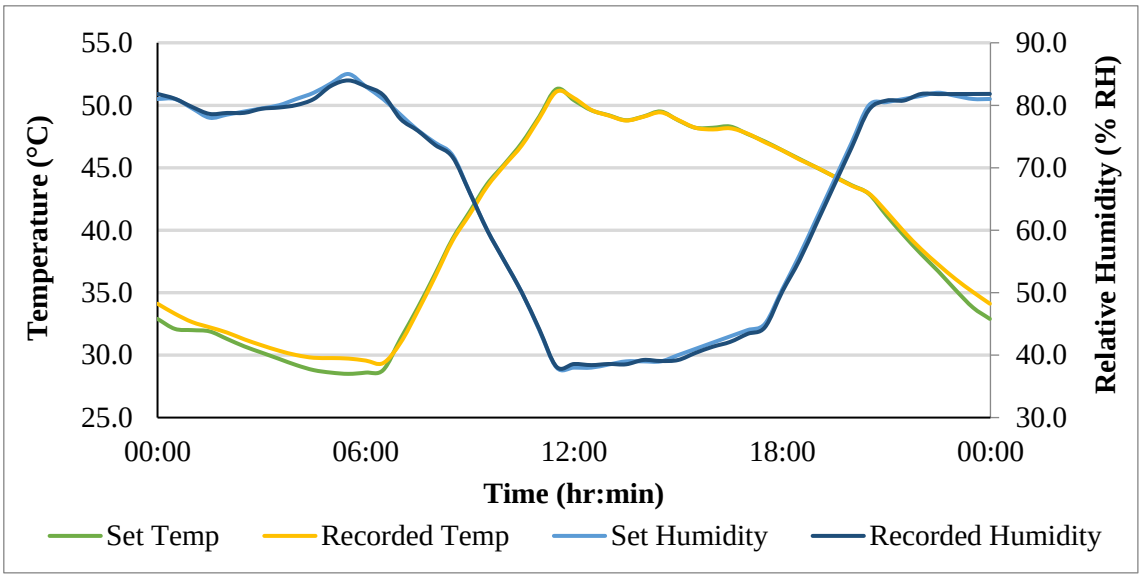


Figure 3.3. The average temperature and RH recorded over 24-hours compared with the set temperature during the cyclic stability test.

3.3. Quality Assessment.

3.3.1. Materials and Chemicals.

The baseline was established by analysing 42 samples in total; comprised of 18 Lumiter tablets (LU, all batch no. 15TAI028A), 12 Coartem tablets (CO, batch nos. K0019, K0020, K0021, K0032, K0034), and 12 Artrin tablets (AR, batch nos. 640256, 640281, 640282). Of the tablets that underwent accelerated stability testing, 42 were assessed for content; comprised of 18 LU tablets (all batch no. 15TAI028A), 12 CO tablets (batch nos. K0019, K0020, K0023, K0033, K0034), and 12 AR tablets (batch nos. 640256, 640281, 640282). All samples contained 20 mg of ATM and 120 mg of LUM. Of the tablets that underwent cyclic stability

testing, 42 were assessed for content; comprised of 18 LU tablets (all batch no. 15TAI028A), 12 CO tablets (batch nos. K0019, K0020, K0021, K0023, K0033, K0034), and 12 AR tablets (batch nos. 640256, 640281, 640282). Due to an analytical error, only the percent content for ATM was recorded for Coartem tablets after cyclic stability testing.

The AR batches and one batch of CO (K0020) had expired at the time of analysis, after accelerated stability testing, while the Lumiter batch had not expired. The Artrin and Coartem batches had expired at the time of analysis, after cyclic stability testing, while the Lumiter batch had not expired. It is believed that stability testing has a more significant impact on the medicines content than the expiration status.

The aqueous mobile phase consisted of 0.1 % *w/v* malonic acid and 0.01 % *v/v* TEA in Millipore water (solvent A), adjust to pH 3.0 with ammonium hydroxide. The organic phase consisted of LiChrosolv® ACN (solvent B). All solutions were then filtered through 0.22 µm Nylon discs under vacuum.

The calibration curve for ATM ranged between 100 – 2500 µg/mL (n = 5) in ACN, with ATS at 450 µg/mL as the IS. The calibration curve for LUM ranged between 5 – 100 µg/mL (n = 5) in 0.1 % malonic acid and ACN (60:40 *v/v*), with LOS at 15 µg/mL as the IS.

3.3.2. Sample Preparation.

Tablets were given a unique code based on the storage condition, tradename, source, blister pack, and position within the blister pack. For example, an Artrin (‘AR’) tablet exposed to the accelerated (‘AC’) stability test would possess the code, ACAR001A07, if it was taken from the first blister pack and occupied the seventh position. The blister packs were orientated and counted in the same manner when allocating the position.

Tablets from each blister packet were selected using the random number generator website, www.random.org (Haahr 1998). The physical measurements included in the assessment were; mass (g), thickness (mm), diameter (mm), and hardness (Newtons, N). These

measurements were included as each measurement is non-destructive and does not impact any proceeding content analysis. Each tablet was weighed using an Mettler Toledo New Classic MF analytical scale (MS105DU, 0.01 mg accuracy). Tablet thickness was measured using a Kinchrome 150 mm Vernier calliper (0.02 mm accuracy). Tablet hardness (1-N accuracy) and diameter (0.01 mm accuracy) were measured using an ERWEKA TBH 220 tablet hardness tester. Tablet mass was measured before and after hardness testing to assess any mass loss during hardness testing.

Tablets were firstly weighed for total mass and then crushed into a fine powder using a Silent Knight® Pill Crusher inside low-density polyethylene pouches. The powdered tablets portions analysed for ATM were dissolved in filtered can, while the powdered tablet portions analysed for LUM were dissolved in a mixture of mobile phase solvents A and B at a 60:40 ratio. The concentration of the extract was altered depending on the experiment. All solutions were then sonicated for 30 minutes, including vortexing three times, before filtration through a 0.45 µm nylon syringe filter. The filtered extract was then diluted to the required concentration of 2000 µg/mL for ATM and 90 µg/mL for LUM; the internal standards were then added to the filtered extracts at a concentration of 450 µg/mL for ATS and 15 µg/mL for LOS.

3.3.3. Instrumental Analysis.

A detailed description of the instrumental methods can be found in Chapter 2. In summary, all samples were separated on the Agilent 1200 series HPLC system using an Agilent Technologies Zorbax SB-C18 (2.1 x 50 mm, 1.8-micron) analytical column held at 30 °C. The injection volume was set at 10 µL. The gradient elution program was initiated at 43 % B, increasing to 75 % B at eight minutes over two minutes, holding for four minutes before re-equilibrating to 43 % B over three minutes and holding for four minutes. ATM and ATS

were best observed on the 200 nm UV-wavelength. LUM and LOS were best observed on the 250 nm UV-wavelength.

Analytes were detected using RTs and shape of the UV-spectra between 200 – 400 nm. The RT and spectra of ATM and LUM were compared against reference standards. The detection of degradants was based on previously reported data from method validation in Chapter 2. Analyte peaks that possess a signal-to-noise ratio (*S/N*) greater than ten were included in the quantitative assessment of the storage conditions.

Quantitative analysis was performed in Microsoft Excel (version 1810). Statistical tests were performed in the Excel Data Analysis Tools add-in. The statistical tests performed to compare the data sets were a *t*-test when comparing the mean of two data sets, and an analysis of variance (ANOVA) for more than two data sets. Visualisation of data sets was performed using a box-and-whisker plot, which indicates the distribution of the data, the upper and lower quartile, and the median value.

Disintegration was assessed using an ERWEKA® Disintegration Tester. The tablets were placed in Millipore water, held at 37 °C (± 2 °C) for 15 minutes. Individual tablets were placed in a single tube of the tester's basket and topped with a plastic disc. The bottom of the tester's basket was a 2 mm wire mesh. The tablets were considered to pass the disintegration testing if no tablet segments remained in the testing basket after the 15-minute period. Each storage condition was treated as a single 'batch' and assessed according to the British Pharmacopeia (Br. Ph.) acceptance criteria: the 'batch' is considered to pass if 16 of 18 tablets completely disintegrated. For the baseline, accelerated, and cyclic storage conditions; six tablets were chosen from each of the three brands (Coartem, Lumiter, and Artrin) to total 18 tablets, representing a Br. Ph. 'batch'.

3.4. Results and Discussion.

The following results present the effect on the medicines after storage in one of three conditions: a) baseline laboratory conditions, b) accelerated stability testing conditions, and c) cyclic stability testing conditions. A summary of the physical measurements and the percent content for each sample is listed in Appendix 3.A.

3.4.1. Physical Measurements.

Tablet thickness and diameter were used to calculate the approximate volume of each tablet. The reason the volume is approximate is that each tablet is not a perfect cylinder with the tablet shape, embossing, etc.

Table 3.1 summarises the physical measurements taken of the tablets before content analysis. The average physical measurements of the tablets after exposure to the different storage conditions were compared with multiple within-brand ANOVAs. A least significant difference (LSD) test was performed on measurements in which the ANOVA indicated a significant difference. The LSD test identified which groups were different.

Table 3.1. The average measurements of mass, volume, and hardness for Lumiter (LU), Coartem (CO), and Artrin (AR) of each storage condition.

Storage	Mass (g)			Volume (mm^3)			Hardness (N)		
Condition	LU	CO	AR	LU	CO	AR	LU	CO	AR
Baseline	0.2536	0.2542	0.2427	51.38	49.35	46.61	53.00	107.25	77.67
Accelerated	0.2532	0.2570	0.2417	**52.74***	51.08	44.58	**55.39**	**96.25**	**83.00**
Cyclic	**0.2560**	0.2604	0.2457	**53.36**	51.63	44.97	**49.83**	**85.25**	**84.67**

***** Bold values are significantly different from the baseline ($p < 0.05$).

The brand that presented as most distinct from the baseline measurements was Lumiter, particularly after cyclic stability testing. The mass and volume of Lumiter increased after exposure to the cyclic stability conditions. The hypothesis for these observations is that the water content of the tablets potentially increased due to the increased humidity. However, as

part of the routine analysis, there was no measurement for water content; therefore, this hypothesis could not be confirmed.

Interestingly, the accelerated samples were also exposed to heightened climate conditions but did not result in a significant difference in both physical measurements as only the volume was larger. The difference between the two conditions were the stagnancy of the accelerated conditions and the variability of the cyclic conditions. This result poses the question as to whether the variances in the cyclic conditions were the cause. The impact of these physical changes is only genuinely significant if the changes are also related to significant content changes.

3.4.2. Content Analysis.

Analysis of the baseline tablets indicated that the tablets were not poor-quality prior to placement in the accelerated or cyclic stability testing, as the average ± SD ATM content was 96 ± 5 % (86 – 108 %) and the average ± SD content for LUM was 104 ± 9 % (81 – 118 %). There was no significant difference between the percent content of ATM from the three manufacturers. There was a significant difference (p 0.017) between the percent content of LUM with Artrin tablets found to contain $\bar{x}$ 109 + 6 %, higher than Coartem ($\bar{x}$ 104 + 8 %) and Lumiter ($\bar{x}$ 100 + 10 %), but all were within acceptable quality limits (± 15 %).

After the baseline content was established and the medicines had completed the stability testing regime, the tablets were once again measured for percent API content and compared against the baseline measurements. For ATM, after the accelerated stability testing, the average ± SD percent content was 95 ± 4 % (89 – 104 %) across all brands. Moreover, there was no significant difference between the average percent content between the three brands (p 0.543). After the cyclic stability testing, the average percent content for ATM was 85 ± 8 % (59 – 100 %) across all brands. Once again, there was no significant difference in content

between the three brands (p 0.138). Figure 3.4 presents a box-and-whisker plot of the percent ATM content of the three storage conditions.

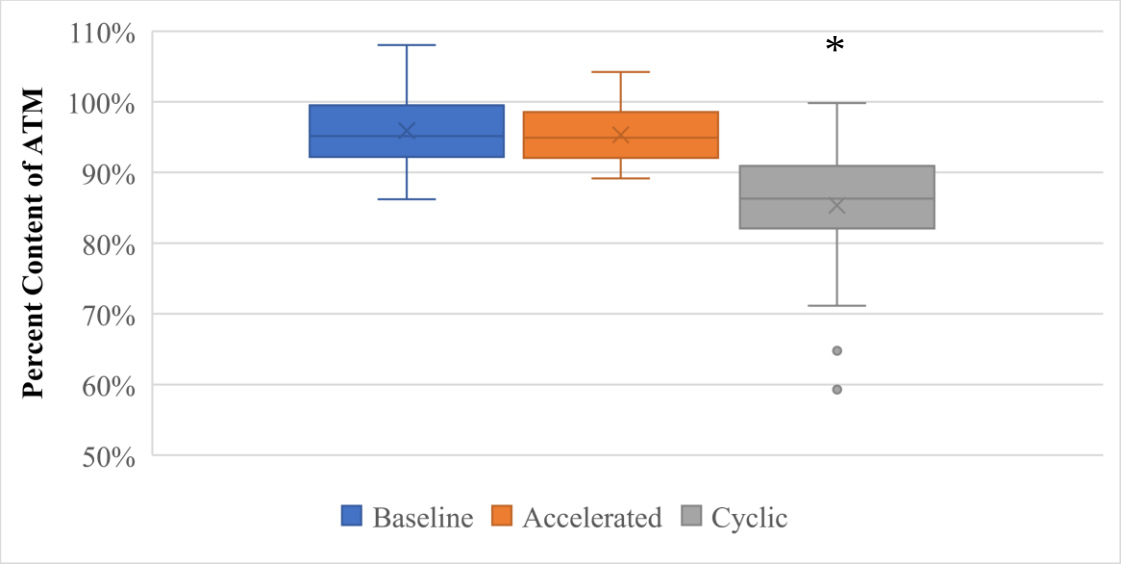


Figure 3.4. A box-and-whisker plot of the percent content of ATM after stability testing (accelerated and cyclic) compared against the baseline across all three brands. Asterix denotes significantly different ($p < 0.05$).

An ANOVA comparing the average percent content of ATM revealed a significant difference (p 2.02 x 10^{-14}) between the three storage conditions. A post hoc LSD confirmed the observation in Figure 3.4 identifying the content after the cyclic stability test as significantly lower than the baseline while after accelerated testing the content is not significantly different.

For LUM, after the accelerated stability testing, the average ± SD percent content was 93 ± 8 % (74 – 112 %) across all brands. However, an ANOVA indicated that there was a significant difference (p 4.2 x 10^{-7}) between the three brands. Coartem had the lowest LUM content at $\bar{x}$ 85 ± 6 % (74 – 93 %) followed by Lumiter at $\bar{x}$ 93 ± 4 % (82 – 100 %) and Artrin at $\bar{x}$ 101 ± 8 % (87 – 112 %). An LSD test identified all as being significantly different from each other. After the cyclic stability testing, the average percent content for LUM was 78 ± 17 % (23 – 99 %) across both Artrin and Lumiter. Due to an analytical error for the CO tablets only AR and LU tablets were analysed; there was no significant difference in content

between the two brands (p 0.260). Figure 3.5 presents a box-and-whisker plot of the LUM percent content of the three storage conditions.

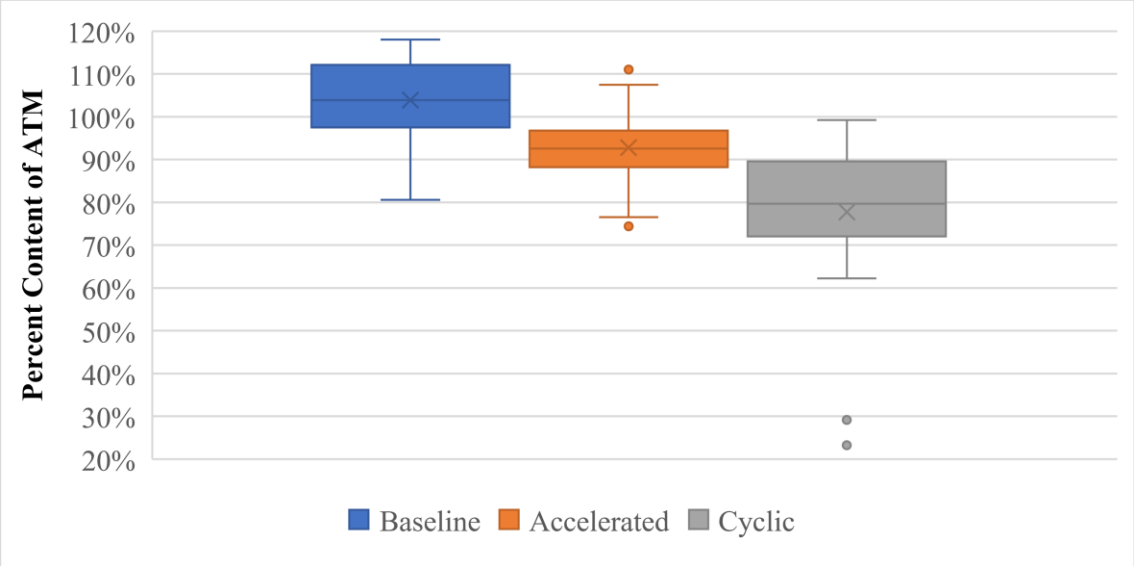


Figure 3.5. A box-and-whisker plot of the percent content of LUM after stability testing (accelerated and cyclic) compared against the baseline across all three brands (except cyclic testing where only AR and LU are included). Asterix denotes significantly different ($p < 0.05$).

An ANOVA was indicated that for LUM there was a significant difference (p 4.45 x 10^{-15}) between the three storage conditions. An LSD test revealed that for LUM both the accelerated and cyclic stability testing resulted in a significant reduction in the percent content of the API.

3.4.3. Degradant Analysis.

The collected samples were assessed for degradation by observing the three degradant products identified in Section 2.5.1. Degradation products were identified if the corresponding HPLC peak possessed a minimum *S/N* ratio of three; those an *S/N* ratio greater than ten were used to assess the degradation profile quantitatively.

In the absence of an IS for the degradants, the degradant peaks were divided by the peak area of the associated API after accounting for the degradant peak purity, referred to as the DPR. The two values were plotted to determine the relationship between the generation of the degradant (represented by the DPR) and the loss of the API. The strength of the relationship is

indicated by the coefficient of determination (r^2) value, with 1.0 indicating a very strong exclusive relationship. The steeper the slope of the data's trendline indicates that more degradant product is generated after the loss of API. A negative correlation is expected between the loss of the API and the generation of a degradant. The identification of the degradant products is described in Chapter 6

3.4.3.1. *Degradant Peak 1.*

Degradant Peak 1 has an average RT of 0.95 minutes, eluting between the solvent front and ATS. Degradant Peak 1 was best identified in ACN, the ATM tablet extraction solution. However, it is acknowledged that this observation may be due to the increased concentration of the tablet in the ATM extract. The API associated with Degradant Peak 1 is ATM.

Out of the 42 samples that were analysed from each storage condition, Degradant Peak 1 was quantified in 36 (85.7 %) from the baseline samples, 37 (88.1 %) from the accelerated stability test, and 38 (90.5 %) from the cyclic stability test (Figure 3.6).

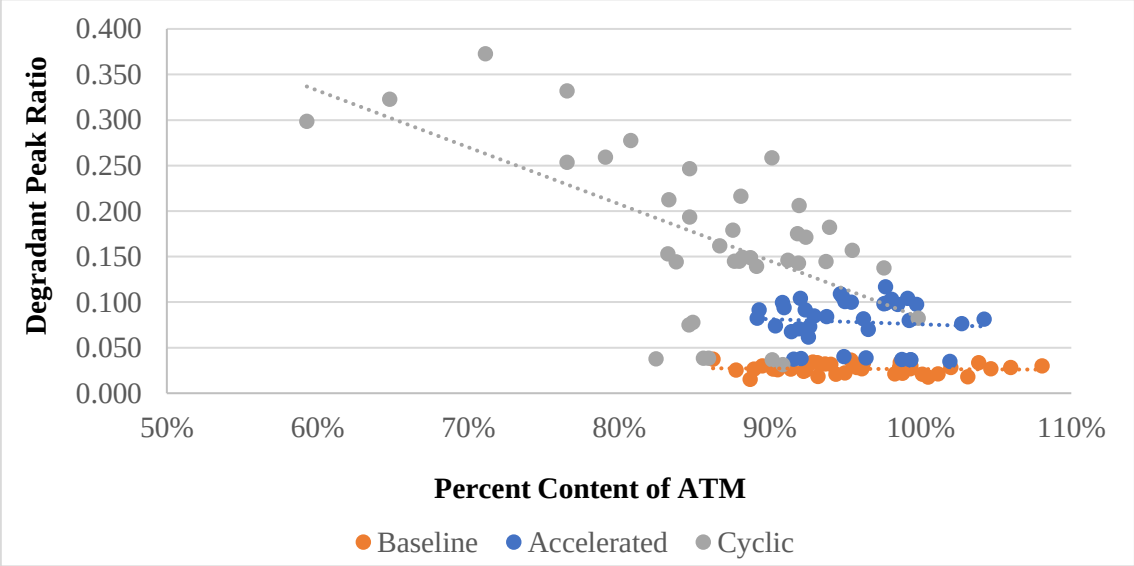


Figure 3.6. Degradant profile of Peak 1 from accelerated and cyclic samples compared against the baseline. Baseline $r^2 = 3.9 \times 10^{-3}$, accelerated $r^2 = 7.5 \times 10^{-3}$, and cyclic $r^2 = 0.35$.

The r^2 values of both the baseline and accelerated samples are negligible, indicating that there is a low relationship between the percent content of ATM and the generation of the degradant in those conditions. However, Figure 3.6 indicates that there was still a higher

amount of degradant detected in the accelerated samples, shown by a cluster of data points between 0.050 and 0.100 DPR, while some remain at the baseline, < 0.050 DPR.

The cyclic samples presented in Figure 3.6 present the strongest relationship (r^2 0.35) between the loss of the API and the generation of the degradant. Moreover, the negative slope is larger the other two storage conditions demonstrating two important observations: a) the expected negative correlation, as API decreases the DPR increases, and b) the rate (slope) of the loss and generation is higher under cyclic conditions.

It is important to consider the conditions of the cyclic and accelerated stability tests that led to the generation of the above degradation profiles. Average temperature was identical, and average RH was 10 % less for the cyclic conditions, but the conditions fluctuated daily in the cyclic testing. As such, the heightened degradation for the cyclic conditions observed in Figure 3.6 can largely be attributed to the daily fluctuations, not to increased climate conditions. Therefore, it is reasonable to conclude that conditions that fluctuate, such as uncontrolled transport and storage in medicine supply chains, may have increased negative effects on the medicines compared with stable, but still heightened, conditions (as demonstrated by the accelerated testing).

3.4.3.2. *Degradant Peak 2.*

Degradant Peak 2 has an average RT of 2.35 minutes, eluting between the ATS and ATM. Degradant Peak 2 was best identified in ACN, the ATM tablet extraction solution. However, it is acknowledged that this observation may be due to the increased concentration of the ATM extract. The API associated with Degradant Peak 2 is LUM based on a previous publication by Verbeken *et al.* (2011) that related the UV spectra (200 – 400 nm) of Degradant Peak 2 to that of a DBK derivative.

Out of the 42 samples that were analysed from each storage condition, Degradant Peak 2 was quantified in none from the baseline samples, 40 (95.2 %) from the accelerated stability test, and 27 (64.3 %) from the cyclic stability test (Figure 3.7).

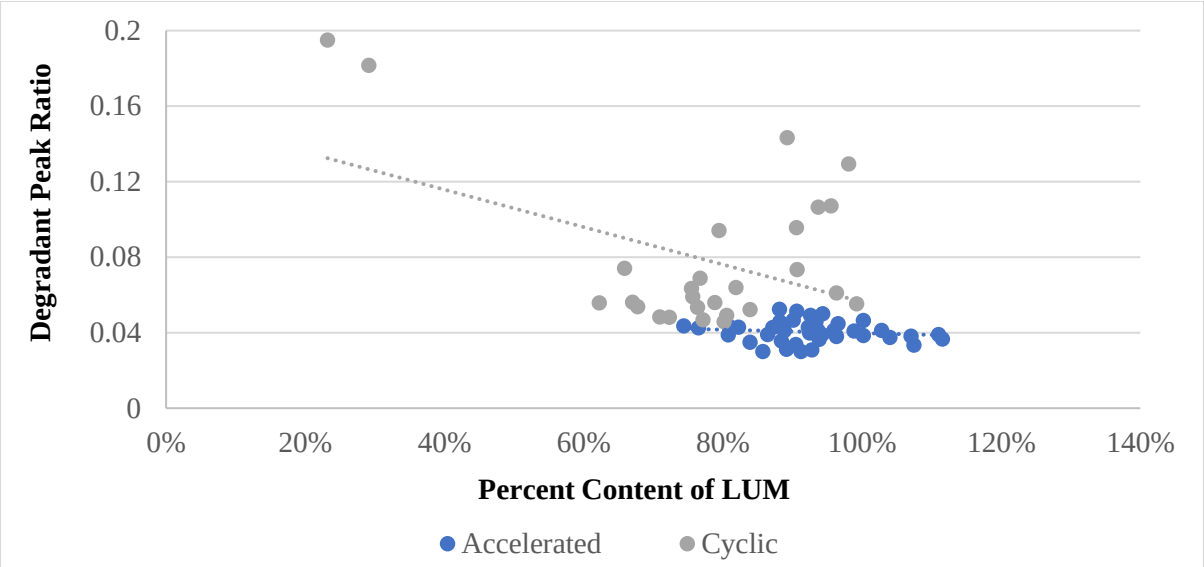


Figure 3.7. Degradant profile of Peak 2 from accelerated and cyclic samples compared against the baseline. Baseline r^2 = nil (as all below LOQ), accelerated r^2 = 0.019, cyclic r^2 = 0.19.

The presence of Degradant Peak 2 after the two stability tests and not in the baseline samples is a clear indication that the peak is significant. While Degradant Peak 2 was detected in nearly all the accelerated samples, the r^2 value relating the generation to LUM loss is weak, at 0.019. For the cyclic data, the r^2 value is 10-fold stronger, 0.19, primarily aided by the two samples in which the percent content for LUM was measured below 30 %. However, the average DPR for the cyclic conditions are higher than those accelerated samples, and the percent content for LUM was also lower comparatively. Interestingly, for the cyclic samples, there are many samples in which the percent content of ATM was measured near 100 %, but the DPR is reported at some of the highest values.

A visual comparison between the two data sets observes that the cyclic conditions led to a more varied relationship between the generation of Degradant Peak 2 and the loss of LUM. Therefore, the information for Degradant Peak 2 indicates that harsher conditions from either

stability test leads to its generation, and more degradant is present after the cyclic stability test than the accelerated study, as a response to LUM loss.

3.4.3.3. *Degradant Peak 3.*

Degradant Peak 3 has an average RT of 3.72 minutes, eluting between the ATS and ATM. Degradant Peak 3 was best identified in ACN, the ATM tablet extraction solution. However, it is acknowledged that this observation may be due to the increased concentration of the tablet in the ATM extract. The API associated with Degradant Peak 3 is thought to be ATM.

Out of the 42 samples that were analysed from each storage condition, Degradant Peak 3 was quantified in 18 (42.9 %) from the baseline samples, six (14.3 %) from the accelerated stability test, and 17 (40.5 %) from the cyclic stability test (Figure 3.8). An important observation for the accelerated samples is that the six samples in which Degradant Peak 3 was quantitated are in samples that returned a percent content of ATM that was below the average (< 95.3 %) for the stability test. This observation suggests that the peak is a degradant or is related to the loss of the API; however, such a trend is only apparent in the accelerated samples.

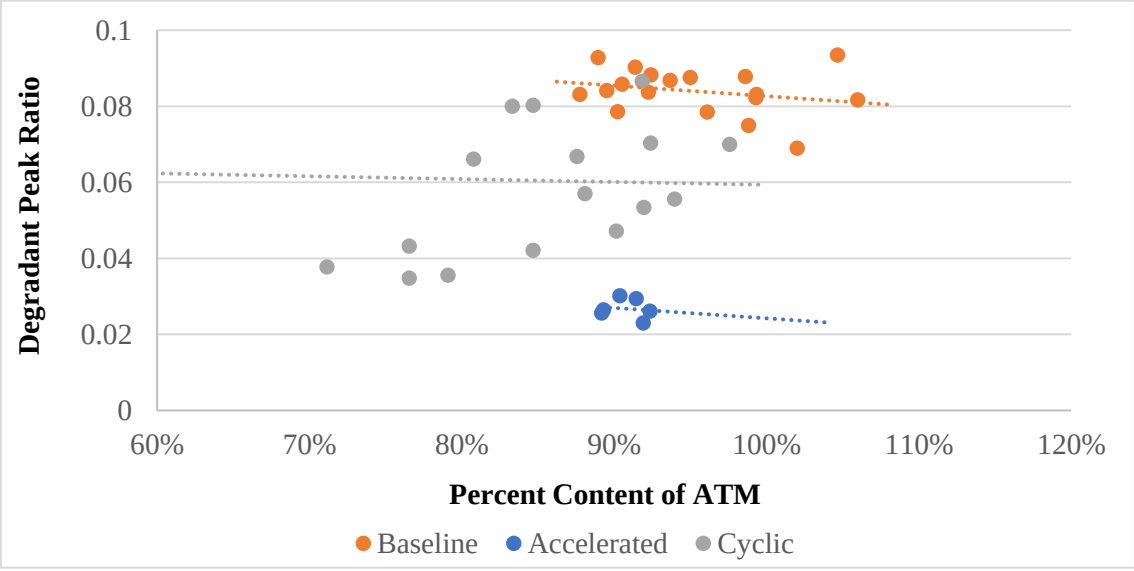


Figure 3.8. Degradant profile of Peak 3 from accelerated and cyclic samples compared against the baseline. Baseline $r^2 = 0.062$, accelerated $r^2 = 0.020$, cyclic $r^2 = 1.3 \times 10^{-3}$.

The information displayed in Figure 3.8 is interesting, as it is contrary to the other two degradant peaks; the DPR for the baseline is consistently higher than after the two stability tests. The relationship between the loss of API and the generation of the degradation product is weak in all three data sets (less than 0.062).

The relationship between Degradant Peak 3 and the API is relatively weak. Therefore, before the peak can be confidentially identified as a known degradant, it is important to collect mass information that would support this conclusion (see Chapter 6).

3.4.4. Disintegration.

In total, 18 tablets from each storage condition were tested, comprising of six tablets from three brands (Coartem, Lumiter, and Artrin). The effect of the storage conditions was allowable if 16 of the 18 tablets tested completely disintegrated in the 15-minute testing period.

For the baseline samples, all the tablets had adequately disintegrated within the 15-minute period. For both the stability tests, accelerated and cyclic, all the 18 tablets disintegrated within the time-period. Therefore, both the baseline and the stability test samples are considered to have passed the disintegration testing.

There was no indication that the medicines were affected by the storage conditions when using disintegration as the measurement. However, additional testing can provide insight into the effect on bioavailability, such as dissolution testing. Dissolution testing was not performed as part of the COPA as the method would require an additionally validated method and was considered beyond the scope of the project.

3.5. Conclusions.

This chapter explored the effect of laboratory-based stability testing on co-formulated ATM and LUM medicines; measuring the physical characteristics, percent content of the API, and the generation of degradants before and after storage. The storage conditions for the

accelerated and cyclic stability tests were designed so that the variable differing the two tests was the daily temperature and RH changes under the cyclic conditions.

After the two stability tests, there was a greater difference in the physical measurements between the baseline samples and the cyclic test samples than the accelerated test samples. Moreover, the percent content for both API was found to be lower in the cyclic test samples than the accelerated test samples. Finally, the degradant profile for the cyclic stability conditions indicated stronger relationships between the loss of the API and the generation of degradant products in the cyclic test samples compared with the accelerated test samples.

The results of the two laboratory-based stability tests consistently demonstrated that the cyclic variation of temperature and RH to reflect a day/night cycle resulted in more severe negative impacts on the medicines stored in those conditions. Therefore, it is recommended that daily cyclic fluctuation should be implemented in routine stability testing given that the conditions closely reflect real-world environmental conditions and the increased impact on the medicines observed.

Appendix 3.A. Details of the laboratory-based stability samples.

Table 3.2 lists the complete source and analysis information for all laboratory-based stability test samples. Abbreviations in Table 3.2: Baseline (BAS), accelerated (ACC), cyclic (CYC), Artrin (AR), Coartem (CO), and Lumiter (LU). Expiry dates denoted with '*' were expired at the time of analysis. Bold content (%) values indicate those samples outside the ± 15 % quality standards.

Table 3.2. The physical and percent content results for the laboratory-based stability tests.

Origin	Brand	Blister Pos.	Batch No.	Exp Date	Mass (g)	Vol. (mm^3)	Hardness (N)	Content (%)	
								ATM	LUM
BAS	AR	A05	640282	Apr-17*	0.2410	43.87	71	96	114
BAS	AR	A07	640282	Apr-17*	0.2449	43.92	77	108	110
BAS	AR	B01	640256	Apr-17*	0.2458	44.74	72	95	112
BAS	AR	B04	640256	Apr-17*	0.2384	44.02	80	93	118
BAS	AR	C02	640281	Apr-17*	0.2397	44.59	69	95	112
BAS	AR	C04	640281	Apr-17*	0.2364	44.02	79	88	113
BAS	AR	D01	640281	Apr-17*	0.2445	44.12	67	86	114
BAS	AR	D08	640281	Apr-17*	0.2372	43.45	80	93	110
BAS	AR	E06	640282	Apr-17*	0.2423	44.30	71	96	108
BAS	AR	E07	640282	Apr-17*	0.2473	44.54	80	94	100
BAS	AR	F03	640256	Apr-17*	0.2500	44.69	97	104	100
BAS	AR	F04	640256	Apr-17*	0.2444	44.64	89	99	102
BAS	CO	A12	K0020	May-17	0.2432	45.39	117	89	108
BAS	CO	A18	K0020	May-17	0.2413	44.82	116	98	114
BAS	CO	B12	K0019	May-17	0.2399	44.54	120	100	105
BAS	CO	B17	K0019	May-17	0.2414	44.54	122	101	98
BAS	CO	C11	K0032	Jul-17	0.2427	44.54	121	94	116
BAS	CO	C18	K0032	Jul-17	0.2402	44.25	119	93	100
BAS	CO	D04	K0034	Jul-17	0.2426	44.59	118	103	96
BAS	CO	D06	K0034	Jul-17	0.2430	44.54	113	100	108
BAS	CO	E06	K0021	Jul-17	0.2887	58.76	88	102	96
BAS	CO	E08	K0021	Jul-17	0.2489	58.48	85	93	95
BAS	CO	F07	K0021	Jul-17	0.2901	59.18	85	92	102
BAS	CO	F09	K0021	Jul-17	0.2883	58.61	83	99	114
BAS	LU	A07	15TAI028A	Sep-18	0.2495	50.30	53	102	101
BAS	LU	A09	15TAI028A	Sep-18	0.2525	50.86	56	105	102
BAS	LU	A13	15TAI028A	Sep-18	0.2535	50.16	68	94	91
BAS	LU	A16	15TAI028A	Sep-18	0.2569	51.49	63	99	96
BAS	LU	A18	15TAI028A	Sep-18	0.2563	51.69	51	106	102
BAS	LU	A23	15TAI028A	Sep-18	0.2536	50.16	63	99	99
BAS	LU	B05	15TAI028A	Sep-18	0.2565	52.57	50	89	95
BAS	LU	B06	15TAI028A	Sep-18	0.2485	50.55	53	91	101

Table 3.2. The physical and percent content results for the laboratory-based stability tests.

Origin	Brand	Blister Pos.	Batch No.	Exp Date	Mass (g)	Vol. (mm^3)	Hardness (N)	Content (%)	
								ATM	LUM
BAS	LU	B07	15TAI028A	Sep-18	0.2518	51.33	52	99	108
BAS	LU	B12	15TAI028A	Sep-18	0.2548	52.23	49	92	91
BAS	LU	B14	15TAI028A	Sep-18	0.2513	50.49	52	89	112
BAS	LU	B21	15TAI028A	Sep-18	0.2554	52.23	50	90	**81**
BAS	LU	C01	15TAI028A	Sep-18	0.2564	51.73	50	99	86
BAS	LU	C03	15TAI028A	Sep-18	0.2558	52.23	52	91	106
BAS	LU	C05	15TAI028A	Sep-18	0.2513	51.45	42	92	**116**
BAS	LU	C20	15TAI028A	Sep-18	0.2529	52.01	50	88	90
BAS	LU	C21	15TAI028A	Sep-18	0.2513	51.45	45	96	107
BAS	LU	C23	15TAI028A	Sep-18	0.2563	51.95	55	95	115
ACC	AR	A01	640282	Apr-17*	0.2420	44.88	83	95	107
ACC	AR	A07	640282	Apr-17*	0.2474	44.83	84	98	100
ACC	AR	B01	640282	Apr-17*	0.2440	44.83	77	100	104
ACC	AR	B06	640282	Apr-17*	0.2395	44.45	83	95	112
ACC	AR	C04	640281	Apr-17*	0.2469	44.88	102	95	96
ACC	AR	C08	640281	Apr-17*	0.2486	45.84	75	95	96
ACC	AR	D04	640281	Apr-17*	0.2419	44.98	73	92	93
ACC	AR	D07	640281	Apr-17*	0.2429	44.17	81	98	111
ACC	AR	E03	640256	Apr-17*	0.2452	45.46	90	98	103
ACC	AR	E08	640256	Apr-17*	0.2554	45.99	94	99	107
ACC	AR	F06	640256	Apr-17*	0.2486	45.03	81	91	87
ACC	AR	F07	640256	Apr-17*	0.2461	44.31	93	98	94
ACC	CO	A17	K0023	Jun-17	0.2443	47.74	74	102	89
ACC	CO	A19	K0023	Jun-17	0.2435	47.51	73	99	91
ACC	CO	B16	K0020	May-17*	0.2442	45.69	88	96	91
ACC	CO	B24	K0020	May-17*	0.2454	46.03	93	95	88
ACC	CO	C10	K0034	Jul-17	0.2464	46.31	95	99	86
ACC	CO	C14	K0034	Jul-17	0.2450	45.69	91	92	93
ACC	CO	D08	K0033	Jul-17	0.2453	46.31	95	99	86
ACC	CO	D13	K0033	Jul-17	0.2441	46.26	91	92	**84**
ACC	CO	E01	K0019	Jun-17	0.2907	62.02	75	91	**81**
ACC	CO	E12	K0019	Jun-17	0.2922	62.02	84	93	**77**
ACC	CO	F06	K0020	Jun-17	0.2927	61.95	88	98	**74**
ACC	CO	F07	K0020	Jun-17	0.2915	61.95	76	92	**81**
ACC	LU	A02	15TAI028A	Sep-18	0.2539	52.58	52	99	97
ACC	LU	A11	15TAI028A	Sep-18	0.2570	53.42	50	104	100
ACC	LU	A12	15TAI028A	Sep-18	0.2568	53.92	46	97	97
ACC	LU	A15	15TAI028A	Sep-18	0.2532	52.64	47	93	91
ACC	LU	A21	15TAI028A	Sep-18	0.2620	53.99	51	103	93
ACC	LU	A24	15TAI028A	Sep-18	0.2583	53.99	46	96	92
ACC	LU	B05	15TAI028A	Sep-18	0.2580	53.48	54	98	93
ACC	LU	B10	15TAI028A	Sep-18	0.2618	53.42	56	99	**82**
ACC	LU	B14	15TAI028A	Sep-18	0.2527	53.48	50	93	94
ACC	LU	B15	15TAI028A	Sep-18	0.2559	53.14	56	91	88

Table 3.2. The physical and percent content results for the laboratory-based stability tests.

Origin	Brand	Blister Pos.	Batch No.	Exp Date	Mass (g)	Vol. (mm^3)	Hardness (N)	Content (%)	
								ATM	LUM
ACC	LU	B20	15TAI028A	Sep-18	0.2520	53.42	48	93	92
ACC	LU	B22	15TAI028A	Sep-18	0.2584	53.54	51	94	94
ACC	LU	C01	15TAI028A	Sep-18	0.2580	53.36	54	92	88
ACC	LU	C06	15TAI028A	Sep-18	0.2507	53.14	48	92	89
ACC	LU	C14	15TAI028A	Sep-18	0.2550	53.42	47	89	94
ACC	LU	C22	15TAI028A	Sep-18	0.2560	53.48	45	90	90
ACC	LU	C23	15TAI028A	Sep-18	0.2539	52.58	52	89	91
ACC	LU	C24	15TAI028A	Sep-18	0.2545	53.42	44	91	99
CYC	AR	A01	640281	Apr-17*	0.2476	44.69	88	95	**76**
CYC	AR	A06	640281	Apr-17*	0.2452	45.21	85	87	**80**
CYC	AR	B03	640281	Apr-17*	0.2396	45.12	60	**83**	**84**
CYC	AR	B07	640281	Apr-17*	0.2323	43.74	82	89	**77**
CYC	AR	C02	640282	Apr-17*	0.2345	43.22	80	84	**72**
CYC	AR	C06	640282	Apr-17*	0.2425	44.54	90	94	**76**
CYC	AR	D03	640282	Apr-17*	0.2456	44.59	87	88	**76**
CYC	AR	D08	640282	Apr-17*	0.2411	44.59	89	88	**79**
CYC	AR	E03	640256	Apr-17*	0.2450	44.97	85	89	**68**
CYC	AR	E08	640256	Apr-17*	0.2421	44.79	83	92	**71**
CYC	AR	F05	640256	Apr-17*	0.2429	45.02	75	88	**62**
CYC	AR	F06	640256	Apr-17*	0.2423	44.45	92	91	**67**
CYC	CO	A03	K0023	Jun-17*	0.2405	47.07	84	100	-
CYC	CO	A08	K0023	Jun-17*	0.2395	47.07	82	85	-
CYC	CO	B12	K0019	May-17*	0.2402	45.59	97	85	-
CYC	CO	B21	K0019	May-17*	0.2435	46.16	111	91	-
CYC	CO	C07	K0033	Jul-17*	0.2418	45.59	100	86	-
CYC	CO	C13	K0033	Jul-17*	0.2440	45.83	107	**82**	-
CYC	CO	D04	K0032	Jul-17*	0.2413	45.59	99	90	-
CYC	CO	D17	K0032	Jul-17*	0.2419	45.36	101	86	-
CYC	CO	E09	K0021	Jul-17*	0.2881	60.45	109	**77**	-
CYC	CO	E10	K0021	Jul-17*	0.2897	60.52	99	**78**	-
CYC	CO	F09	K0020	Jun-17*	0.2867	61.74	82	**84**	-
CYC	CO	F11	K0020	Jun-17*	0.2871	62.02	84	**81**	-
CYC	LU	A02	15TAI028A	Sep-18	0.2540	52.57	61	**71**	89
CYC	LU	A03	15TAI028A	Sep-18	0.2554	52.29	60	**77**	98
CYC	LU	A10	15TAI028A	Sep-18	0.2549	52.51	59	**79**	91
CYC	LU	A13	15TAI028A	Sep-18	0.2566	52.79	61	**77**	96
CYC	LU	A19	15TAI028A	Sep-18	0.2541	52.57	56	85	91
CYC	LU	A20	15TAI028A	Sep-18	0.2509	52.23	55	90	94
CYC	LU	B04	15TAI028A	Sep-18	0.2492	52.68	53	92	**29**
CYC	LU	B06	15TAI028A	Sep-18	0.2500	52.52	48	**81**	96
CYC	LU	B11	15TAI028A	Sep-18	0.2537	53.02	50	92	66
CYC	LU	B13	15TAI028A	Sep-18	0.2494	51.34	63	85	88
CYC	LU	B19	15TAI028A	Sep-18	0.2561	53.52	55	88	99
CYC	LU	B24	15TAI028A	Sep-18	0.2538	53.30	55	**83**	85

Table 3.2. The physical and percent content results for the laboratory-based stability tests.

Origin	Brand	Blister Pos.	Batch No.	Exp Date	Mass (g)	Vol. (mm^3)	Hardness (N)	Content (%)	
								ATM	LUM
CYC	LU	C03	15TAI028A	Sep-18	0.2534	53.30	55	**59**	**80**
CYC	LU	C04	15TAI028A	Sep-18	0.2476	52.40	48	98	**81**
CYC	LU	C06	15TAI028A	Sep-18	0.2503	52.52	52	88	**82**
CYC	LU	C09	15TAI028A	Sep-18	0.2596	53.80	60	92	**23**
CYC	LU	C12	15TAI028A	Sep-18	0.2523	52.74	51	94	**77**
CYC	LU	C23	15TAI028A	Sep-18	0.2564	53.30	55	**65**	**79**

Chapter 4. Atypical Stability Testing.

4.1. Background.

In the previous chapter, the effects on medicine quality after laboratory-based stability studies were assessed where the conditions were controlled and measured. This chapter explores an atypical stability test, in which medicines are stored in conditions that are exposed to natural temperature and humidity variations where the conditions were measured but not controlled. To achieve this, medicines were stored in two locations in Australia: Darwin in the Northern Territory and Perth in Western Australia.

4.1.1. The Climate Regions of Australia

Australia experiences a wide-ranging climate environment, ranging from a tropical climate to a desert climate, to an oceanic climate (Peel *et al.* 2007). The two selected locations represent different climate regions. On the Köppen-Geiger classification, Darwin is considered in the Tropical Savanna 'Aw/As' category. The climate criteria typical of a Tropical Savanna 'Aw/As' classification are: the temperature in the coldest month is above 18 °C and the driest month has less than 60 mm of rain and less than [100 – (mean annual precipitation/25)] mm of rainfall (Peel *et al.* 2007). Perth is placed in the Hot-summer Mediterranean 'Csa' climate classification. The climate criteria typical of a Hot-summer Mediterranean 'Csa' classification are: temperature of the coldest month is between 0 – 18 °C, the hottest month is more than 22 °C, and the rainfall in the driest summer month is less than 40 mm and is less than one-third the rainfall in the wettest month (Peel *et al.* 2007).

Figure 4.1 presents maps of the Köppen-Geiger climate classification of Africa and Australia. Of note is the similarity between Darwin in the north and Uganda, both coloured in light blue signifying the Tropical Savanna 'Aw/As' classification. With Uganda as the target

country in Chapter 5, this climate commonality is important as the conditions of Darwin are presumed to reflect the conditions of the Ugandan supply chain.

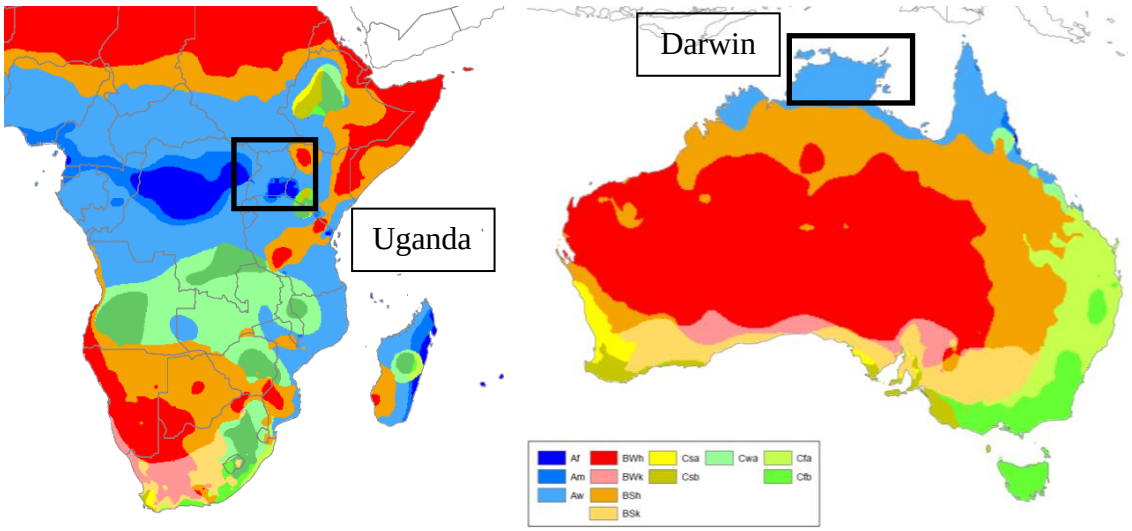


Figure 4.1. Köppen-Geiger climate classification of Africa and Australia (Peel *et al.* 2007).

The similarities between the Darwin atypical stability study and Uganda is beneficial in drawing relationships between the results of the quality assessment.

4.1.2. Pilot Atypical Storage Experiment.

A pilot study in the project measured the temperature (°C) and relative humidity (% RH) inside a shipping container stored at Qube Ports & Bulk terminal located in Berrimah, Northern Territory, between 16/07/2015 and 30/07/2015. Typically, it would be expected that some medicines may be transferred in shipping containers during the supply chain. Measurements inside the shipping container were taken every five minutes.

The short study aimed to determine whether there was a difference between the climate conditions inside the shipping container and the external environment, and whether the conditions could potentially lead to the accelerated degradation of medicines stored inside.

The first assessment measured the difference between the conditions at the wall of the shipping container, compared with the conditions near the centre. The hypothesis was that heat would be retained inside the metal of the container and would influence the recordings through

heat radiation. Two key observations are apparent in Figure 4.2: that each day there is a cyclic fluctuation of temperature and humidity, and that there was a measurable difference between the two locations within the container.

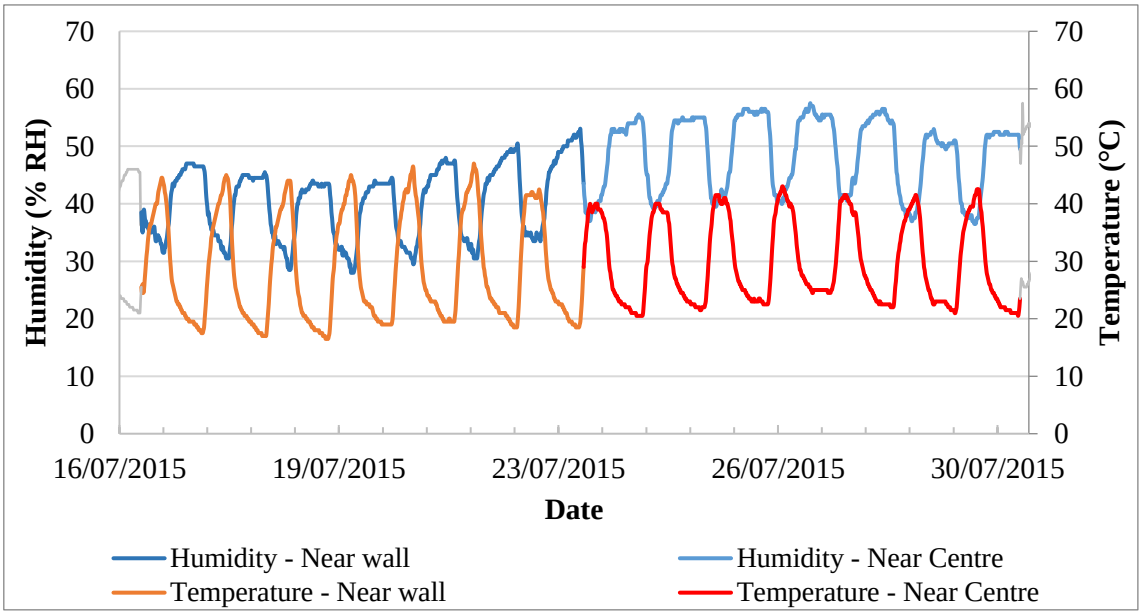


Figure 4.2. Temperature (°C) and relative humidity (% RH) inside a shipping container, near the wall and in the centre, stored at Qube Ports & Bulk terminal located in Berrimah, Northern Territory, between 16/07/2015 and 30/07/2015.

The maximum temperature when located near the wall (16/07/2015 – 23/07/2015) was 5.5 °C higher than when located near the centre (23/07/2015 – 30/07/2015). Moreover, the temperature range near the wall was 30 °C (17.0 – 47.0 °C) compared with 21 °C (20.5 – 41.5 °C) at the centre. These results indicate that not only were the conditions hotter near the wall, but the temperature was also variable. The RH, when located near the wall, was on average 8.6 % RH less than when located near the centre. However, while the RH ranges were similar, the maximum near the wall (52.0 % RH) was lower than at the centre (57.5 % RH). An increase in relative humidity at the centre of the container is consistent with the increase in temperature near the wall.

The second assessment compared the temperature within the storage container with the ambient temperature in Darwin, to measure the effect of confinement within the shipping

container. Darwin weather information was sourced from a weather reporting website (www.worldweatheronline.com) and was reported in 20-minute intervals (World Weather Online® 2015). Figure 4.3 compares the two temperature data sets and highlights the difference between the two. During the day, the temperature inside the container was up to 16 °C higher than the ambient temperature of Darwin. Furthermore, at night, the temperature was continuously colder within the container, resulting in a larger temperature cycle each 24-hour period.

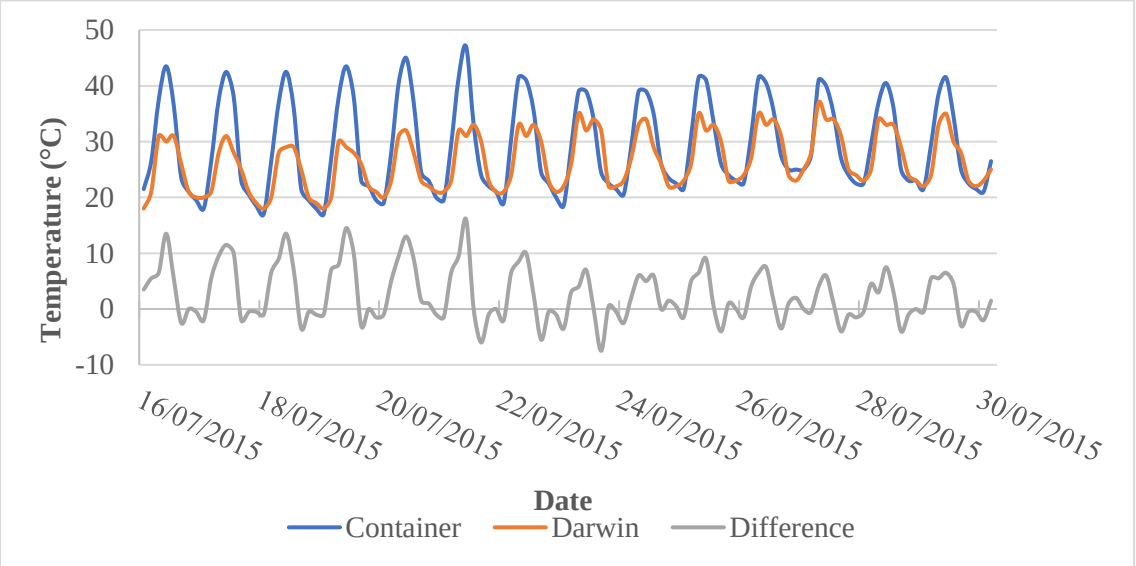


Figure 4.3. The temperature of Darwin (ambient), inside the shipping container, and the difference between the two measurements for shipping container stored at Qube Ports & Bulk terminal located in Berrimah, Northern Territory, between 16/07/2015 and 30/07/2015.

The information gathered in the pilot study justified the further investigation of the consequences of medicines' prolonged storage in heightened conditions. Of note was the distinct cyclic variations of both temperature and RH. This observation raised the questions, is the effect common to different climate regions, and do the conditions in different climate regions result in a different impact on the quality of the exposed medicines?

Therefore, the aim of this chapter was to assess whether the degradation profile of medicines that were stored in uncontrolled environmental conditions were more similar to the cyclic stability testing than the accelerated stability testing presented in Chapter 3. The

medicines were measured for percent content of the API, the generation of any degradation products, and response to disintegration testing.

4.2. Storage of Medicines in Uncontrolled but Measured Conditions.

After the successful completion of the pilot experiment, the scope was extended to implement atypical stability tests in two uncontrolled conditions, Darwin and Perth. The medicines were stored in a lockable, non-insulated Craftright 385 mm metal toolbox (product no. CR365TB, Bunnings Group Limited, Perth, Australia) (Figure 4.4). A Lascar EasyLog temperature and humidity logger was placed inside each medicine box. Temperature (5 - 60 °C, ± 0.5 °C) and humidity (0 - 100 % RH, ± 2 % RH) recordings were taken every hour for the period of storage.

Figure 4.4. A lockable, non-insulated metal toolbox in which the medicines were stored.

Three batches of Lumiter blister packs (manufactured by Macleods Pharmaceuticals Limited) were included in the medicine boxes with the details: A) 15TAI068A exp. 11/2018, B) 15TAI074A exp. 11/2018, and C) 15TAI078A exp. 11/2018. All three blister packs were

made of a transparent plastic and aluminium foil. Blister pack A and B contained 24 tablets and blister pack C contained 18.

Three batches of Artrin blister packs (manufactured by Medreich Limited) were included in the medicine boxes with the details: A) 1640217 exp. 03/2017, B) 640219 exp. 03/2017, and C) 640219 exp. 03/2017. Blister pack A was entirely made of aluminium foil while blister packs B and C were made of a transparent plastic and aluminium foil. All three blister packs contained eight tablets each. There was no indication of recommended storage conditions on the blister packs of either the Lumiter or Artrin products used in the atypical stability test.

4.2.1. Darwin Study.

In Darwin, the metal box containing the medicines was stored in a non-refrigerated shipping container, located at the Qube Ports & Bulk terminal in Berrimah, Northern Territory (Figure 4.5).

Figure 4.5. Map of the location of Qube Ports & Bulk in Darwin.

The metal box was placed in the location between 22/08/2016 and 17/03/2017 (208 days, 6 months and 24 days). During this period, the average temperature was 33.3 °C, and the humidity was 58.6 % RH. The MKT for inside the container at Darwin was calculated to be 37.1 °C (refer to Equation 3.1). The maximum and minimum recordings were 59.5 °C

and 84.5 % RH, and 21.5 °C and 33.0 % RH, respectively. On average throughout the day, the temperature changed 19.9 °C, and the RH changed 12.9 % RH. The average temperature and humidity recording each 30-minute increment over the day is presented in Figure 4.6, demonstrating the average day/night cycle.

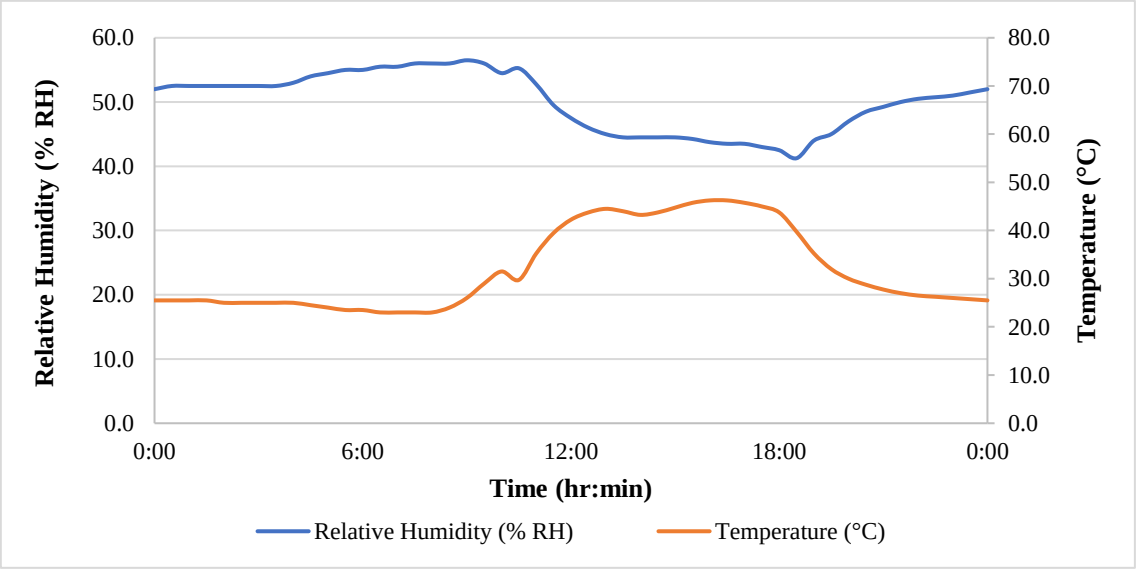


Figure 4.6. Darwin average daily temperature and RH fluctuation measured within the storage container between 22/08/2016 and 17/03/2017.

Darwin experiences a yearly 'wet season' between November and April, in which ambient humidity significantly increases. As observed in Figure 4.7, mid-way through November there is a visible increase in the average RH from approximately 50 % RH raising to approximately 80 % RH, reflecting this seasonal change. However, the temperature remained quite stable, 9.0 % coefficient of variance (% CV) for the average daily temperature compared with 19.9 % CV for RH.

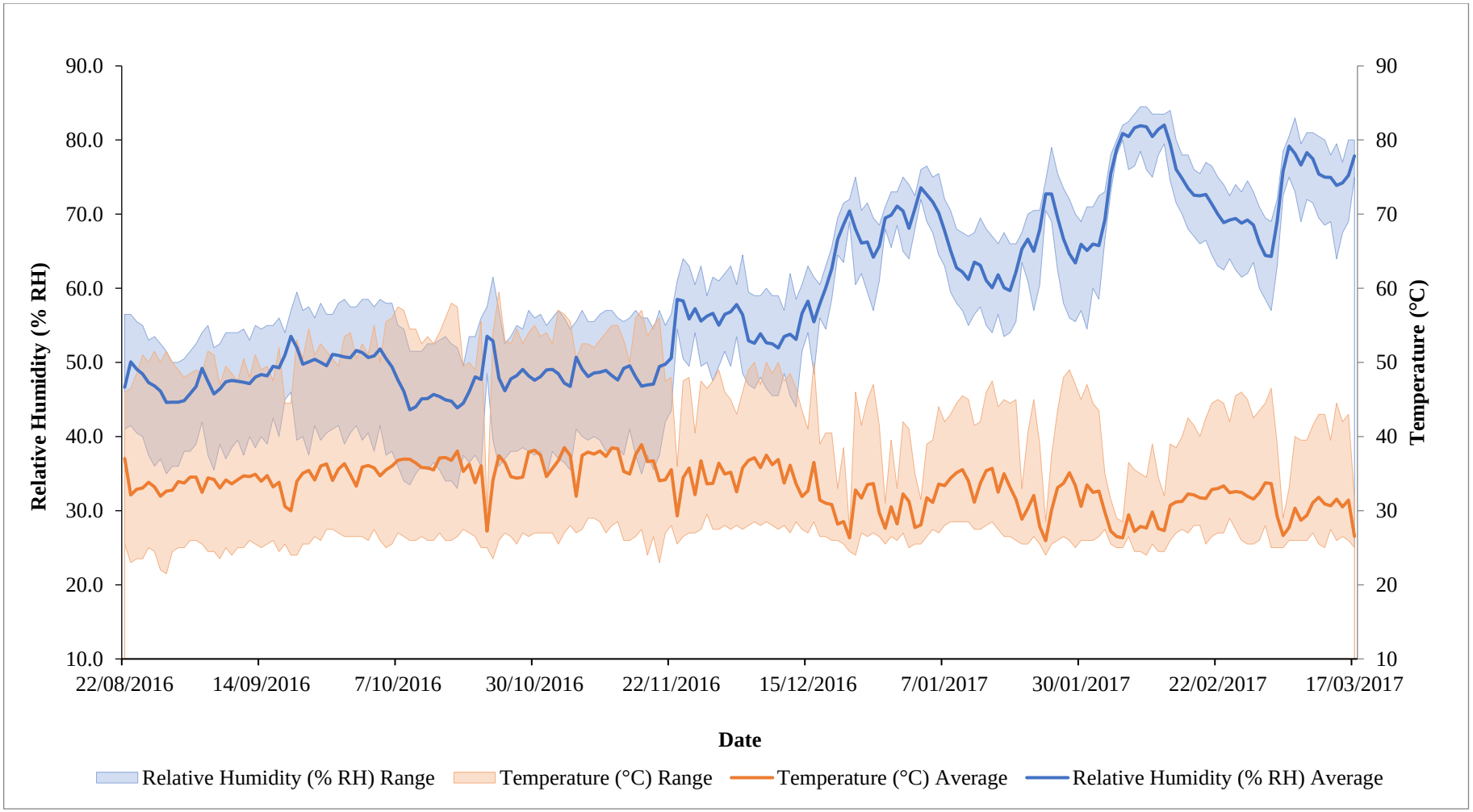


Figure 4.7. Temperature (°C) and relative humidity (% RH) readings at the point of storage within a shipping container in Darwin, between 22/08/2016 and 17/03/2017. The solid line indicates the average recording for that day, the transparent shading represents the range of recordings for that day.

4.2.2. Perth Study.

The metal box containing the medicine was stored in northern Perth, Western Australia, in an open, outside area, which was exposed to direct sunlight during the day (Figure 4.8).

Figure 4.8. Map of the location in North Perth where the metal box was stored.

The medicine box was placed in the location between 28/10/2016 and 15/05/2017 (200 days, 6 months and 18 days). During this period, the average temperature was 22.9 °C and RH was 54.9 %; the MKT for Perth was calculated to be 25.0 °C. The maximum and minimum recordings were 49.5 °C and 86.5 % RH, and 12.5 °C and 15.0 5% RH, respectively. On average throughout the day, the temperature changed 17.3 °C, and the RH changed 26.9 % RH. The average temperature and RH recording each 20-minute increment over the day is presented in Figure 4.9, demonstrating the average day/night cycle.

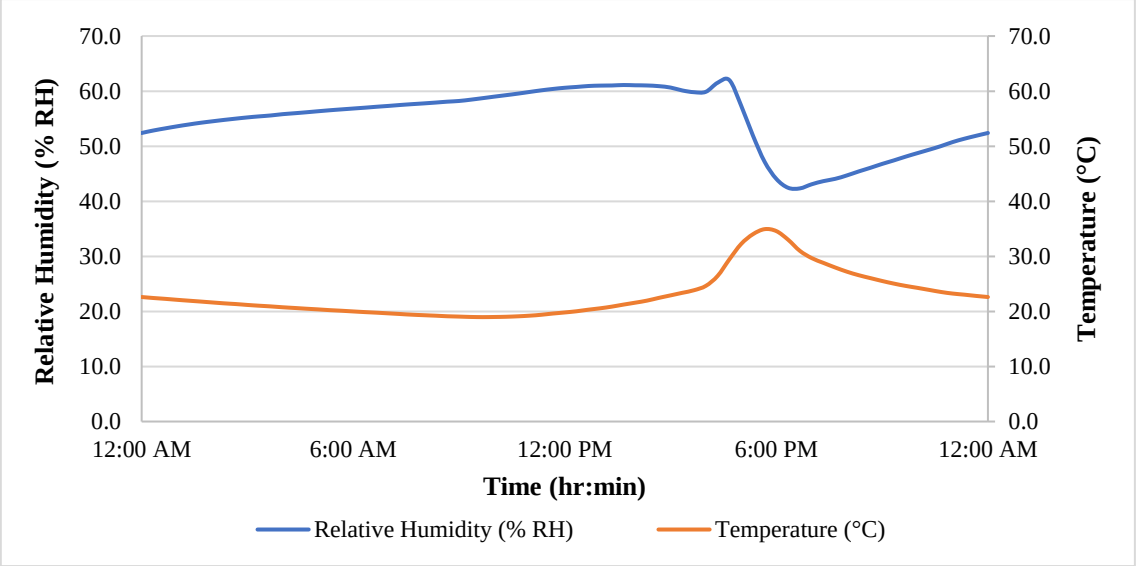


Figure 4.9. Perth average daily temperature and relative humidity fluctuation recorded while stored outside between 28/10/2016 and 15/05/2017.

The medicine box was placed on the west side of the building and received direct sunlight in the afternoon. This location likely caused the recorded trend at approximately 6:00 pm. Figure 4.10 presents the temperature and humidity conditions that the medicine box was exposed to while stored outside in Perth.

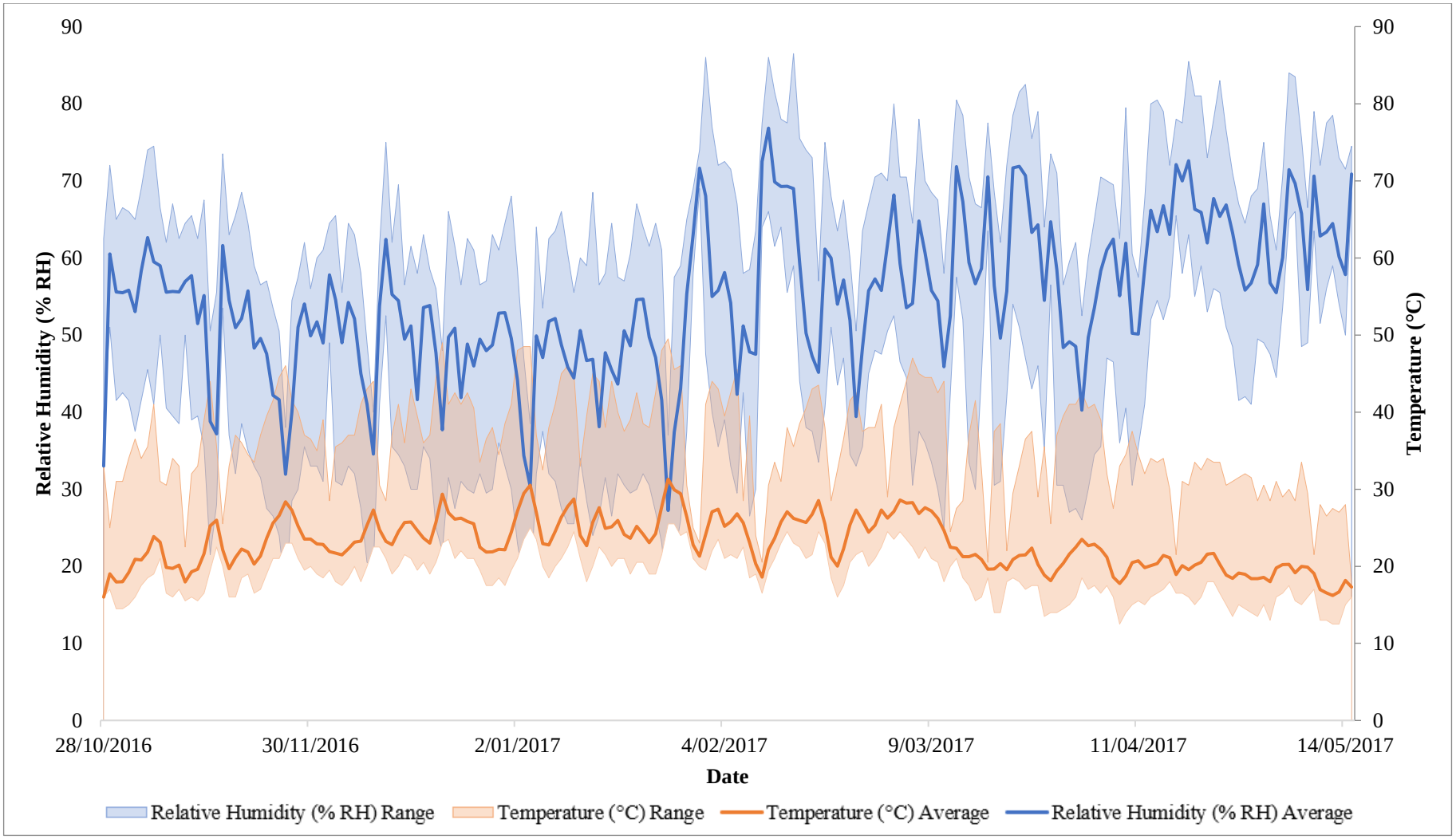


Figure 4.10. Temperature (°C) and relative humidity (% RH) readings at the point of storage in Perth, between 28/10/2016 and 14/05/2017. The solid line indicates the average recording for that day. The transparent shading represents the range of recordings for that day.

4.3. Quality Assessment.

After storage in the two atypical conditions, the contained medicines were analysed using the COPA protocol to establish the quality of the conditions. With the knowledge that the medicines were sourced directly from the manufacturer and not falsified, the medicines still underwent quality assessment of the API and any potential degradant products using the validated HPLC-PDA method.

4.3.1. Materials and Chemicals.

All tablets, both Lumiter (Macleods) and Artrin (Medreich), were co-formulated ATM and LUM formulations at a 20/120 mg strength. The baseline was established by analysing 30 samples in total, comprising of 18 Lumiter tablets (all batch no. 15TAI028A) and 12 Artrin tablets (batch nos. 640256, 640281, 640282). Of the tablets stored in Darwin, 30 were assessed for content, comprising of 18 Lumiter tablets (batch nos. 15TAI068A, 15TAI074A, 15TAI078A) and 12 Artrin tablets (batch nos. 640217, 640218, 640219). Of the tablets stored in Perth, 42 were assessed for content, comprising of 30 Lumiter (batch nos. 15TAI068A, 15TAI074A, 15TAI078A) and 12 Artrin tablets (batch nos. 640217, 640218, 640219). An instrumental error for 12 Lumiter tablets resulted in ATM and LUM being measured from different tablets within the same blister pack. At the time of analysis, the Artrin tablets had expired.

The aqueous mobile phase consisted of 0.1 % *w/v* malonic acid and 0.01 % *v/v* TEA in Millipore water (solvent A), adjust to pH 3.0 with ammonium hydroxide. The organic phase consisted of LiChrosolv® ACN (solvent B). All solutions were then filtered through 0.22 µm Nylon discs under vacuum.

The calibration curve for ATM ranged between 100 – 2500 µg/mL (n = 5) in ACN, with ATS at 450 µg/mL as the IS. The calibration curve for LUM ranged between 5 – 100

µg/mL (n = 5), dissolved in 0.1 % malonic acid and ACN (60:40 *v/v*), with LOS at 15 µg/mL as the IS.

4.3.2. Sample Preparation.

Tablets were given a unique code based on the storage condition, tradename, source, blister pack, and position within the blister pack. For example, an Artrin ('AR') tablet stored in Perth ('PE') would possess the code, PEAR001A07, if it was taken from the first blister pack and occupied the seventh position. The blister packs were orientated and counted in the same manner when allocating the position.

Tablets from each blister packet were selected using the random number generator website, www.random.org (Haahr 1998). The physical measurements included in the assessment were: mass (g), thickness (mm), diameter (mm), and hardness (Newtons, N). These measurements were included as each measurement is non-destructive and does not impact any proceeding content analysis. Each tablet was weighed using an Mettler Toledo New Classic MF analytical scale (MS105DU, 0.01 mg accuracy). Tablet thickness was measured using a Kinchrome 150 mm Vernier calliper (0.02 mm accuracy). Tablet hardness (1-N accuracy) and diameter (0.01 mm accuracy) were measured using an ERWEKA TBH 220 tablet hardness tester. Tablet mass was measured before and after hardness testing to assess any mass loss during hardness testing.

Tablets were firstly weighed for total mass and then crushed into a fine powder using a Silent Knight® Pill Crusher inside low-density polyethylene pouches. The powdered tablets portions analysed for ATM were dissolved in filtered ACN, while the powdered tablet portions analysed for LUM were dissolved in a mixture of mobile phase solvents A and B at a 60:40 ratio. The concentration of the extract was altered depending on the experiment. All solutions were then sonicated for 30 minutes, including vortexing three times, before filtration through a 0.45 µm nylon syringe filter. The filtered extract was then diluted to the required

concentration of 2000 µg/mL for ATM and 90 µg/mL for LUM; the internal standards were then added to the filtered extracts at a concentration of 450 µg/mL for ATS and 15 µg/mL for LOS.

4.3.3. Instrumental Analysis.

A detailed description of the instrumental methods can be found in Chapter 2.

4.3.3.1. *HPLC-PDA Analysis.*

All samples were separated on the Agilent 1200 series HPLC system using an Agilent Technologies Zorbax SB-C18 (2.1 x 50 mm, 1.8-Micron) analytical column. The gradient elution program was initiated at 43 % B, increasing to 75 % B at eight minutes over two minutes, holding for four minutes before re-equilibrating to 43 % B over three minutes and holding for four minutes. ATM and ATS were best observed on the 200 nm UV wavelength. LUM and LOS were best observed on the 250 nm UV-wavelength.

Analytes were detected using RTs and shape of the UV-spectra between 200 – 400 nm. The RT and spectra of ATM and LUM were compared against reference standards. The detection of degradants was based on previously reported data from method validation in Chapter 2. Analyte peaks that possess a S/N greater than ten were included in the quantitative assessment of the storage conditions.

Quantitative analysis was performed in Microsoft Excel (version 1810). Statistical tests were performed in the Excel Data Analysis Tools add-in. The statistical tests performed to compare the data sets were a t-test when comparing the mean of two data sets, and an ANOVA when more than two data sets. Visualisation of data sets was performed using a box-and-whisker plot, which indicates the distribution of the data, the upper and lower quartile, and the median value.

4.3.3.2. *Disintegration.*

Disintegration was assessed using an ERWEKA® Disintegration Tester. The tablets were placed in Millipore water, held at 37 °C (± 2 °C) for 15 minutes. Individual tablets were placed in a single tube of the tester's basket and topped with a plastic disc. The bottom of the tester's basket was a 2 mm wire mesh. The tablets were considered to pass the disintegration testing if no tablet segments remained in the testing basket after the 15-minute period.

Each storage condition was treated as a single 'batch' and assessed according to the Br. Ph. acceptance criteria: the 'batch' is considered to pass if 16 of 18 tablets completely disintegrated. For the baseline, Darwin, and Perth stored medicines, nine tablets were chosen from the two brands (Lumiter and Artrin) to total 18 tablets, representing a Br. Ph. 'batch'.

4.4. Results and Discussion.

The following results present the effect on the medicines after storage in one of three conditions: a) baseline laboratory conditions, b) stored in a shipping container in Darwin, and c) stored outside in Perth. A summary of the physical measurements and the percent content for each sample is listed in Appendix 4.A.

4.4.1. Physical Measurements.

Tablet thickness and diameter were used to calculate the approximate volume of each tablet. The reason the volume is approximate is that each tablet is not a perfect cylinder with the tablet shape, embossing, etc.

Table 4.1 summarises the physical measurements taken of the tablets before content analysis. The average physical measurements of the tablets after exposure to the different storage conditions were compared with multiple within-brand ANOVAs. An LSD test was performed on measurements in which the ANOVA indicated a significant difference. The LSD test identified which groups were different.

Table 4.1. The average measurements of mass, volume, and hardness for Lumiter (LU), Coartem (CO), and Artrin (AR) of each storage condition. Bold values are significantly different from the baseline ($p < 0.05$).

Storage	Mass (g)		Volume (mm^3)		Hardness (N)	
Condition	LU	AR	LU	AR	LU	AR
Baseline	0.25358	0.24265	51.38	44.24	53.00	78.67
Darwin	0.25146	0.24332	**50.47**	44.19	54.72	78.00
Perth	0.25248	0.24101	**50.03**	**43.83**	54.13	75.79

There were minimal physical changes after storing the medicines in the two conditions in Darwin and Perth. The only physical characteristic to see a significant loss was in the volume of the tablets, with the Lumiter tablets found to be significantly lower after both conditions. For Artrin, the results were less consistent. There was a significantly lower volume following storage in Perth, compared to the baseline. Moreover, there was no obvious relationship between the volume and the mass of the Artrin tablets.

4.4.2. Content Analysis.

Analysis of the baseline tablets indicated that the tablets were not poor-quality before placement in the atypical stability tests, as the average ± SD ATM content was 96 ± 5 % (86 – 108 %) and the average content for LUM was 104 ± 9 % (81 – 118 %). There was no significant difference between the percent content of ATM from the two manufacturers. There was a significant difference (p 0.017) between the percent content of LUM with Artrin tablets found to contain $\bar{x}$ 109 ± 6 %, higher than Lumiter ($\bar{x}$ 100 ± 10 %), but within acceptable limits.

The baseline measurements were used as a comparison to measure the effect of storing the medicines in Darwin and Perth. For ATM, after storage in Darwin, the average percent content was 85 ± 6 % (7 – 95 %) across the two brands. Furthermore, there was a significant difference (p 4.3 x 10^{-3}) between the Artrin ($\bar{x}$ 89 ± 4 %) and Lumiter ($\bar{x}$ 83 ± 6 %). After storage in Perth, the average percent content for ATM was 95 ± 6 % (84 – 109 %) for Artrin and Lumiter. However, for the Perth samples, there was no significant difference (p 0.302)

after storage. Figure 4.11 presents a box-and-whisker plot of the percent content of the three storage conditions.

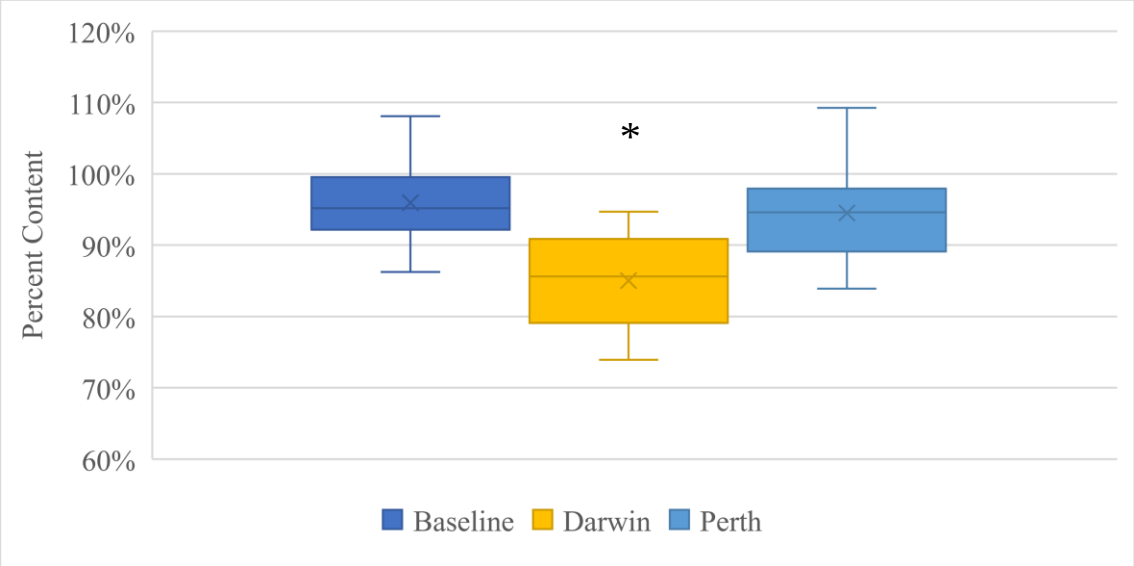


Figure 4.11. A box-and-whisker plot of the percent content of ATM after the unconventional stability testing in Darwin and Perth compared against the baseline. Asterix denotes significantly different ($p < 0.05$).

An ANOVA comparing the average percent content of ATM revealed a significant difference (p 1.08 x 10^{-11}) between the three storage conditions. A post-hoc LSD confirmed the observation in Figure 4.11, identifying that storing the medicines in the Darwin conditions resulted in a significantly lower (p 2.37 x 10^{-11}) content than the baseline, while after storing the medicines in Perth there was not significant difference (p 0.315).

For LUM, after storage in Darwin, the average ± SD percent content was 99 ± 15 % (78 – 126 %) for the two brands. A t-test indicated that there was no significant difference (p 0.300) between Artrin ($\bar{x}$ 95 %, 78 – 107 %) and Lumiter ($\bar{x}$ 102 %, 78 – 126 %). However, the similarity between the means is likely due to the high degree of variability within the analysed sample data sets and may not accurately indicate the two data sets are similar. After exposure to the storage conditions in Perth, the percent content for LUM was 83 ± 17 % (22 – 105 %). There was no significant difference in the content between the Lumiter and

Artrin brands (*p* 0.584). Figure 4.12 presents a box-and-whisker plot of the percent content of the three storage conditions.

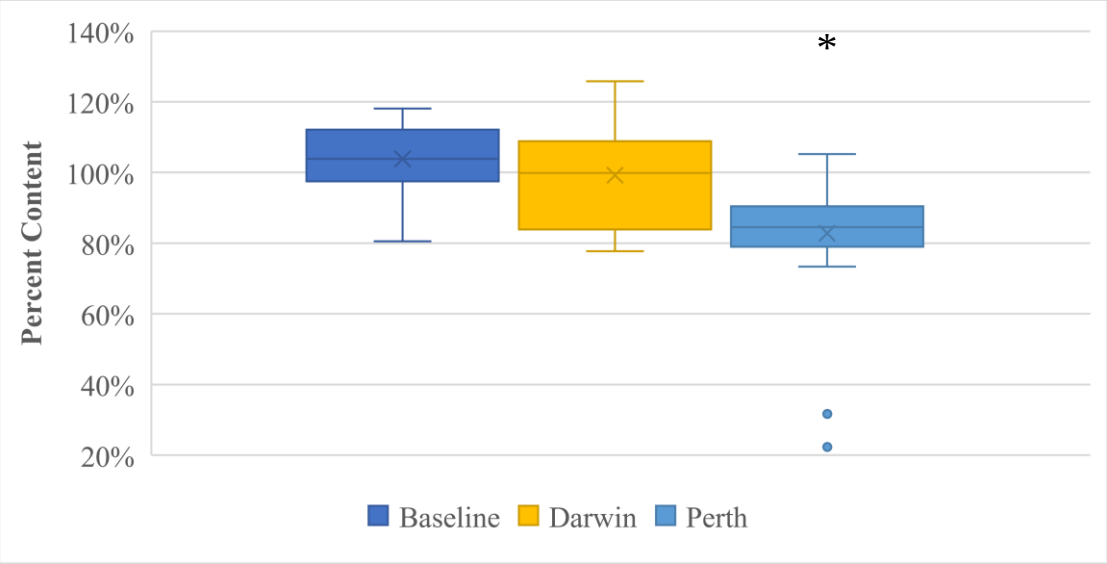


Figure 4.12. A box-and-whisker plot of the percent content of LUM after the atypical stability testing compared against the baseline. Asterix denotes significantly different ($p < 0.05$).

An ANOVA comparing the average percent content of ATM revealed a significant difference (*p* 1.20 x 10^{-8}) between the three storage conditions. A post-hoc LSD confirmed the observation in Figure 4.12, identifying that storing the medicines in the Perth conditions resulted in a significantly lower (*p* 2.46 x 10^{-7}) content than the baseline, while after storing the medicines in Darwin there was no significant difference (*p* 0.144).

4.4.3. Degradant Analysis.

The collected samples were assessed for degradation by observing the three degradant products identified in Section 2.5.1. Degradation products were identified if the HPLC peak possessed a minimum *S/N* ratio of 3; those with an *S/N* ratio greater than ten were used to assess the degradation profile quantitatively.

In the absence of an IS for the degradants, the degradant peaks were divided by the peak area of the associated API after accounting for the degradant peak purity, calculating the DPR. The API % and DPR were plotted, with the correlation observed and measured, to determine the relationship between the generation of the degradant (represented by the DPR) and the loss

of the API. The strength of the relationship is indicated by the r^2 value, with 1.0 indicating a very strong exclusive relationship. The steeper the slope of the data's trendline indicates that more degradant product is generated after the loss of API. A negative correlation is expected between the loss of the API and the generation of a degradant. The identification of the degradant products is described in Chapter 6

4.4.3.1. *Degradant Peak 1*

Degradant Peak 1 has an average RT of 0.95 minutes, eluting between the solvent front and ATS (refer to Section 3.4.3.1). The API associated with Degradant Peak 1 is ATM.

Out of the samples that were analysed from each storage condition, Degradant Peak 1 was quantified in 36 (85.7 %) from the baseline samples, 30 (100.0 %) from the Darwin stability test, and 30 (100.0 %) from the Perth stability test (Figure 4.13).

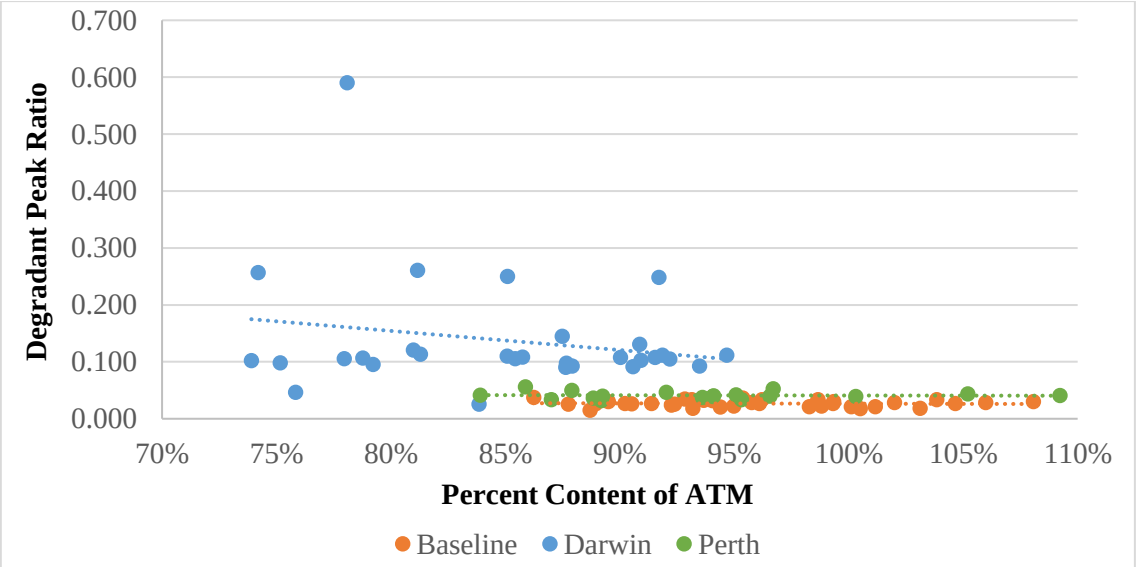


Figure 4.13. Degradant profile of Peak 1 from Darwin and Perth samples compared against the baseline. Baseline $r^2 = 3.9 \times 10^{-3}$, Darwin $r^2 = 0.042$, Perth $r^2 = 1.7 \times 10^{-3}$.

The r^2 values for both the baseline and the Perth samples are very low, demonstrating that there is a low relationship between the percent content of ATM and the generation of the degradant product. The r^2 value for the Darwin samples is slightly higher, r^2 0.042, and the slope is more negatively inclined. However, with the weak relationship, it appears that the negative slope is primarily related to the single data point with the DPR at approximately 0.600.

The remaining data points indicate that the range of ATM percent content is lower in the Darwin samples compared with the baseline and Perth samples.

While the relationship is not a strong negative linear relationship, there are two important observations in the Darwin data: ATM is reduced, and there is the production of a potential degradant product. When compared with the laboratory-based stability tests of Chapter 3, the degradant profile for Degradant Peak 1 is more like the cyclic stability testing profile than the accelerated stability test based on the slope of the trendline. However, the Darwin samples did not degrade to as low percent content for ATM.

4.4.3.2. *Degradant Peak 2*

Degradant Peak 2 has an average RT of 2.35 minutes, eluting between the ATS and ATM (refer to Section 3.4.3.2). The API associated with Degradant Peak 2 is LUM based on a previous publication by Verbeken *et al.* (2011) that related the UV spectra (200 – 400 nm) of Degradant Peak 2 to that of a DBK derivative.

Out of the samples that were analysed from each storage condition, Degradant Peak 2 was quantified in none from the baseline samples, 25 (83.3 %) from the Darwin stability test, and 24 (80.0 %) from the Perth stability test (Figure 4.14).

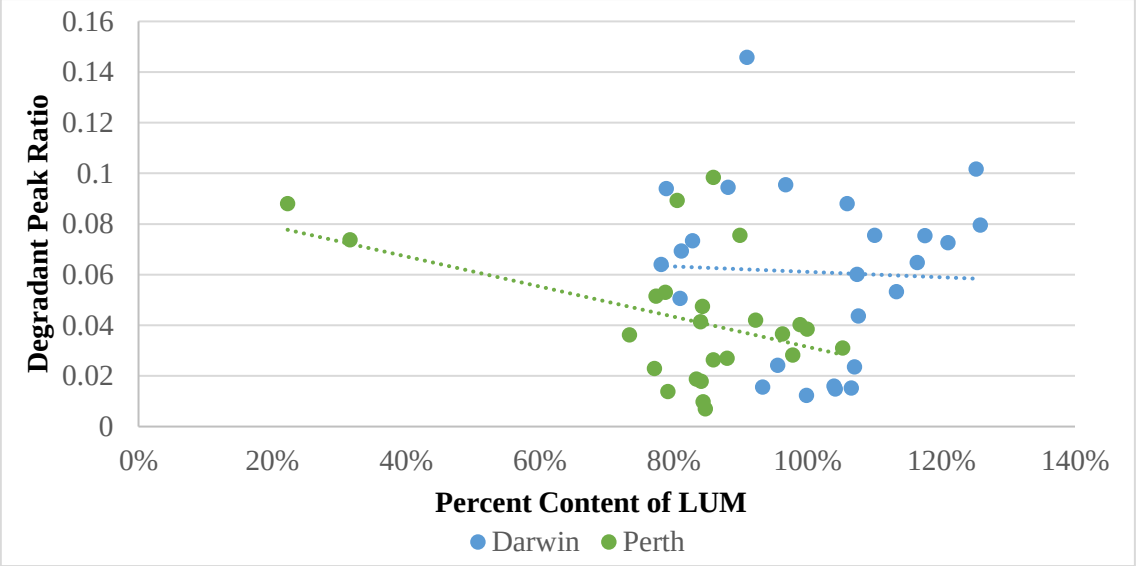


Figure 4.14. Degradant profile of Peak 2 from Darwin and Perth samples. Darwin $r^2 = 2.1 \times 10^{-3}$, Perth $r^2 = 0.188$.

Of the two atypical stability tests, the r^2 value for the Perth samples is the highest at 0.188. However, the stronger relationship is due primarily to the two samples in which the content percent of LUM was less than 40.0 %. It seems that the relationship of the Darwin samples is largely affected by the concentration of LUM, with multiple samples returning content above 120 %. Furthermore, there is a larger spread of the DPR for the Darwin samples, ranging between approximately 0.01 and 0.14 (~ 0.13 DPR), more than the Perth samples (~ 0.09 DPR).

Again, however, the presence of Degradant Peak 2 in the Darwin and Perth samples yet an absence in the baseline samples is in of itself a significant occurrence, suggesting that there is the production of the degradant after the atypical stability test. Moreover, the degradant profiles for the Darwin and Perth samples (Chapter 3) reflected those for Degradant Peak 2 in the laboratory-based stability testing. The percent content was largely within the acceptable limits (± 15 %) but the DPR was greater, and more variable, than seen for Degradant Peak 1 in any storage conditions.

4.4.3.3. *Degradant Peak 3*

Degradant Peak 3 has an average RT of 3.72 minutes, eluting between the ATS and ATM (refer to Section 3.4.3.3). Out of the 42 samples that were analysed from each storage condition, Degradant Peak 3 was quantified in 18 (42.9 %) of the baseline samples, 29 (96.7 %) from the Darwin storage conditions, and 12 (28.6 %) from the Perth storage conditions (Figure 4.15).

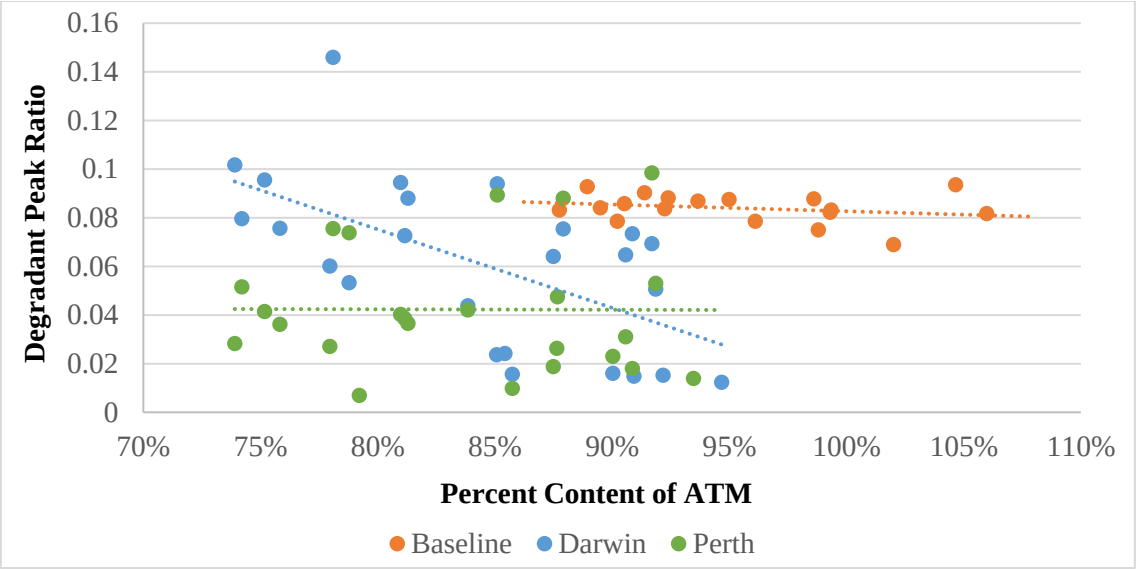


Figure 4.15. Degradant profile of Peak 3 from Darwin and Perth samples compared against the baseline. Baseline $r^2 = 0.062$, Darwin $r^2 = 0.361$, Perth $r^2 = 2.0 \times 10^{-5}$.

The percent content for ATM after the three storage conditions presents what is to be expected (Figure 4.15). The percent content for the baseline samples ranges within the acceptable quality limits of ± 15 % and the two atypical storage conditions indicate a loss of API, with the percent content of ATM for both sets of samples ranging between approximately 74 % and 95 %. However, the DPR relationships are unexpected. The baseline samples exhibit a higher average DPR, ranging between 0.07 and 0.10, while for both the Darwin and Perth samples, the DPR ranges between 0.01 and 0.10. Furthermore, for Darwin specifically, the relationship between the loss of API and the generation of Degradant Peak 3 is relatively strong at 0.361, far greater than the baseline (r^2 0.062) and the Perth samples (r^2 2.0×10^{-5}).

The DPR relationships for the two atypical stability tests are not suggestive of the loss of API and the generation of a degradant product since the two data sets have a lower DPR than the baseline. Importantly, similar results were seen for Degradant Peak 3 in Chapter 3; the baseline samples had a higher DPR than the degraded samples, the peak was quantifiable in only a low number of samples, and there was a relatively weak coefficient of determination for the relationship.

It is conceivable that the peak may be related to a system peak or tablet impurity rather than a degradant peak. The low trendline slopes and the weak r^2 relationships observed in the Perth samples, and the baseline, irrespective of the percent content of the tablet suggests Degradant Peak 3 is alternate to a degradant product. Mass spectrum information is required to confirm that Degradant Peak 3 is a degradant product (see Appendix 5.A).

4.5. Conclusions.

The purpose of this chapter was to observe the effects of uncontrolled climate conditions on the quality of co-formulated ATM and LUM antimalarials during atypical stability tests. The consequences of the storage conditions were measured through the loss of the API and the generation of associated degradant products.

The medicines stored in the shipping container in Darwin were significantly affected by the heightened conditions to which they were exposed. After storage, samples were found to contain less than the acceptable levels of both ATM and LUM, 85 %. Moreover, the profiles of the degradant peaks indicated the generation of degradant products, particularly Degradant Peaks 1 and 2.

The medicines that were stored outside in Perth were also affected but not to the same degree as those in Darwin. Only a single sample returned a concentration of ATM below 85 %, while conflictingly most samples returned LUM below 85 %. However, the degradant profiles of the Perth samples support the conclusion that the medicines were only slightly affected, with

a smaller amount of degradation products formed; suggesting that the significant loss of LUM may have been an analytical consequence.

The analytical observations of the degradant profiles of the two set of samples were indicative of the conditions to which the medicines were exposed. For the atypical stability tests, the conditions were not controlled but were monitored. Therefore, a climate profile was created for each location. The temperature and RH conditions in Darwin were seen to be higher and more variable than the conditions in Perth. The heightened conditions correlate to the increased consequences on the quality of medicines.

In Chapters 3 and 4, there was greater indications of degradation in the storage conditions that experienced the larger variance in temperature and RH, i.e. cyclic stability testing and the Darwin samples. This correlation suggests that the different climate conditions observed in two Köppen-Geiger regions do have the potential to negatively affect the quality of stored medicines. Such a result is foreboding for the distribution of medicines in tropical regions in the absence of climate control (e.g. air conditioning). However, the experiments do not reflect the entire medicine distribution supply chain for tropical regions, and therefore, further insight into the conditions along the supply chain should be sought (see Future Directions in Chapter 7).

Appendix 4.A. Details of the atypical stability samples.

Table 4.2 lists the complete source and analysis information for all laboratory-based stability test samples. Abbreviations in Table 4.2, Baseline (BAS), accelerated (ACC), cyclic (CYC), Artrin (AR), Coartem (CO), and Lumiter (LU). Expiry dates denoted with '*' were expired at the time of analysis. Bold content (%) values indicate those samples outside the ± 15 % quality standards.

Table 4.2. The physical and percent content results for the atypical stability tests.

Origin	Brand	Blister Pos.	Batch No.	Exp Date	Mass (g)	Vol. (mm^3)	Hardness (N)	Content (%)	
								ATM	LUM
BAS	AR	A05	640282	Apr-17*	0.2410	43.87	71	96	114
BAS	AR	A07	640282	Apr-17*	0.2449	43.92	77	108	110
BAS	AR	B01	640256	Apr-17*	0.2458	44.74	72	95	112
BAS	AR	B04	640256	Apr-17*	0.2384	44.02	80	93	118
BAS	AR	C02	640281	Apr-17*	0.2397	44.59	69	95	112
BAS	AR	C04	640281	Apr-17*	0.2364	44.02	79	88	113
BAS	AR	D01	640281	Apr-17*	0.2445	44.12	67	86	114
BAS	AR	D08	640281	Apr-17*	0.2372	43.45	80	93	110
BAS	AR	E06	640282	Apr-17*	0.2423	44.30	71	96	108
BAS	AR	E07	640282	Apr-17*	0.2473	44.54	80	94	100
BAS	AR	F03	640256	Apr-17*	0.2500	44.69	97	104	100
BAS	AR	F04	640256	Apr-17*	0.2444	44.64	89	99	102
BAS	CO	A12	K0020	May-17	0.2432	45.39	117	89	108
BAS	CO	A18	K0020	May-17	0.2413	44.82	116	98	114
BAS	CO	B12	K0019	May-17	0.2399	44.54	120	100	105
BAS	CO	B17	K0019	May-17	0.2414	44.54	122	101	98
BAS	CO	C11	K0032	Jul-17	0.2427	44.54	121	94	116
BAS	CO	C18	K0032	Jul-17	0.2402	44.25	119	93	100
BAS	CO	D04	K0034	Jul-17	0.2426	44.59	118	103	96
BAS	CO	D06	K0034	Jul-17	0.2430	44.54	113	100	108
BAS	CO	E06	K0021	Jul-17	0.2887	58.76	88	102	96
BAS	CO	E08	K0021	Jul-17	0.2489	58.48	85	93	95
BAS	CO	F07	K0021	Jul-17	0.2901	59.18	85	92	102
BAS	CO	F09	K0021	Jul-17	0.2883	58.61	83	99	114
BAS	LU	A07	15TAI028A	Sep-18	0.2495	50.30	53	102	101
BAS	LU	A09	15TAI028A	Sep-18	0.2525	50.86	56	105	102
BAS	LU	A13	15TAI028A	Sep-18	0.2535	50.16	68	94	91
BAS	LU	A16	15TAI028A	Sep-18	0.2569	51.49	63	99	96
BAS	LU	A18	15TAI028A	Sep-18	0.2563	51.69	51	106	102
BAS	LU	A23	15TAI028A	Sep-18	0.2536	50.16	63	99	99
BAS	LU	B05	15TAI028A	Sep-18	0.2565	52.57	50	89	95
BAS	LU	B06	15TAI028A	Sep-18	0.2485	50.55	53	91	101

Table 4.2. The physical and percent content results for the atypical stability tests.

Origin	Brand	Blister Pos.	Batch No.	Exp Date	Mass (g)	Vol. (mm^3)	Hardness (N)	Content (%)	
								ATM	LUM
BAS	LU	B07	15TAI028A	Sep-18	0.2518	51.33	52	99	108
BAS	LU	B12	15TAI028A	Sep-18	0.2548	52.23	49	92	91
BAS	LU	B14	15TAI028A	Sep-18	0.2513	50.49	52	89	112
BAS	LU	B21	15TAI028A	Sep-18	0.2554	52.23	50	90	**81**
BAS	LU	C01	15TAI028A	Sep-18	0.2564	51.73	50	99	86
BAS	LU	C03	15TAI028A	Sep-18	0.2558	52.23	52	91	106
BAS	LU	C05	15TAI028A	Sep-18	0.2513	51.45	42	92	**116**
BAS	LU	C20	15TAI028A	Sep-18	0.2529	52.01	50	88	90
BAS	LU	C21	15TAI028A	Sep-18	0.2513	51.45	45	96	107
BAS	LU	C23	15TAI028A	Sep-18	0.2563	51.95	55	95	115
DAR	AR	A02	640219	Mar-17*	0.2413	43.93	100	92	107
DAR	AR	A04	640219	Mar-17*	0.2418	44.31	79	91	104
DAR	AR	B01	640218	Mar-17*	0.2441	44.12	79	95	100
DAR	AR	D03	640219	Mar-17*	0.2500	44.98	85	92	98
DAR	AR	E01	640218	Mar-17*	0.2380	43.60	79	85	96
DAR	AR	E08	640218	Mar-17*	0.2463	44.74	75	86	93
DAR	AR	F06	640218	Mar-17*	0.2404	44.17	91	85	107
DAR	AR	F08	640218	Mar-17*	0.2460	44.21	83	90	104
DAR	AR	G01	640217	Mar-17*	0.2425	44.17	50	88	**78**
DAR	AR	G04	640217	Mar-17*	0.2427	43.74	68	88	85
DAR	AR	H01	640217	Mar-17*	0.2455	44.31	70	93	
DAR	AR	H05	640217	Mar-17*	0.2414	43.98	77	**79**	**80**
DAR	LU	A01	15TAI068A	Nov-18	0.2509	51.29	56	85	**79**
DAR	LU	A02	15TAI068A	Nov-18	0.2485	51.57	48	92	**81**
DAR	LU	A13	15TAI068A	Nov-18	0.2587	51.96	63	91	**83**
DAR	LU	A15	15TAI068A	Nov-18	0.2546	51.63	50	**78**	91
DAR	LU	A19	15TAI068A	Nov-18	0.2525	51.17	53	92	**81**
DAR	LU	A22	15TAI068A	Nov-18	0.2482	51.17	51	87	**78**
DAR	LU	B07	15TAI074A	Nov-18	0.2476	48.82	55	**74**	**125**
DAR	LU	B12	15TAI074A	Nov-18	0.2491	49.43	59	88	**118**
DAR	LU	B14	15TAI074A	Nov-18	0.2550	49.66	63	91	**116**
DAR	LU	B16	15TAI074A	Nov-18	0.2510	49.38	61	**79**	113
DAR	LU	B20	15TAI074A	Nov-18	0.2552	49.43	68	**78**	107
DAR	LU	B23	15TAI074A	Nov-18	0.2451	48.60	55	**76**	110
DAR	LU	C02	15TAI078A	Nov-18	0.2520	52.23	34	**84**	108
DAR	LU	C07	15TAI078A	Nov-18	0.2470	49.43	49	**75**	97
DAR	LU	C08	15TAI078A	Nov-18	0.2542	52.34	48	**74**	**126**
DAR	LU	C12	15TAI078A	Nov-18	0.2473	48.88	57	**81**	106
DAR	LU	C14	15TAI078A	Nov-18	0.2548	52.40	53	**81**	**121**
DAR	LU	C15	15TAI078A	Nov-18	0.2545	48.99	62	**81**	88
PER	AR	A05	640219	Mar-17*	0.2400	43.64	78	97	88
PER	AR	A06	640219	Mar-17*	0.2423	44.12	86	96	97
PER	AR	A07	640219	Mar-17*	0.2388	43.69	77	102	86
PER	AR	A08	640219	Mar-17*	0.2434	43.64	76	97	**84**

Table 4.2. The physical and percent content results for the atypical stability tests.

Origin	Brand	Blister Pos.	Batch No.	Exp Date	Mass (g)	Vol. (mm^3)	Hardness (N)	Content (%)	
								ATM	LUM
PER	AR	B02	640218	Mar-17*	0.2417	43.36	75	93	87
PER	AR	B03	640218	Mar-17*	0.2454	44.17	85	92	**84**
PER	AR	B05	640218	Mar-17*	0.2421	43.36	82	102	**77**
PER	AR	B07	640218	Mar-17*	0.2421	43.64	72	96	**77**
PER	AR	C02	640217	Mar-17*	0.2380	43.98	76	87	86
PER	AR	C06	640217	Mar-17*	0.2390	43.74	77	101	**84**
PER	AR	C07	640217	Mar-17*	0.2461	45.07	71	88	**79**
PER	AR	C08	640217	Mar-17*	0.2370	43.60	73	101	85
PER	LU	A04	15TAI074A	Nov-18	0.2547	48.71	66	94	**80**
PER	LU	A07	15TAI074A	Nov-18	0.2576	49.82	59	87	86
PER	LU	A11	15TAI074A	Nov-18	0.2448	47.93	61	89	**84**
PER	LU	A19	15TAI074A	Nov-18	0.2449	47.93	57	95	90
PER	LU	A21	15TAI074A	Nov-18	0.2415	48.60	46	97	**79**
PER	LU	A22	15TAI074A	Nov-18	0.2495	48.77	60	94	**83**
PER	LU	B01	15TAI068A	Nov-18	0.2513	50.83	47	-	98
PER	LU	B05	15TAI068A	Nov-18	0.2447	49.71	44	-	**22**
PER	LU	B08	15TAI068A	Nov-18	0.2521	50.33	41	-	105
PER	LU	B12	15TAI068A	Nov-18	0.2539	50.10	57	-	**32**
PER	LU	B21	15TAI068A	Nov-18	0.2557	50.89	53	-	88
PER	LU	B22	15TAI068A	Nov-18	0.2506	50.33	47	-	**73**
PER	LU	C09	15TAI078A	Nov-18	0.2550	50.27	56	-	92
PER	LU	C10	15TAI078A	Nov-18	0.2553	49.55	59	-	**84**
PER	LU	C12	15TAI078A	Nov-18	0.2550	49.77	60	-	**77**
PER	LU	C14	15TAI078A	Nov-18	0.2544	49.71	61	-	96
PER	LU	C15	15TAI078A	Nov-18	0.2507	49.49	56	-	100
PER	LU	C17	15TAI078A	Nov-18	0.2490	49.38	53	-	99
PER	LU	B09	15TAI078A	Nov-18	0.2619	51.84	50	105	-
PER	LU	B13	15TAI078A	Nov-18	0.2548	51.06	49	89	-
PER	LU	B14	15TAI078A	Nov-18	0.2558	50.89	46	94	-
PER	LU	B15	15TAI078A	Nov-18	0.2545	50.33	60	109	-
PER	LU	B16	15TAI078A	Nov-18	0.2458	50.33	47	88	-
PER	LU	B23	15TAI078A	Nov-18	0.2575	51.28	54	**84**	-
PER	LU	C01	15TAI078A	Nov-18	0.2553	49.55	56	97	-
PER	LU	C05	15TAI078A	Nov-18	0.2505	49.38	59	86	-
PER	LU	C07	15TAI078A	Nov-18	0.2559	50.05	55	89	-
PER	LU	C08	15TAI078A	Nov-18	0.2536	49.55	56	92	-
PER	LU	C11	15TAI078A	Nov-18	0.2503	49.55	49	95	-
PER	LU	C16	15TAI078A	Nov-18	0.2579	50.10	60	100	-

Chapter 5. Field Survey of Antimalarials in Uganda.

5.1. Background

The purpose of the following chapter was to implement the COPA protocol to assess the prevalence of poor-quality antimalarial medicines in Uganda over a four-month period in 2016/2017. The COPA protocol has been successfully applied to controlled and semi-controlled stability testing and was found capable. However, to demonstrate the capability of the protocol, a real-world application was required.

The chapter will connect the previous stability testing, both conventional and non-conventional, to a real-world situation. Antimalarials medicines that are being sold as chemotherapeutic treatments in Uganda will undergo the same instrumental analysis and assessment as the previous chapters. The storage of medicines collected in this chapter has not been controlled nor monitored. However, there will be indications to the previous storage conditions of the medicines by measuring their quality.

There are two forms of data collected in this chapter. The results collected using the COPA protocol, including authenticity and the content of API and degradants. Then, further utilising the opportunity to collect information about each pharmacies' practices, a series of questionnaires provides insight into the storage history of the collected medicines.

5.2. Field Survey Design.

The sampling protocol aimed to achieve coverage of the population of registered private PODs through stratified random sampling (SRS). The recommended sampling approach to achieve coverage of such a large area and to remain statistically representative with limited capacity is to perform an SRS approach.

Registered private PODs were selected as information pertaining to their locations around Uganda was readily available, whereas a complete list of public PODs was not

available. Furthermore, a 2011 survey of six sub-Saharan countries indicated that in all but one occasion, most antimalarials were sold or distributed in private, for-profit dispensaries (O'Connell *et al.* 2011). For Uganda specifically, public health centres or not-for-profit dispensaries occupy less than a quarter of the market share, further supporting the primary focus on the private PODs.

Traditionally, geographical administrative boundaries in Uganda are confined within provinces (1st level) and then districts (2nd level), as defined in the country's 2014 census data (Uganda Bureau of Statistics 2016). Uganda has four administrative provinces: Central, Eastern, Northern, and Western, which are subdivided into 24, 32, 30, and 26 districts, respectively (Figure 5.1). Uganda was divided at the district level for this research project. To determine which districts contained a POD, a complete list of PODs was first acquired in 2015 and was further updated in 2016 (National Drug Authority 2016). As of June 2018, statistics on the licensed outlets have been available on the Ugandan National Drug Authority website.

5.2.1. Stratification.

Stratification involves dividing the total area into smaller zones (strata) and apply random sampling within some randomly selected strata (Lance and Hattori 2016). With the novel aspect of this research involving antimalarial stability, stratification of Uganda into smaller areas was defined through climate-related information. A total of 51 of the 112 (46 %) districts contained PODs of which, adequate climate information could be retrieved for 47 districts. The four districts without climate information were excluded from the sampling method. Districts were also excluded from sampling if there were no PODs in the district, no weather stations were reported for the district or that the related weather station was erroneously reported. These exclusion criteria removed less than 1 % of possible PODs.

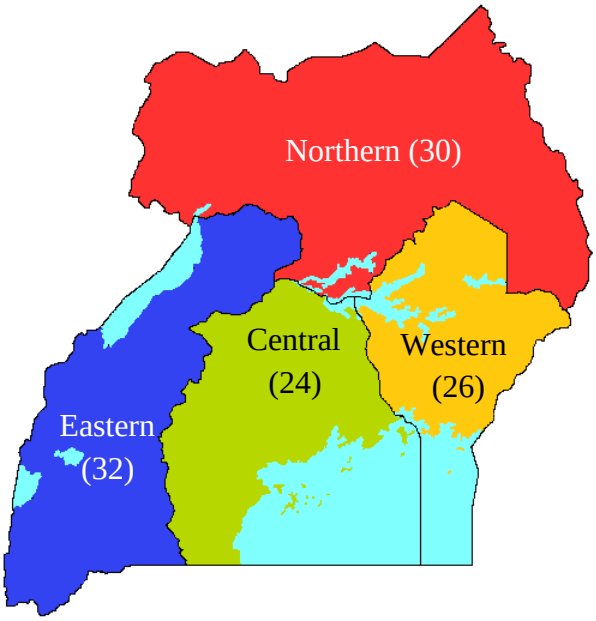


Figure 5.1. Map of Uganda separated into the four administrative provinces (Eastern, Central, Northern and Western); the number of districts contained in each province is indicated in brackets.

For each district with a pharmacy and weather station, daily (n = 365) maximum and minimum temperatures (°C) were obtained and used to calculate daily change (Δ) in temperature; relative humidity (% RH) at midday was also collected. Using these values, the following variables (n = 13) were calculated: highest, lowest, and yearly average recorded maximum daily temperature, highest and lowest recorded minimum daily temperature, yearly average Δ temperature, number of days with a maximum temperature between 30 and 35 °C, number of days with a maximum temperature between 35 and 40 °C, number of days with a maximum temperature above 40 °C, and highest, lowest and average recorded % RH at midday.

Using Statistical Package for the Social Science (SPSS, IBM Corporation), a factor analysis was performed on these variables. The dataset passed the Kaiser-Meyer-Olkin measure of sampling adequacy (KMO = 0.581), and Bartlett's sphericity test was significant ($p < 0.05$). Three components were utilised based upon eigenvalues greater than one; these explained 78.8 % cumulative variance between districts using a principal component analysis (PCA) method. The component indices for each variable were then applied to the recorded

variable measurements for each district to allow the district to be graphed on a PCA plot (Figure 5.2). As seen in Figure 5.2, the PCA scores for each district form an apparent gradient. This trend is to be expected as climate data for each district would typically transition slowly across a geographical area rather than a cluster. It can be concluded that the Northern and Eastern provinces experience less variability in the climate when compared with the Central and Western provinces.

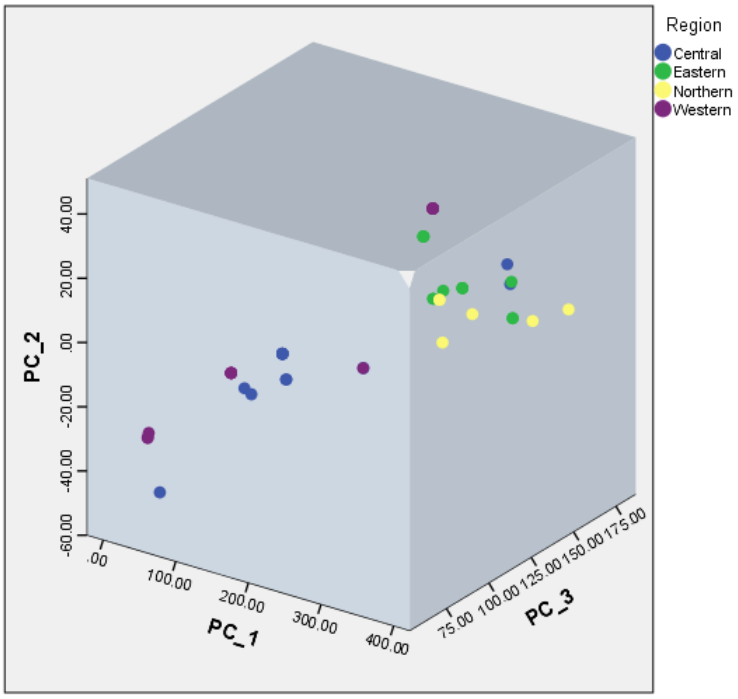


Figure 5.2. Three-dimensional principal component analysis (PCA) scatterplot illustrating the distribution of Ugandan districts using climate information.

Districts were then forced into five clusters using agglomerative hierarchical cluster analysis based upon the three PCA scores (see Figure 5.3). The combined number of PODs reported within these five clusters in 2015/2016 were used to allocate the proportion of sampling sites. The total number of required sampling sites allocated to each cluster was estimated using the formula detailed in Equation 5.1 (Newton *et al.* 2009). The estimated prevalence of poor-quality antimalarials (18 %) was determined through the accumulation of field survey data collected from 61 published articles published between 1994 – 2016 (Grech

et al. 2017). The determined sample size of 100 PODs will allow the reporting of PQMs in Uganda with the precision of ± 7.5 % with 95 % confidence intervals.

$$\boldsymbol{n = \frac{4\,prevalence\,(1-prevalence)\,z^2}{precision^2}}$$

where z = z-score at 95 % confidence level.

Equation 5.1. Estimation of required sample size for random sampling surveys.

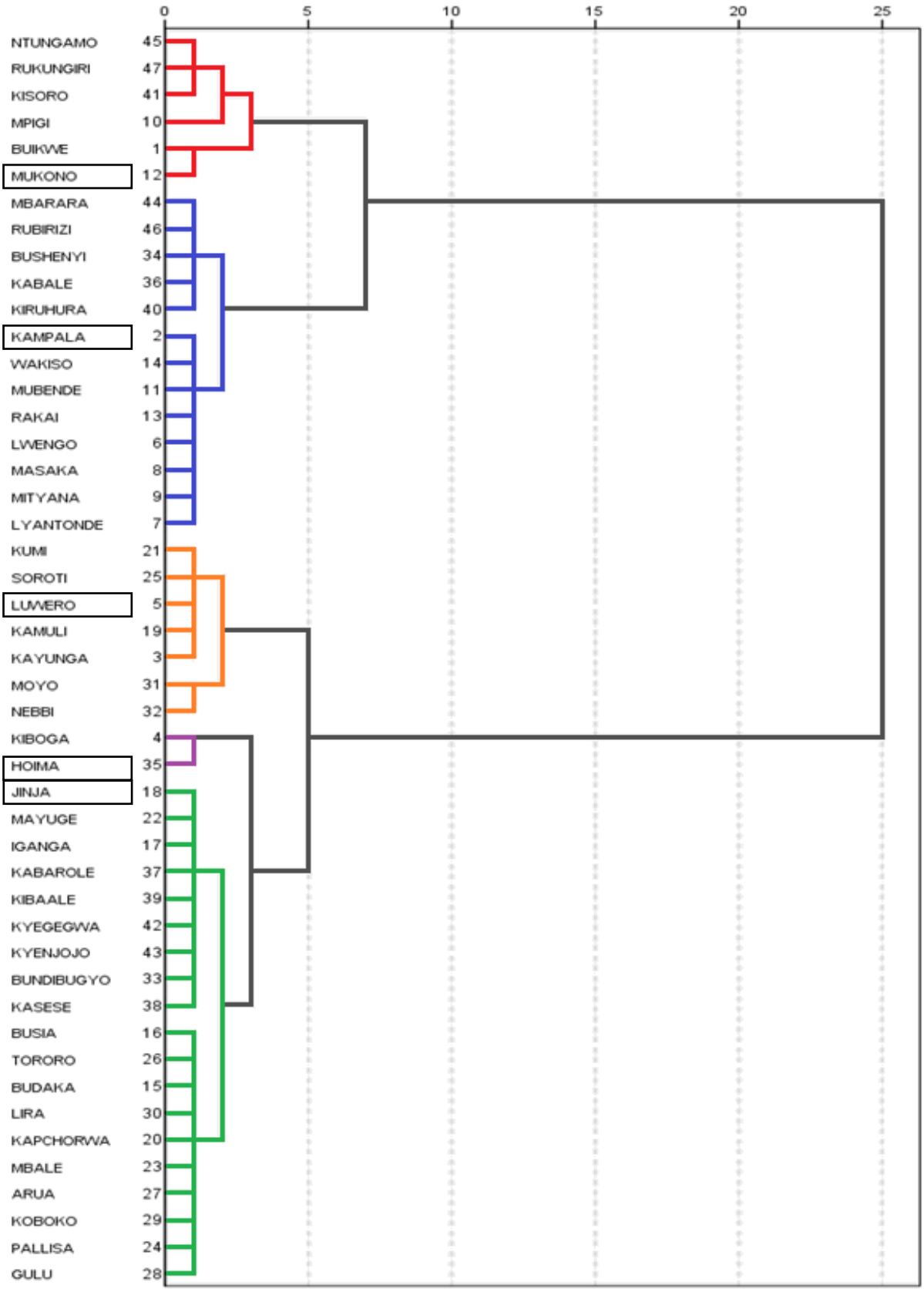


Figure 5.3. A dendrogram indicating agglomerative hierarchical clusters of Ugandan districts using three principal components. The selected districts are bordered. (Cluster 1 - Red, Cluster 2 - Blue, Cluster 3 - Orange, Cluster 4 - Purple, Cluster 5 - Green).

5.2.2. Random Allocation.

The allocation of PODs as sampling sites was performed in two rounds of SRS. The first round guaranteed the sampling of each cluster while avoiding over-representing a cluster through selecting a minimum of five pharmacies or 20 % of the total pharmacies, whichever was less. The second-round allocations distributed the remaining sampling sites based upon a 'population proportional to size' method in which clusters which contained most of the pharmacies (i.e. clusters 2 and 5) were allocated most of the remaining sampling sites (Table 5.1).

Table 5.1. Distribution of districts, PODs, and simple random sampling (SRS) sample sites within each formed cluster

Cluster	n Districts	n of PODs (% of total)		n of SRS Sample Sites (1^{st} + 2^{nd} Round)	
1	6	26	(3.3)	8	(5 + 3)
2	13	615	(78.0)	67	(5 + 62)
3	7	19	(2.4)	6	(4 + 2)
4	2	9	(1.1)	3	(2 + 1)
5	19	119	(15.1)	17	(5 + 12)

Additionally, non-registered dispensaries were conveniently sampled based upon close geographical proximity to the selected PODs. Through this application, it is intended that the convenience sampling will share the coverage area of SRS.

The list of the PODs was sorted alphabetically using the name of the pharmacy. Using the information from the Ugandan NMRA list of registered PODs, a list was compiled detailing a unique, four-digit code, the name, address, and district for each POD. A National Institute of Standards and Technology random number table was then used to allocate every POD a number between 0 and 10,000 (National Institute of Standards and Technology 2016). Random numbers were then arranged in ascending order to determine the order of preference for sampling.

For the five clusters, one district was chosen as a representative of the cluster. The choice of the district was based on the number of PODs within the district and was necessary for sampling logistics. It was logistically impractical to collect 100 samples from a total list of Uganda registered PODs, as a single POD selected in a distant district would require multiple days and excessive funding to sample. Therefore, the district with the largest number of registered PODs from within each cluster was chosen as the sole sampling district. The following districts were chosen to represent each cluster: Mukono (Cluster 1), Kampala (Cluster 2), Luwero (Cluster 3), Hoima (Cluster 4), Jinja (Cluster 5).

A Ugandan collaborator, located in Kampala, was contacted through The International Association of Forensic Toxicology. The collaborator had previous experience in forensic chemistry and an interest in international health. The Ugandan collaborator was responsible for sample collection and was provided with the list along with the standard operating procedure detailed in Appendix 5.A, and sampling was initiated in the Kampala district in October 2016. It became apparent that the initial list of registered PODs from May 2014 was outdated as many PODs were closed. Two actions were taken to counter this: 1) if a POD was closed, the selected POD was replaced with a nearby registered POD, and 2) an updated list of registered PODs from October 2016 was applied to the remaining four districts. A revised sampling list for the Kampala district was not implemented, so the sample sites already visited did not become invalid.

In two situations, additional convenience sampling occurred outside the sampling protocol. In districts outside of Kampala, the Ugandan collaborator continued to sample down the list, beyond the number allocated to the Cluster. These extra samples were a logistical decision to capitalise on the travel to the outside district. Inside Kampala, a convenience sampling method was used to sample 25 additional registered PODs. The samples collected

from these sites were analysed using the same protocol as those collected using the SRS method but are reported separately.

5.2.3. Ethics.

The project received prior approval for the field survey from the University of Canberra Human Research Ethics Committee, project number 16-65.

Many ethical decisions were made when designing and implementing the field survey research. The research implemented an overt sampling method, whereby the collaborator identified themselves as researchers and communicated the intentions of the project prior to sample procurement. The benefit of an overt sampling method compared with a covert sampling method was the ability to collect a coinciding survey at the time of sampling, which provided an opportunity to gather information regarding the PODs practices such as the typical on-site storage time before sale which could help inform the interpretation of the field survey results with respect to observed degradation. However, the clear disadvantage is that the POD participant is aware of the research and can select samples known to be of high quality, potentially biasing the collection. For the COPA project, ethics approval was contingent on an overt sampling method being applied. After ethics approval was granted, further research surrounding the ethics of medicine quality surveys was published addressing the decisions surrounding overt and covert sampling, highlighting the challenges in this space (Tabernero *et al.* 2016).

A key ethical consideration was confirming the willingness to participate from all persons involved. Letters of consent were completed and signed by: a) the local collaborator (Purchaser) prior sampling occurring, and b) the POD participants (Seller) after first being informed about the research (Appendix 5.B).

The information provided to the POD participants at the time of sampling did not contain identifiable information of the local collaborator to reduce the risk of potential

consequence on them. The contact information was that of the University of Canberra research group, and all external email correspondence was performed with a University of Canberra email address so that it would be automatically recorded. All research reporting was assured to de-identify information concerning the visited PODs to mitigate negative risks on the participants. All participants were offered to opt-in to be informed on the outcomes of the results for their own POD. Finally, the research data (collected antimalarials and the associated surveys) are stored in a secured room at University of Canberra for at least five years. The survey was performed using these ethical guides.

5.3. Field Survey Implementation.

5.3.1. Creating a Custom Survey Tool.

The aim of the custom survey tool was to create a hand-held device capable of recording critical information of the field survey, including: date and time, temperature (°C), humidity (% RH), GPS coordinates, and to capture photos. The survey tool was custom-built by a student member of Forensic Studies at the University of Canberra Forensic (Alex Spence) to address the specific needs of the COPA project. An important feature of the survey tool was a simple user-interface that would encourage user uptake (see Appendix 5.C). The survey tool used a combination of touch-screen and manual buttons to navigate the sampling function (Figure 5.4).

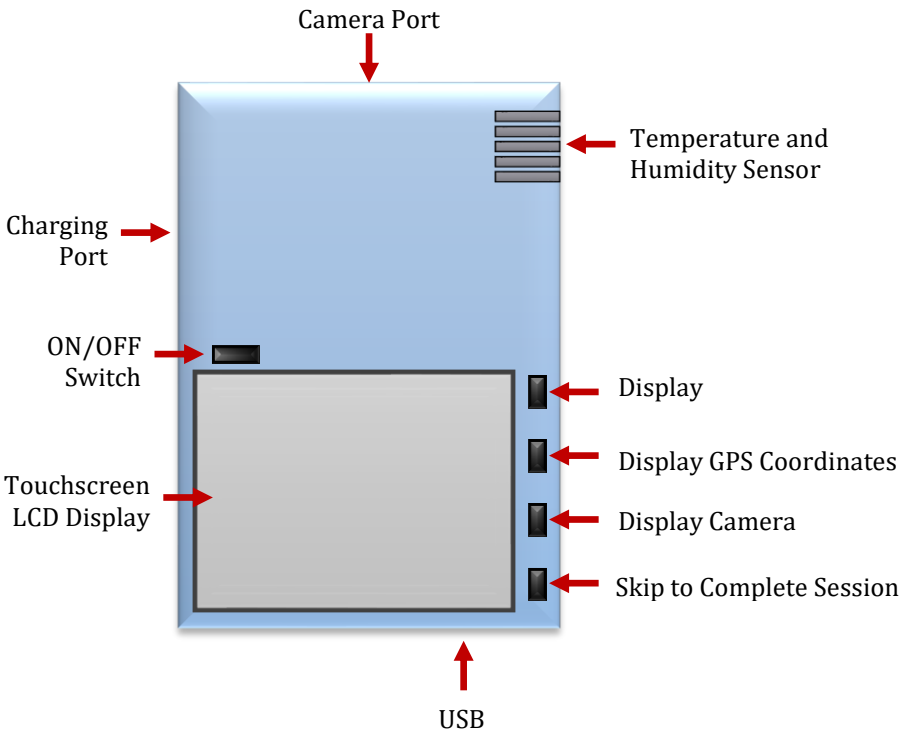


Figure 5.4. Schematic Diagram of the Custom Survey Tool (Annotated).

The camera port contained an eight-megapixel camera capable of 3280 x 2464-pixel static images. The temperature (-40 to 80 °C, 0.5 °C accuracy) and humidity (0 to 100 % RH, 2.5 % RH accuracy) sensor made recordings every two seconds. The sensor was protected from heat from the battery by a silicon encasement. The internal GPS unit recorded time, date, longitude, latitude, and height every 15 seconds. A list of the components in the survey tool is presented in Appendix 5.D; the cost associated with the construction of the device was less than $ 250.00 USD.

The pilot implementation of the survey tool was overall successful. Much of the information reported in Section 5.3.3 was recorded with the survey tool in addition to insightful images such as that displayed in Figure 5.5.

Figure 5.5. An image captured using the survey tool, presenting the inside a POD.

5.3.2. Sample Receipt Processing.

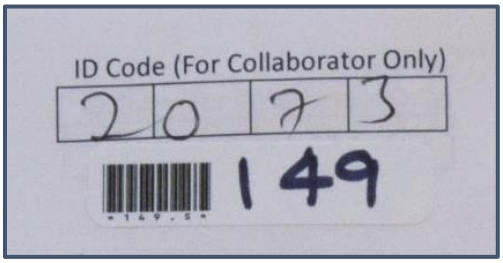
Between 22/10/2016 and 01/02/2017, the local collaborator responsible for sample procurement collected sample packs from 182 PODs from the five administrative districts using both SRS (n = 91) and convenience sampling (n = 91). The convenience sampling samples are consisted of 33 collected due to their proximity to an SRS POD and 58 samples due to opportunistic or additional sampling.

Once the samples were sent to the University of Canberra for analysis, sample packs were selected at random from the cardboard box by a University associate, allocated a sequential Pack code (1 through 182), and logged in an exhibit store.

A Lascar data logger (EL-USB-2-LCD) was placed in with samples upon arrival at the University, measuring in 20-minute increments. The average temperature was 20.1 °C and 40.3 % RH, the MKT for the stored Ugandan samples was calculated to be 20.7 °C. The maximum and minimum recordings were 25.5 °C and 57.5 % RH, and 17.5 °C and 28.5 % RH, respectively. On average throughout the day, the temperature changed 1.7 °C and the humidity changed 3.7 % RH.

The 182 sample packs were received unsorted in a large cardboard box. Each sample pack contained the completed questionnaire form(s) and the purchased antimalarial sample packet. A sticker (indicating the sequential Pack code, and a barcode used for exhibit management) was placed over the POD ID code for the sample pack to blind any bias regarding location or registration status; the survey questionnaires were not blinded (see Figure 5.6).

a)



b)

Figure 5.6. a) A representative survey questionnaire form showing both the POD ID code and sequential Pack code, b) the sample pack showing the POD ID code covered by the sequential Pack code.

All blister packs, the patient information leaflet, and the cardboard packet, which was cut and splayed, were photographed on both sides. From this point, the questionnaires and the sample pack containing the antimalarials were separated and stored independently.

5.3.3. Cost Breakdown of Field Survey.

To increase transparency of the research and to provide insight into the viability of future research, the costs to perform the Ugandan field survey are reported (Visser *et al.* 2014). Purchases were made in Ugandan Shillings and were converted to $ USD for reporting. The applied conversion rate was 3400 Ugandan Shillings per $ USD. Costs to analyse each tablet were approximated in Australian dollars and converted to $ USD at a rate of $ 0.75 Australian Dollars per $ USD for reporting.

The local collaborator volunteered for the collection and absorbed no costs. This greatly reduced the cost of the collection. Partial funding for the Ugandan field survey was donated to

the University of Canberra Faculty of Health by the Tall Foundation. The total cost of the field survey was $5707 USD; the breakdown of the total cost is included in Appendix 5.D.

5.4. Field Survey Results.

5.4.1. Questionnaire Results.

The following results combine the answers to two surveys, herein referred to as Questionnaire 1 and 2, that was distributed at the visited PODs as part of the field survey (Appendix 5.E). Questionnaire 1 refers to the survey completed by the local collaborator, which collected information detailing the medicines collected and the POD at the time of collection. Questionnaire 2 refers to the optional questionnaire completed by the POD participant.

2.3.3.2. *Questionnaire 1 – Local Collaborator.*

In total, 12 formulations of ATM and LUM co-formulated tablets were collected, differing either by brand or strength (Table 5.2). The strength of the tablets is reported as the amount, in milligrams, of ATM and LUM in each tablet (ATM/LUM).

Table 5.2. Brand names, manufacturer information, and number of collected samples in Ugandan field survey.

Brand Name	Manufacturer (Location)	Strength ATM/LUM (mg)	n of Samples
Artefan	Ajanta Pharma Limited (Mumbai, India)	20/120	30
		80/480	2
Cach-ART	Cachet Pharmaceutical Private Limited (Mumbai, India)	20/120	1
Coartem®	Novartis (Basel, Switzerland)	20/120	8
Combiart	Strides Shasun Limited, formerly Strides Arcolab Limited. (Bangalore, India)	20/120	22
Komefan 280	Mylan Laboratories Limited (Maharashtra, India)	40/240	1
Laritem	Ipca Laboratories Limited (Mumbai, India)	80/480	1
Lonart	Bliss GVS Pharma Limited (Maharashtra, India)	20/120	26
Lonart Forte		40/240	4
Lonart-DS		80/480	4
Lumartem	Cipla Limited (Pithampur, India)	20/120	82
Lumiter	Macleods Pharmaceuticals Limited (Mumbai, India)	20/120	1

Of interest was the different costs of procuring each of the formulations listed in Table 5.2 and identifying whether variables such as cluster, strength or brand affected the price. Figure 5.7 presents the average cost of procuring each formulation of AM within each administrative district (representing each cluster).

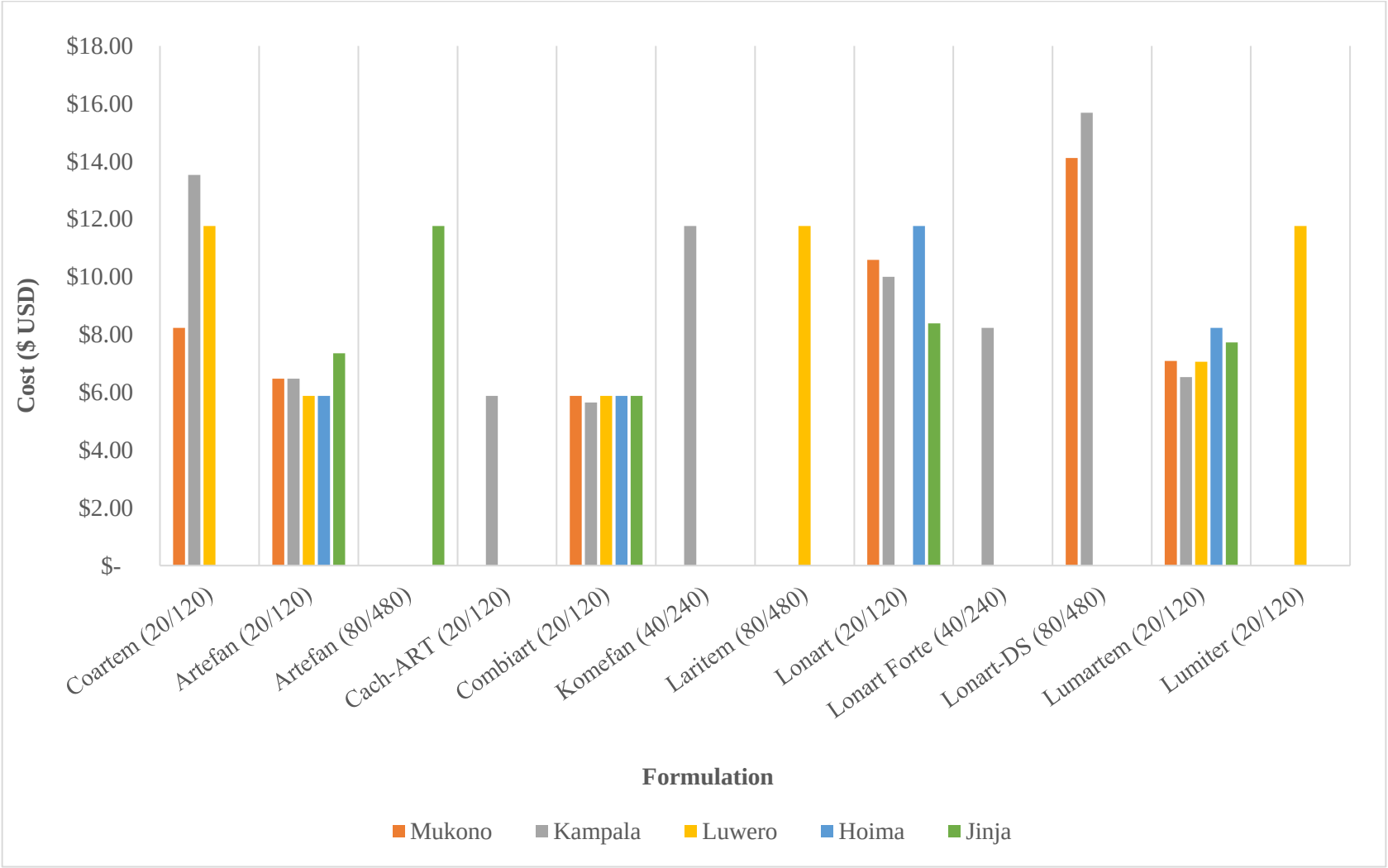


Figure 5.7. The average cost of all the collected antimalarials reported as the average of each administrative district.

There was a significant difference between the total average price of the innovator brand, Coartem ($\overline{x}$ $ 11.76 USD), and the remaining generic versions ($\overline{x}$ ~ $ 7.94 USD) (*p* 3.8 x 10^{-3}) (Figure 5.7). This supports the common conception that the innovator brand remains more expensive than generic versions. Moreover, there was a significant difference between the price of all versions with 20/120 strength ($\overline{x}$ ~ $ 7.65 USD) compared with the other two strengths, 40/240 and 80/480 ($\overline{x}$ ~ $ 12.35 USD) (*p* 1.8 x 10^{-4}). It is important to note that while the strength of each tablet is different, the higher strength packets contained less tablets, resulting in an identical total dose delivered. Therefore, patients who purchase higher strength tablets at the higher price perhaps do so for the convenience of being required to consume less tablets.

Three versions were common to all five administrative districts that were surveyed: Artefan, Combiart, and Lumartem (all 20/120 strength). These versions (totalling 134 samples, 74 % of sampled packs) were selected to identify whether there was a difference in price based on district. The difference in the average price was assessed with an ANOVA test and concluded there was no significant difference in the average price of the three versions (*p* 0.35).

Another variable identified as potentially affecting the purchase price was the type of POD. At the time of collection, the local collaborator allocated the selected POD one of the following POD descriptions: commercial dispensary (Chain), independent dispensary, large-scale retailer (pharmacy), non-specialised general store, small-scale retailer, or wholesaler. A primary POD description was provided in 97 % of the surveys, and for some PODs (5 %) the collaborator provided a secondary description, typically as a wholesaler.

The two most common descriptions in all five administrative districts (n = 182) were small-scale (38 %) and large-scale (36 %) retailers. In Kampala (n = 103), there was also a high percentage of commercial dispensaries (13 %) and independent dispensaries (15 %). Figure 5.8 presents the proportion of POD descriptions for the 182 PODs sampled in the field survey.

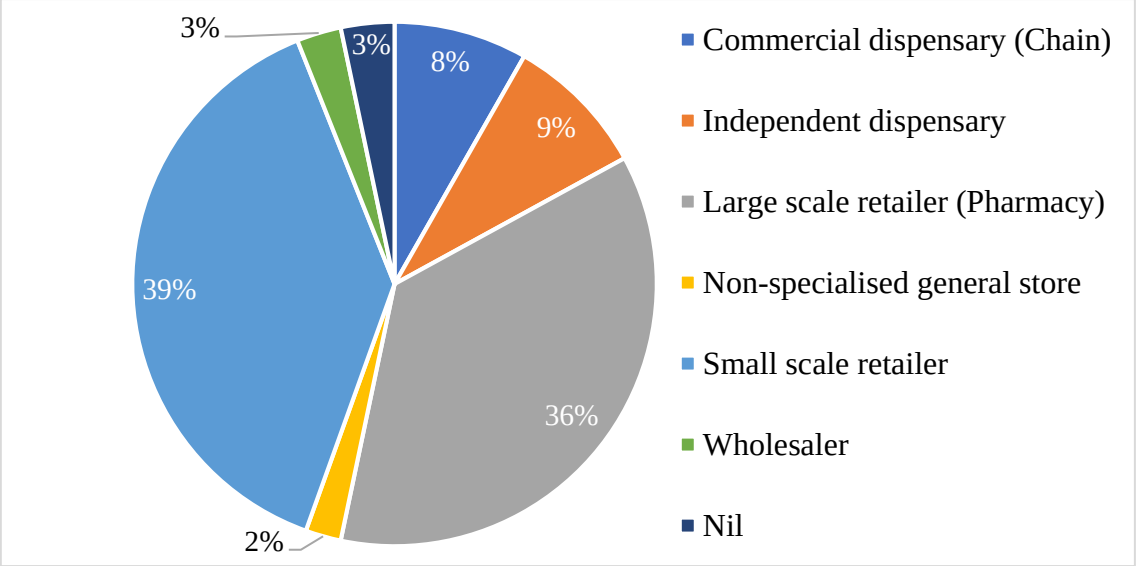


Figure 5.8. A pie chart demonstrating the distribution of POD descriptions of 182 PODs surveyed throughout Uganda in the field survey.

An ANOVA was used to compare the average cost of antimalarials within the above POD descriptions. The test indicated a significant difference *(p* 0.011) between the descriptions. The average price ranged between $ 4.94 USD for those described as wholesalers and $ 9.67 USD for those described as a commercial dispensary (chain).

As described in Section 5.2.1, the five sampled administrative districts were selected based on climate-related variables. The collaborator recorded the temperature (°C) and % RH within the POD at the time of sampling, at 82 % and 37 % of PODs, respectively. For PODs in which both temperature and humidity were recorded (n = 67, 37 %), the survey tool detailed in Section 5.3.1 was utilised. For the PODs in which only temperature was recorded, the temperature was measured by a Brannan thermometer with an accuracy of 0.5 °C (product no. 14/410/3, Cumbria, England).

The mean POD temperature and % RH collected in the five sampled administrative districts was compared using an ANOVA with a post-hoc LSD analysis. The average temperature was found to be significantly different *(p* 1.3 x 10^{-16}) between all five administrative districts. However, some districts were found to be similar based on their average temperature (demonstrated in Figure 5.9). Therefore, the 5 surveyed districts could be

assigned into one of three categories based on average temperature at the time of sampling: a) Jinja ($\bar{x}$ 28.6 °C) and Kampala ($\bar{x}$ 28.2 °C), b) Hoima ($\bar{x}$ 31.8 °C) and Mukono ($\bar{x}$ 31.9 °C), and c) Luwero ($\bar{x}$ 34.8 °C). Of the four administrative districts in which humidity data were recorded, Luwero ($\bar{x}$ 38.9 % RH) was found to be significantly different (*p* 3.3 x 10^{-3}) to Kampala ($\bar{x}$ 44.4 % RH), Hoima ($\bar{x}$ 47.4 % RH), and Mukono ($\bar{x}$ 47.4 % RH).

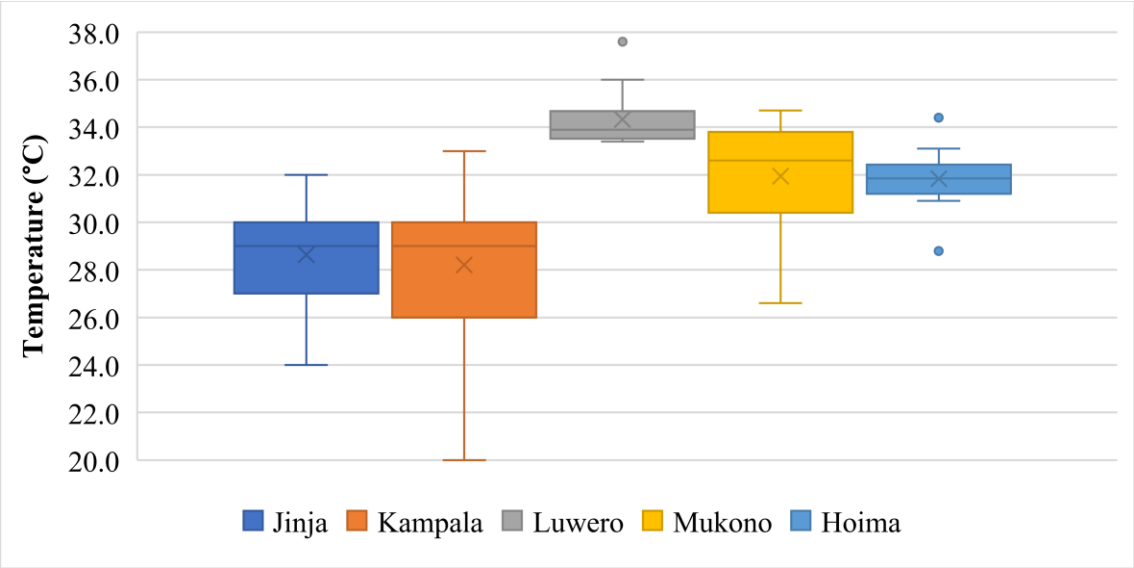


Figure 5.9. Box and Whisker plot demonstrating the difference in the recorded temperatures of the five administrative districts at the time of sampling.

The heightened temperature and humidity recordings can be mitigated through adequate regulation, e.g. air-conditioning systems. The survey included a free-text response querying whether the POD possessed a form of temperature control. Approximately 20 % of the visited PODs had a temperature control, either; a fan (n = 27), a thermometer (n = 13), both a fan and thermometer (n = 2), or an air-conditioning system (n = 1). When arranged by administrative district, the PODs visited in Jinja contained the highest percentage of temperature control (50 %), followed by Kampala (20 %). In support of the higher recorded temperature above, none of the PODs in Luwero possessed a form of temperature control. When arranged by POD description, the POD more likely to contain a form of temperature control was a large-scale retailer (32 %), followed by a commercial dispensary. None of the

non-specialised general stores (n = 4) possessed a form of temperature control, presumably as their commercial interests were not solely the sale of medicines.

The knowledge of the conditions that are required to store medicine properly before sale may correspond with the presence of a trained pharmacist. Each POD that was visited was queried whether a pharmacist was present. Of the 182 stores, only 32 % (n = 59) had a pharmacist present at the time of sample collection. The highest response by far was from PODs in Kampala (n = 58, 98 %), with only one other POD in Mukono having a pharmacist present. The POD description with the highest percentage of pharmacists present was a wholesaler (n = 4, 80 %), followed by independent dispensaries (n = 11, 69 %). As expected, none of the non-specialised general stores had a pharmacist present at the time of the survey.

The highest qualification of the person in charge during the purchase was also surveyed; secondary school, a Bachelor degree, Master degree, or a Doctoral degree, and a free-text response to the discipline was available. Of the 21 respondents, 16 (76 %) reported a Bachelor degree. Large-scale retailers appeared to have the highest concentration of tertiary qualifications. With two of the four respondents possessing a Master degree and one respondent possessing a doctoral degree.

The presence or absence of a pharmacist did not contribute to the requirement of a prescription to purchase the antimalarials, as none of the PODs that were visited requested a prescription for sale. This result contrasts the statement printed on the cardboard packaging of some medicines as being a prescription-only medicine.

2.3.3.3. *Questionnaire 2 – POD Participant.*

At the time of sampling, the participants were requested to complete an optional questionnaire regarding their purchasing, storage, and disposal practices. Of the 182 PODs that were visited, 31 opted to complete the questionnaire; however, not all respondents completed all the questions.

The respondents were asked to select one of six timeframes indicating how often the POD procured medicines: < 3 months, 3 – 6 months, 6 – 9 months, 9 – 12 months, 12 - 8 months, and > 18 months. Of the 25 respondents, 23 (92 %) reported procuring medicines in less than three months with only two procuring medicines within a 3 – 6 month timeframe. No trends regarding the administrative districts nor the POD description were apparent.

The number of treatment courses procured each time ranged between 3 – 100 courses. The highest average number of treatment courses procured at each time was reported by a wholesaler ($\bar{x}$ 90.0 courses), followed by the large-scale retailer ($\bar{x}$ 46.3 courses). The lowest number of courses procured each time was by a small-scale retailer ($\bar{x}$ 11.7 courses), aligning the size of the POD and the treatment courses.

The respondents were asked how confident they were that the procured medicines were high quality: not confident, somewhat confident, confident, and highly confident. Of the 26 respondents, 10 (38 %) were highly confident, 15 (58 %) were confident, and only one (4 %) was somewhat confident. The POD description that responded as somewhat confident was an independent dispensary. The POD description with the highest level of confidence was the commercial dispensary (n = 3, 60 %), followed by large-scale retailers (n = 2, 50). The results indicate that the presumed purchasing power may result in more confidence that the medicines are high quality.

Regarding their disposal practices, most of the respondents indicated the POD did not dispose of any expired medicines as the product typically sold before the expiration date. However, those who did report their method of disposal (n = 19) strongly reported surrendering the expired medicine to the Ugandan National Drug Authority (NDA, 42.8 %), followed by incineration (28.5 %). Two respondents did not state their method of disposal.

The responses addressing the typical storage time of the medicines before sale supports the idea that most medicines sell-out before the expiry date. The respondents were asked to

select one of the following timeframes indicating the typical on-site storage time before sale: < 3 months, 3 – 6 months, 6 – 9 months, 9 – 12 months, 12 - 8 months, and > 18 months. Out of the 25 respondents, 22 (88 %) reported storing medicines on-site for less than three months before sale. The POD description with the longest storage time, > 18 months, was an independent dispensary in Kampala; however, 87.5 % (n = 7) of the independent dispensaries visited reported a storage time of fewer than three months. These data suggest the long storage time of the independent dispensary is an anomaly.

When queried whether the temperature at which the medicines were stored was controlled during the months before sale, the respondents were asked whether the temperature was controlled (yes/no) and given a free-text response to indicate the temperature (°C). The free-text responses fell largely within two temperature ranges, < 25 °C and between 26 – 40 °C. Of the 25 respondents, 15 (60.0 %) indicated a form of temperature control. However, of those that indicated yes, 60 % (n = 9) reported a temperature range between 26 – 40 °C. This response supports the earlier observations in the measurement of temperatures. However, it is worrisome as the storage conditions for most of the antimalarials is below 30 °C.

Finally, as a measure of validity in the responses to the survey and to ensure that the respondents were comfortable with the survey, the final question queried the ease of understanding of the survey. Respondents were given the option to select, “very easy”, “easy”, “difficult”, or “I did not understand” to explain their level of understanding. The results were overwhelmingly positive, with 35 % (n = 9) respondents stating the survey was “very easy” and 65 % (n = 17) stating the survey was “easy”.

5.4.2. Assessment of Packaging and Blister Pack.

Packaging analysis was multifaceted, achieved through collecting physical measurements and validating information on the cardboard packaging and the blister pack.

The measurements of the packaging followed the example provided in Newton *et al.* (2011a), which compared suspected falsified medicines against known reference packaging of the same brand and type. As this field survey could not acquire known reference packaging from all the collected manufacturers, deviations from the average measurements of the packaging were used to identify falsified antimalarials. A total of eight measurements for each cardboard package were collected for all 182 collected samples (Figure 5.10 a)). Table 5.3 presents the average recorded measurements of the cardboard packaging for the 12 types of collected medicines. All measurements presented a standard deviation (SD) of less than 1.2 mm, except for Combiart (20/120). Two height measurements for Combiart (20/120) demonstrated high standard deviation. On investigation, the cause for this deviation was that one sample was marketed for children, which required fewer tablets and therefore, was a smaller 12-tablet packet. However, the 12-tablet product is a genuine Combiart product. Based on these observations it was concluded that there were no falsified medicines procured in the Ugandan field survey based on the measurements of the cardboard packaging.

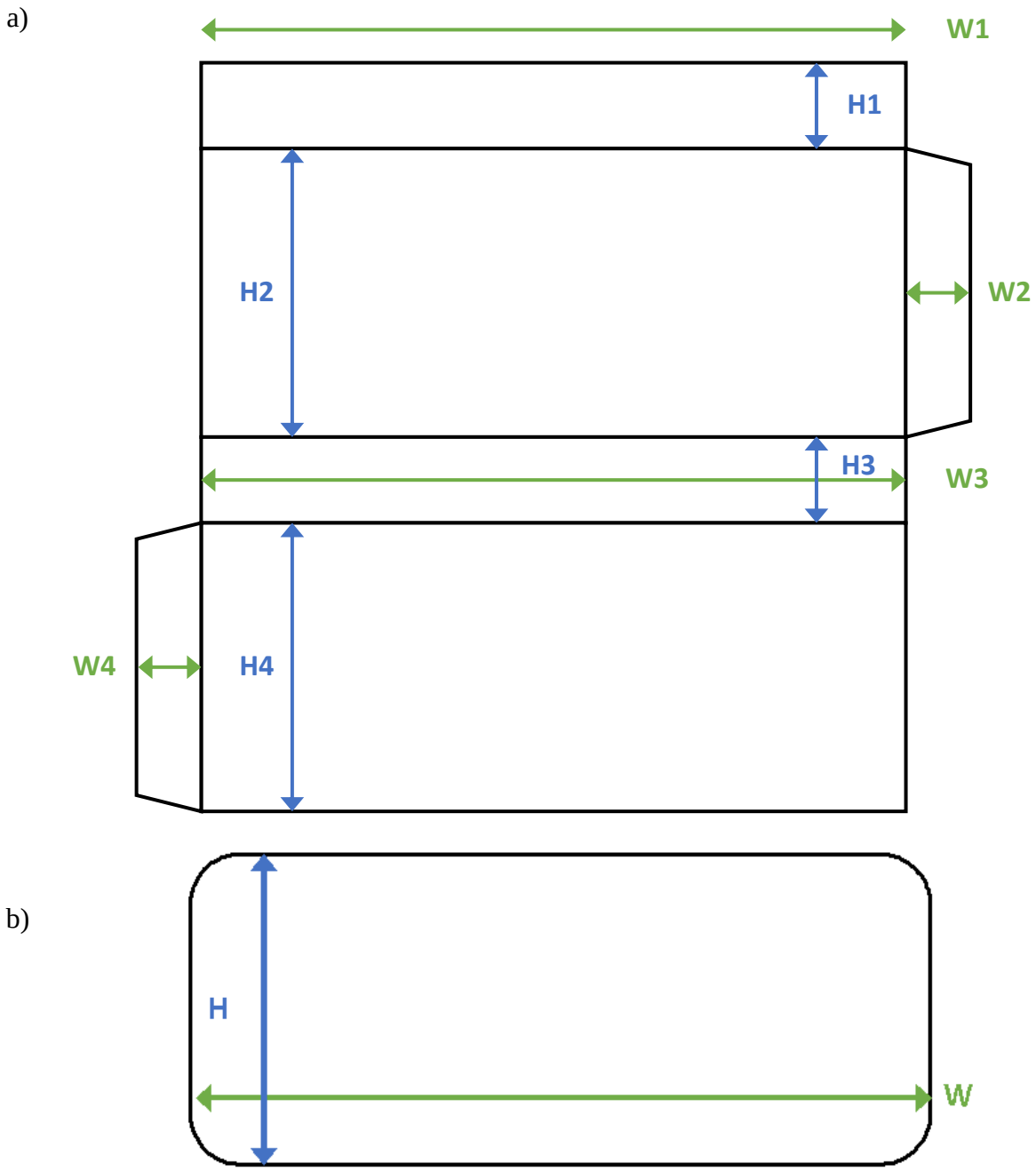


Figure 5.10. Details of the measurements for a) the cardboard packaging and b) the blister packs. All dimensions were measured in mm.

Table 5.3. The observed measurements for the cardboard packaging for all samples collected in Ugandan field survey.

Brand (Strength)	n	Average measurements in mm (SD)							
		W1	**W2**	**W3**	**W4**	**H1**	**H2**	**H3**	**H4**
Artefan (20/120)	30	107.0 (0.8)	16.4 (0.6)	16.2 (0.6)	107.1 (0.7)	16.9 (1.1)	89.1 (0.8)	17.3 (0.5)	89.1 (0.7)
Artefan (80/480)	2	111.8 (0.4)	11.5 (0.0)	11.3 (0.4)	112.0 (0.0)	11.3 (0.4)	62.8 (0.4)	11.5 (0.0)	63 (0.7)
Cach-ART (20/120)	1	105.5	20.0	20.0	106.0	21.5	44.0	21.0	44.5
Coartem (20/120)	8	106.6 (0.8)	28.6 (0.4)	28.5 (0.3)	106.6 (0.7)	29.4 (0.5)	44.7 (0.4)	29.3 (0.5)	44.7 (0.5)
Combiart (20/120)	22	106.9 (0.4)	14.3 (0.6)	14.3 (0.5)	106.9 (0.3)	15.0 (0.8)	**72.9 (5.6)**	15.1 (0.6)	**73.0 (5.6)**
Komefan (40/240)	1	98.0	27.5	28.0	96.5	19.0	63.5	20.0	63.0
Laritem (80/480)	1	72.0	14.5	15.0	71.0	16.5	59.5	17.0	59.0
Lonart (20/120)	26	106.8 (0.9)	18.8 (0.6)	18.7 (0.5)	106.7 (0.6)	19.2 (0.6)	45.0 (0.6)	19.3 (0.5)	44.7 (0.6)
Lonart Forte (40/240)	4	108.4 (0.9)	18.3 (0.5)	18.0 (0.4)	108.3 (1.2)	18.9 (0.5)	64.3 (0.5)	18.9 (0.6)	64.3 (0.3)
Lonart-DS (80/480)	4	92.8 (0.5)	13.4 (0.3)	13.3 (0.3)	92.5 (0.7)	14.8 (0.3)	84.4 (0.9)	14.6 (0.6)	84.1 (0.9)
Lumartem (20/120)	82	106.9 (0.5)	16.9 (0.5)	16.8 (0.5)	106.8 (0.6)	17.1 (0.5)	76.8 (0.4)	17.2 (0.6)	76.6 (0.5)
Lumiter (20/120)	1	108.0	13.5	13.5	107.5	15.0	72.5	14.5	72.0

Similar measurements to the cardboard packing were recorded of the blister packs (Figure 5.10 b)). Table 5.4 presents the average measurements for the blister packs for the 12 types of collected medicines. There was very little deviation found within the measurements indicating genuine packaging across the collected medicines. Again, Combiart (20/120) presented a high standard deviation for one measurement due to the reduced size of the 12-tablet product. However, Lonart (20/120) also presented measurements with large standard deviations. The reasons for this outcome is discussed below.

Table 5.4. The observed measurements for the blister packs for all samples collected in Ugandan field survey.

Brand (Strength)	POD (n)	Blisters (n)	Average Width in mm (SD)		Average Height in mm (SD)	
			Total	Within-POD range	Total	Within-POD range
Artefan (20/120)	30	1	101.2 (0.5)	100.5 - 103.0	84.7 (0.8)	81.5 - 85.5
Artefan (80/480)	2	1	108 (0.0)	108.0 - 108.0	59.5 (0.7)	59.0 - 60.0
Cach-ART (20/120)	1	3	98.0 (0.0)		38.3 (0.3)	
Coartem (20/120)	8	4	98.1 (0.2)	98.0 - 98.4 (0.0 - 0.3)	39.0 (0.1)	39.0 - 39.1 (0.0 - 0.3)
Combiart (20/120)	22	1	100 (0.2)	99.5 - 100.5	**68.4 (5.4)**	44.5 - 70.5
Komefan (40/240)	1	1	90.0		59.0	
Laritem (80/480)	1	1	64.0		55.5	
Lonart (20/120)	26	3	97.4 (5.6)	**85.5 - 98.2 (0.0 - 25)**	38.9 (0.3)	38.0 - 39.2 (0.0 - 0.6)
Lonart Forte (40/240)	4	2	96.0 (0.7)	95.5 - 96.3 (0.0 - 1.4)	60.1 (0.4)	59.8 - 60.3 (0.4 - 0.7)
Lonart-DS (80/480)	4	1	84.9 (0.3)	84.5 - 85.0	79.5 (0.6)	79.0 - 80.0
Lumartem (20/120)	82	1	100.1 (0.3)	100.0 - 101.0	70.0 (0.4)	69.0 - 70.5
Lumiter (20/120)	1	1	99.0		68.0	

The following questions were used to assess the validity of the information on the cardboard packaging and blister packs:

- Does the cardboard show sign of poor handling?
- Is the trade/brand name spelt correctly?
- Is the active ingredient name spelt correctly?
- Is the trade name related to the active ingredient?
- Is the manufacturer name and/or logo legible and correct?
- Does the logo or hologram (if applicable) look authentic?
- Does the hologram change colour at different angles?
- Is the manufacturer's full address listed and legible?
- Is the dosage strength clearly stated on the label?
- Does the number of units listed on the label match the contents?
- Does the label clearly state the batch number and expiry date?
- What is the expiry date on the label?
- What is the batch number on the label?
- What are the listed recommended storage conditions?
- What is the primary package colour?
- What is the secondary package colour? (World Health Professions Alliance 2007)

The importance of these questions lies in the ability to cross-reference pieces of information to identify whether a sample has been tampered with. For example, expired medicines may be placed in the used cardboard packaging of a medicine that is within its expiry date. Therefore, the expiry date and batch number will differ between the blister pack and the cardboard packaging.

For all but one sample packet, the packaging and blister pack did not present indications of being falsified. Chaccour *et al.* (2012) identified a falsified medicine through spelling mistakes. No sample packets had any spelling mistakes in either the trade name nor the API. All sample packets had a trade name that related to the API and a legible manufacturer name and logo.

However, a falsified antimalarial was identified based on the above questions. A packet of Lonart® (manufactured by Bliss GVS Pharma Ltd.) 20 mg ATM + 120 mg LUM tablets was collected from a POD in Kampala. The packet indicated that three blisters of eight tablets were contained inside. Upon opening, it was witnessed that the packet contained three full blister packs from two batches (batch numbers E1AFJ070 and E1AFJ044) and another that was cut in half with the batch number removed (see Figure 5.11). The altered blister pack size would increase the standard deviation of the collective measurements and therefore, would aid in identifying the falsified packaging. However, while the sample is concluded as falsified in this instance, it is not possible to determine whether the inclusion was intentionally malicious.

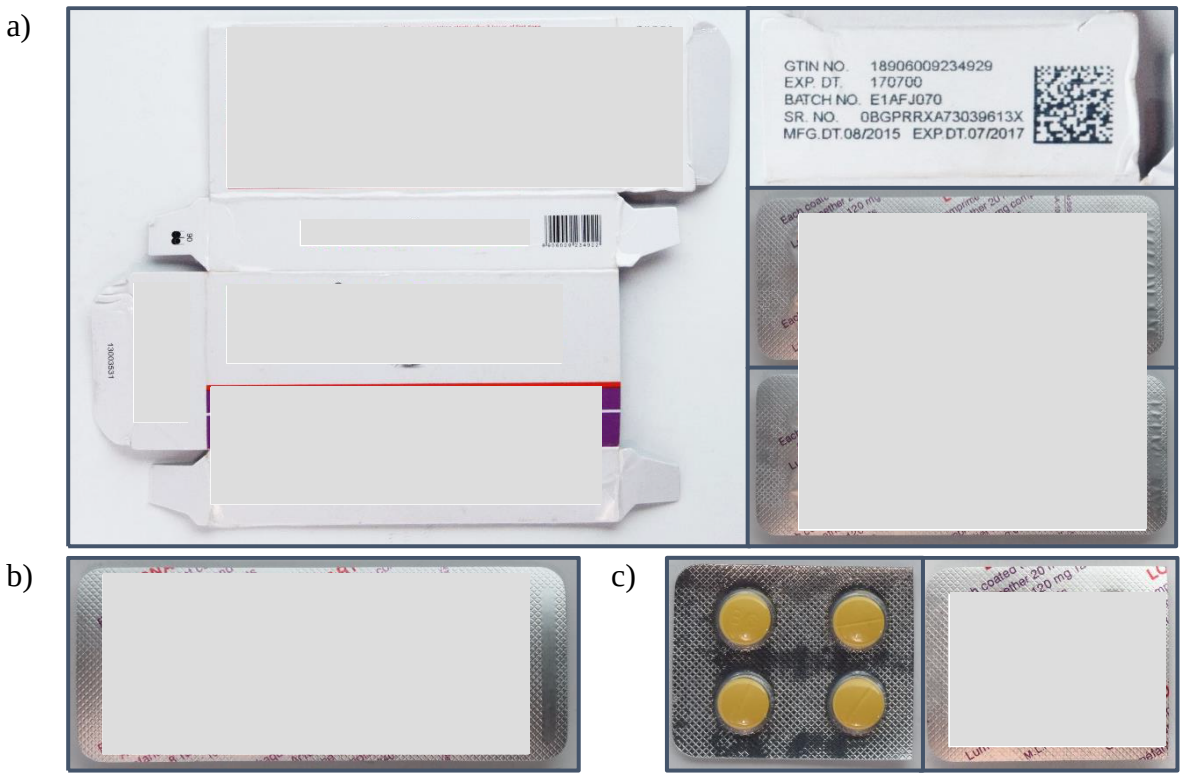


Figure 5.11. Contents of a Lonart packet of which the batch numbers vary between; a) matching batch numbers on two of three blister packs with packaging (E1AFJ070), b) a different batch number on one of three blister packs (E1AFJ044), and c) a cut blister pack (front and back) with the batch number removed.

Other valuable information on the packaging was the recommended storage conditions. The recommended storage conditions were stipulated on the cardboard packaging for all collected samples. The typical storage conditions were: "Do not store above 30°C. Protect from light." In one instance the recommended conditions were below 25°C. The recommended storage conditions were compared to the temperature recorded at the time of sampling. A comparison between the recommended storage conditions and those temperatures recorded as part of the survey indicated that 34.6 % (n = 63) of the medicines were stored in temperatures above the recommended storage conditions.

Other features of the packaging included reflective holograms, which were present on 19 Lonart ® sample packets whose batch number began with "E1AFJ" (see Figure 5.12).

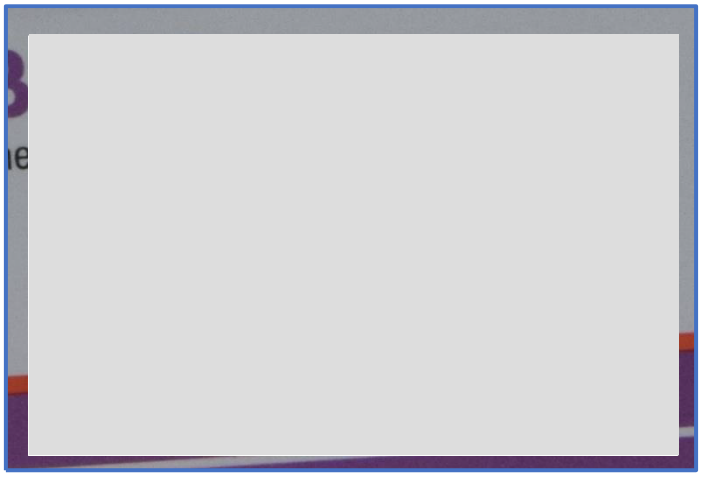


Figure 5.12. Example hologram of Lonart ® packaging.

All the sample packets with holograms were found to present reflective changes in response to moving the light, a response that is indicative of authenticity. Packets of Lonart ® tablets with a batch number beginning with "F1AFJ" did not present with a hologram. Without reference packaging from the manufacturers, it is not possible to determine if these observations indicate authenticity.

5.5. High Performance Liquid Chromatography – Photo Diode Array Analysis of Collected Samples.

Of the 182 sample packs collected, 124 sample packs were assessed for content using high performance liquid chromatography-photo diode array (HPLC-PDA). The 124 samples included: 91 selected as part of the stratified random sampling (SRS) method and 33 collected from PODs not on the registered private pharmacy list (National Drug Authority 2016). In addition, the three blister packs from the sample packet identified as falsified as part of packaging analysis were also analysed for content. The sample allocation between the five sampled administrative districts was: 12 from Mukono, 71 from Kampala, 12 from Luwero, 7 from Hoima, and 23 from Jinja, totalling 125 PODs.

5.5.1. Experimental.

5.5.1.1. *Materials and Chemicals.*

The reference standards for the target APIs, ATM and LUM, were manufactured by Tokyo Chemical Industries. The IS' were ATS and LOS. ATS was donated by Guilin Pharmaceuticals. The LOS reference standard was provided by the WWARN Reference Standards Programme.

The aqueous mobile phase consisted of 0.1 % *w/v* malonic acid and 0.01 % *v/v* TEA in Millipore water (solvent A), adjust to pH 3.0 with ammonium hydroxide. The organic phase consisted of LiChrosolv® ACN (solvent B). All solutions were then filtered through 0.22 µm Nylon discs under vacuum. Full details of all reference material and chemicals can be found in Chapter 2.

The calibration curve for ATM ranged between 100 – 2500 µg/mL (n = 5) in ACN, with ATS at 450 µg/mL as the IS. The calibration curve for LUM ranged between 5 – 100 µg/mL (n = 5) in 0.1 % malonic acid and ACN (60:40 *v/v*), with LOS at 15 µg/mL as the IS.

5.5.1.2. *Sample Preparation.*

Tablets were given a unique code based on the storage condition, tradename, source, blister pack, and position within the blister pack. For example, an Artrin ('AR') tablet collected from Uganda ('UG') would possess the code, ACUG107A07, if the POD had the de-identified code of 107 and it was taken from the first blister pack and occupied the seventh position. The blister packs were orientated and counted in the same manner when allocating the position.

Duplicate tablets from each sample packet were selected using the random number generator website, (Haahr 1998). The physical measurements included in the assessment were: mass (mg), thickness (mm), diameter (mm), and hardness (N). These measurements were included as each measurement is non-destructive and does not impact any proceeding content

analysis. The collected samples were allocated to a version based on brand and strength, e.g. Artefan (20/120).

Each tablet was weighed using an Mettler Toledo New Classic MF analytical scale (MS105DU, 0.01 mg accuracy). Tablet thickness was measured using a Kinchrome 150 mm Vernier calliper (model no. 2310, Supplier, Victoria, Australia), with 0.02 mm accuracy. Tablet hardness (with 1-newton accuracy) and diameter (with 0.01 mm accuracy) were measured using a TBH 220 tablet hardness tester (ERWEKA, Heusenstamm, Germany). Tablet mass was measured once again after hardness testing to assess any mass loss during hardness testing.

Tablets were firstly weighed for total mass and then crushed into a fine powder using a Silent Knight® Pill Crusher inside low-density polyethylene pouches. The powdered tablets portions analysed for ATM were dissolved in filtered ACN. The powdered tablet portions analysed for LUM were dissolved in a mixture of mobile phase solvents A and B at a 60:40 ratio. The concentration of the extract was altered depending on the experiment. All solutions were then sonicated for 30 minutes, including vortexing three times, before filtration through a 0.45 µm nylon filter. The filtered extract was then diluted to the required concentration of 2000 µg/mL for ATM and 90 µg/mL for LUM; the internal standards were then added to the filtered extracts at a concentration of 450 µg/mL for ATS and 15 µg/mL for LOS.

5.5.1.3. *HPLC Conditions.*

All samples were separated on the Agilent 1200 series HPLC system using an Agilent Technologies Zorbax SB-C18 (2.1 x 50 mm, 1.8-Micron) analytical column. The gradient elution program was initiated at 43 % B, increasing to 75 % B at eight minutes over two minutes, holding for four minutes before re-equilibrating to 43 % B over three minutes and holding for four minutes. ATM and ATS were best observed on the 200 nm UV-wavelength. LUM and LOS were best observed on the 250 nm UV-wavelength.

Analytes were detected using RTs and shape of the UV-spectra between 200 – 400 nm. The RT and spectra of ATM and LUM were compared against reference standards. The detection of degradants was based on previously reported data from method validation in Chapter 2. Analyte peaks that possess a S/N greater than 10 were included in quantitative assessment of the storage conditions.

Quantitative analysis was performed in Microsoft Excel (version 1810). Statistical tests were performed in the Excel Data Analysis Tools add-in. The statistical tests performed to compare the data sets were a t-test when comparing the mean of two data sets, and an ANOVA when more than two data sets. Visualisation of data sets was performed using a box-and-whisker plot, which indicates the distribution of the data, the upper and lower quartile, and the median value.

5.5.2. Results and Discussion.

The results for a POD are reported as an average of two replicate analyses, including: physical measurements, percent of API content (ATM and LUM separately), and percent of degradant content.

5.5.2.1. *Physical Assessment of the Tablets*

The average measurements and standard deviation for each sample version are reported in Table 5.5. These values were used to calculate the % CV for each measurement to determine which measurement may indicate if a medicine is of poor quality. If a collected sample was measured far outside the accepted variance of a version it can indicate poor quality. The maximum % CV for mass was 2.4 % CV, for thickness was 7.4 % CV, for diameter was 0.4 % CV, and for hardness was 15.9 % CV. These results suggest that mass and diameter may assist in detecting PQMs, while hardness may not.

Table 5.5. The average measurement of physical characteristics for each sample category.

Version	n	Average measurements (SD)			
		Mass (mg)	Thickness (mm)	Diameter (mm)	Hardness (N)
Artefan (20/120)	22	242.3 (2.2)	3.2 (0.06)	9.11 (0.03)	82 (12)
Lonart (20/120)	17	332.9 (3.1)	4.27 (0.32)	10.26 (0.04)	64 (6)
Lumartem (20/120)	59	355.1 (8.6)	3.7 (0.26)	10.07 (0.03)	73 (11)
Combiart (20/120)	11	404.9 (2.6)	4.3 (0.06)	10.15 (0.03)	65 (9)
Coartem (20/120)	6	242.8 (0.6)	3.16 (0.02)	9.08 (0.02)	99 (6)
Lonart (80/480)	3	684.4 (4.7)	5.98 (0.04)	12.16 (0.04)	156 (25)
Komefan (40/240)	1	605.1	5.40	11.69	104
Lumiter (20/120)	1	251.4	3.54	8.86	44
Lonart (40/240)	3	572.2 (7.4)	5.42 (0.06)	12.32 (0.05)	75 (8)
Cach-Art (20/120)	1	343.1	5.04	9.60	91
Laritem (80/480)	1	989.5	6.00	9.21	287

5.5.2.2. API Content

The complete information of the 125 samples that were assessed for API content is presented in Appendix 5.F. The information includes: the source district, registration status of the POD, the strength of the analysed tablets from which the percent content was measured, the batch number, and expiry date information presented on the packaging. Importantly, the percent content of both ATM and LUM is reported in Appendix 5.F, which is the average of the two tablets randomly selected from the collected packet. The standards by which the percent content was within quality standards was ± 15 %. The quality standards reflect the allowable accuracy and precision limits of the HPLC method (detailed in Chapter 2) and the British Pharmacopeia (British Pharmacopoeia Commission 2016).

The overwhelmingly positive outcome of the field survey was that only one out of the 125 (0.8 %) were identified as poor quality based on content analysis. The recorded content for ATM ranged between 87 – 111 %, with an average of 97 % (SD 5 %). The recorded content

for LUM ranged between 79 – 111 %, with an average of 97 % (SD 6 %). Such results instil a high-degree of confidence in the antimalarial products supplied in Uganda. The results indicated similar content results despite the sample packets being assorted brands, being collected from different climate-related clusters, being expired at the time of analysis, being collected from both registered and non-registered PODs, and the POD participants having varying levels of confidence in the products.

The information of the nine brands of samples, including the number of samples, are presented in Table 5.5. To determine whether there was a difference in percent content for ATM and LUM between these brands, the average content for those brands with more than one sample was compared. An ANOVA indicated a significant difference in the average percent content for ATM (p 1.33 x 10^{-5}). An LSD post-hoc analysis indicated that the brand Lumartem ($\overline{x}$ = 94.1 %) was lower than the four remaining brands ($\overline{x}$ = 99.0 %). However, for LUM, an ANOVA indicated no significant difference between the five brands ($\overline{x}$ = 97.8 %, p 0.086). Importantly, these results are still within the content requirement of high quality, which is ± 15 %.

The average content for ATM and LUM from each cluster were compared to determine whether the climate-related differences identified in Section 5.2.1 resulted in a difference in the API content of the collected samples. An ANOVA comparing the average ATM content concluded there was no significant difference (p 0.472) between the groups despite the average ATM content ranging between 93 and 97 %. There was also no significant difference (p 0.219) in the average content of LUM between the five clusters.

Of the 125 PODs tested for content, 23 (18 %) had expired at the time of analysis. The typical time between manufacturing and expiry was three years. To determine whether samples that pass expiration impacts content analysis, the percent content for ATM and LUM were compared between expired and non-expired samples. A *t*-test indicated for ATM there was no

significant difference (*p* 0.168) between expired ($\overline{x}$ = 98 %) and non-expired ($\overline{x}$ = 97 %) samples. However, for LUM, a *t*-test indicated a very slight difference (*p* 0.048) between expired ($\overline{x}$ = 97 %) and non-expired ($\overline{x}$ = 100 %) samples. These two results indicate that samples surpassing the expiry date does not negatively impact the content analysis in the context of this field survey.

Of the 125 PODs tested for content, 33 (26.4 %) were collected from PODs not included in the registered private pharmacies list in 2016 (see Section 5.2.2). It is important to indicate that PODs not listed as registered private pharmacies, on which the SRS method is based, are not by default illegal or improper, e.g. public, not-for-profit pharmacies. The samples from each set were compared to determine whether presence/absence from the list correlates with percent content for ATM and LUM. A *t*-test indicated there was no significant difference between the two types for ATM (96 %, *p* 0.507) nor LUM (97 %, *p* 0.954).

Of the 26 participants who responded to Questionnaire 2 regarding their level of confidence, eight PODs were subject to content analysis. The PODs were distributed as five that were confident and three that were highly confident. The average content for ATM and LUM were compared using a *t*-test. The t-tests indicated that there was no significant difference between either level of confidence for ATM ($\overline{x}$ 98 %, *p* 0.921) or LUM ($\overline{x}$ 97 %, *p* 0.804).

Unlike the stability data reported in Chapters 3 and 4, the conditions in which the antimalarials were stored before content was assessed is unknown. The temperature and % RH information are only representative of the POD at the time of sampling. However, comparing the recorded content of the Ugandan samples against the baseline data from Chapter 3 can provide further insight into storage history of the Ugandan samples.

The average percent content for the Ugandan samples (n = 125) was compared with the baseline samples (n = 42) using a *t*-test. For ATM, the t-test indicated there was no significant difference (*p* 0.382) between the Ugandan samples ($\overline{x}$ 97 %) and the baseline ($\overline{x}$ 96 %). For

LUM, the *t*-test indicated there was a significant difference (p 7.07 x 10^{-5}) between the Ugandan samples ($\bar{x}$ 97 %) and the baseline ($\bar{x}$ 104 %). While there was an observed difference between the Ugandan samples and the baseline, both data sets are within the acceptable limits of quality (± 15%) and is therefore a positive result.

However, there was single packet found to contain a percent content of LUM below the allowable specification (i.e. < 85.0 %). Both duplicate samples were initially analysed and identified as below specification and therefore, two other tablets were analysed for LUM content to confirm the finding was not an analytical error. The average percent content for the new tablets was 78.6 %. Therefore, the detection of degradation products in the samples is required to determine whether the packet is substandard or degraded.

5.5.2.3. *Degradant Products*

The collected samples were assessed for degradation by observing the three degradant products identified in Section 2.5.1. Degradation products were identified if the HPLC peak possessed a minimum *S/N* ratio of 3. Degradant product peaks with an *S/N* ratio of above 10 were used to assess the degradation profile quantitatively. The noise calculation involved measuring the baseline height deviations of a corresponding blank standard and was performed using the EZChrom Elite HPLC software.

Unlike the identified API, ATM and LUM, there was no certified reference material of the degradation products to generate a calibration curve from which the concentration of the degradation product could be derived. So, to create a metric that measured the degradant product generation, the peak area of the degradant product was compared with the API. Quantitative assessment involved calculating the DPR; the portion of degradant produced relative to the loss of the associated API.

The DPR and the percent content of the API were compared to assess the correlation between the loss of API and the generation of a degradant product. A negative correlation

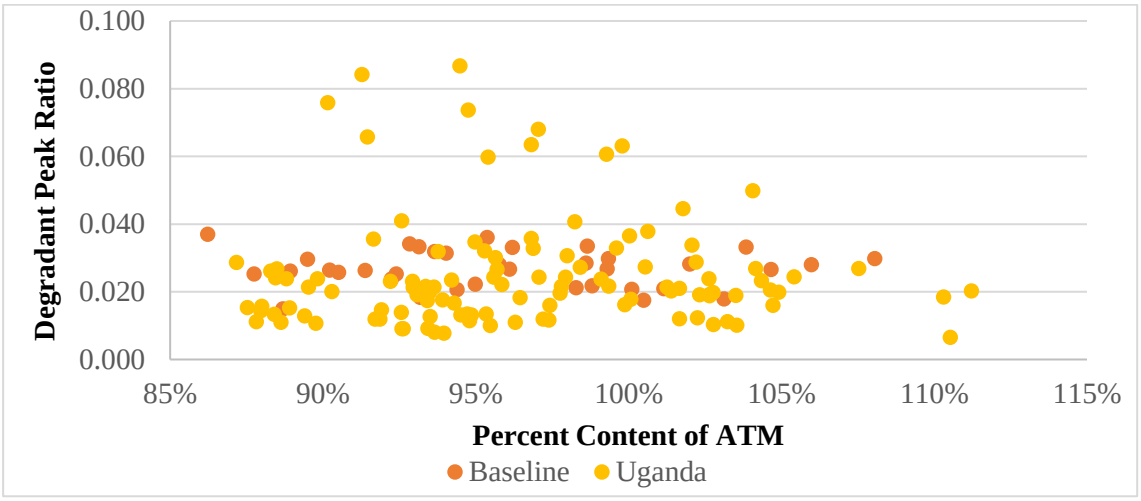
between the two parameters was expected if the relationship is indeed loss of API leads to the production of the degradant product.

However, it is highly implausible to expect an r^2 value of 1.0 correlating the production of the degradation products and the loss of the API. This expectation would assume that the degradation products interact with the detector identically to the API and that no other external factors influenced the measurement, e.g. instrument precision and accuracy.

Figure 5.13 presents the degradation profiles of the three degradation peaks observed in the Ugandan samples compared with those of the baseline. The r^2 value for the three degradant peaks indicates the proportion of degradant product that is dependent on the loss of API. The r^2 values are, 1.0×10^{-5} for Peak 1, 0.030 for Peak 2, and 0.027 for Peak 3, indicating a very low relationship. For all the peaks, the slope was less than 0.1, when a larger, negative slope was expected for the proposed relationship. However, one insightful observation was presented in Peak 2, which was not able to be quantified in the baseline (refer to Section 3.4.3.2) but was quantified in over 50 samples collected from Uganda.

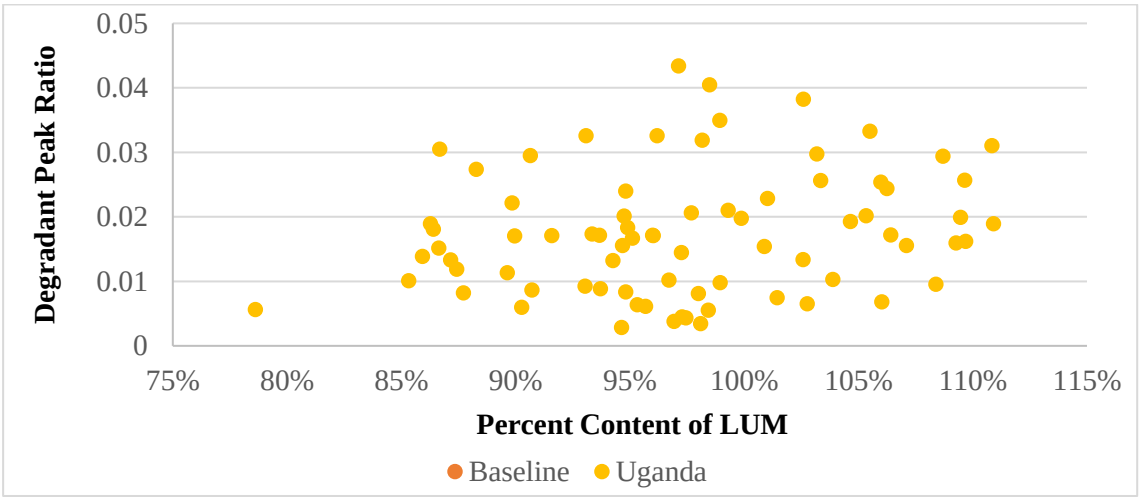
For the sample identified as below specification for the percent content of LUM, the initial duplicate samples did indicate the presence of the LUM-related degradant presented as Peak 2. The results of other packets from the same manufacturing batch collected from other locations (identified by identical batch numbers) found that while Degradant Peak 2 was detected, the percent content of LUM was within quality specifications. Therefore, it is concluded that the sample found to be below specification is a degraded sample.

a) Degradant Peak 1 $\bar{x}$ RT = 0.95 minutes



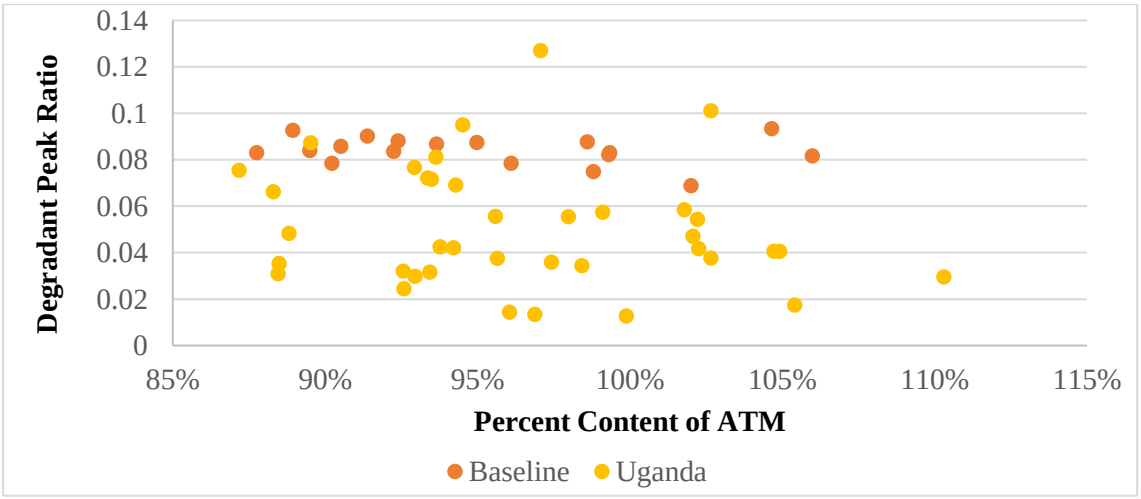
Baseline: $R^2 = 3.9x10^{-3}$. Slope = $-6.3x10^{-3}$. Uganda: $r^2 = 2.0x10^{-4}$. Slope = $-4.6x10^{-3}$.

b) Degradant Peak 2 $\bar{x}$ RT = 2.35 minutes



Baseline: No quantative information. Uganda: $r^2 = 0.044$. Slope = 0.028.

c) Degradant Peak 3 $\bar{x}$ RT = 3.72 minutes



Baseline: $R^2 = 0.062$. Slope = -0.028. Uganda: $r^2 = 0.037$. Slope = 0.093.

Figure 5.13. A comparison of the degradant profile of Ugandan samples with Baseline samples.

5.6. Disintegration Analysis of the Collected Samples.

Disintegration testing was performed as an indication of bioavailability (Schirmer *et al.* 1973). With all the Ugandan samples, except for one, analysed and reported as being within quality specifications, the collection was considered as one group for disintegration testing.

5.6.1. Experimental.

Disintegration was assessed using an ERWEKA® Disintegration Tester (model no. ZT224, Heusenstamm, Germany). The tablets were placed in Millipore water, held at 37 °C (± 2 °C) for 15 minutes. Individual tablets were placed in a single tube of the tester's basket and topped with a plastic disc. The bottom of the tester's basket was a 2 mm wire mesh.

Analysis conditions were based around the British Pharmacopeia specifications (British Pharmacopoeia Commission 2016). For all laboratory-based stability testing, 18 tablets were tested for disintegration; the 18 tablets were equally distributed between brands and blister packs. The requirements of the test were met if more than 16 of the 18 tablets tested had disintegrated.

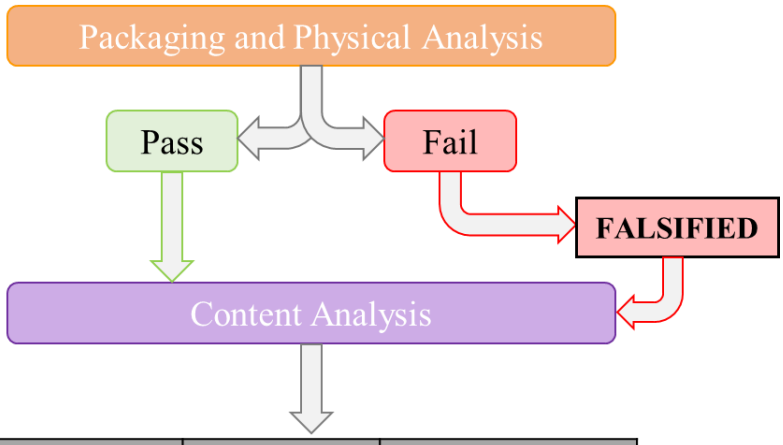
For the samples collected during the Ugandan survey, duplicate tablets were selected from ten POD sample packs that were chosen at random. In addition, duplicate tablets were selected from the PODs found to be falsified and degraded, totalling 12 PODs in total from Uganda.

5.6.2. Results and Discussion.

The Ugandan field survey samples selected for disintegration testing represented all five districts: six from Kampala, three from Luwero, and one from Hoima, Jinja, and Mukono. The brands represented were; eight of Lumartem, two of Artefan, and one of Lonart and Combiart. All 24 tablets, including those identified as poor-quality, passed the disintegration test within the 15-minutes timeframe.

5.7. Conclusions.

The COPA protocol was successfully implemented in the assessment of AM quality for medicines collected as part of a field survey in five administrative districts of Uganda, Africa. The AMs were assessed for authentic packaging, percent content of the API, and the presence of degradation products. The assessment was able to identify a single falsified and a single degraded AM (Figure 5.14). The falsified medicine contained blister packs from two manufacturing batches and a partial blister pack that did not indicate the manufacturing batch nor an expiry date. The degraded medicine was identified by the low percent content of LUM and the presence of the LUM-related degradation product, DBK. Furthermore, other packets from the same manufacturing batch (identified by identical batch numbers) collected from other locations were within quality specifications, which excluded the sample from being substandard. However, the quality of the samples from Uganda was high, as all other medicines were assessed as within quality specifications and not significantly different from medicines procured directly from the manufacturers and stored within an air-conditioned setting.



Content (± 15 %)	Degradation markers	Result
PASS	PRESENT	**Within Standard**
PASS	ABSENT	**Within Standard**
FAIL	PRESENT	**DEGRADED**
FAIL	ABSENT	**SUBSTANDARD**

Figure 5.14. The criteria and decision tree to accurately characterise poor-quality medicines.

The survey of pharmacy practices in Uganda provided great insight into the conditions in which the medicines were stored. In brief, while all but one of PODs did not contain an air-conditioning system the medicines were only stored for a short period (typically < 3 months). This suggests that the supply chain is adequately protected from adverse temperature and humidity excursions and that the medicines do not remain in uncontrolled conditions long enough to degrade the medicines to an unacceptable level.

The success of the Ugandan survey, while only identifying two poor-quality antimalarials, serves as a strong proof of concept that the COPA protocol can be applied to field surveys of antimalarial medicine quality.

Appendix 5.A. Sample Collection Process.

The Sampling Collaborator was instructed to follow a standard operating procedure (SOP) when visiting the PODs (also see flow chart, Figure 5.15). The purpose of the SOP was to ensure the Sampling Collaborator acted within the ethical considerations of the project, relating to informed consent, and to reduce any participant bias. The procedure is outlined in Figure 5.15 as a decision chart.

Standard Operating Procedure for Sample Collection

1. Upon entry, identify to the POD attendee as a researcher investigating antimalarial medicine.
    a. If there is more than one person in the shop, introduce yourself to the one who serves customers first and then ask for the owner or person responsible for selling medicines.
    b. *Explain* that you are involved in the project, which is about assessing the quality of medicine in private registered pharmacies and non-registered sellers in the region.
2. Provide a brief summary of what the project is all about and why the private provider is asked to participate in the study.
    a. *Explain* that you are collecting information and samples from registered-private pharmacies and non-registered sellers in that area.
    b. **Ask:** Do you stock antimalarial medicines containing the active pharmaceutical ingredients (API) Artemether and Lumefantrine (e.g. Coartem)? If yes, proceed with following steps.
3. <u>If the shop is eligible for inclusion</u>, hand the person the *"Participant Information Sheet"* and the *"Participant Consent (Seller of Medication)"* documents allow them to read carefully and complete as required.
    a. Be sure to write the POD ID code on <u>both</u> documents and the POD pack label.
    b. If no consent to participate is given, leave the POD and instead visit the next reserve POD, in order, as listed on the sampling list.

4. If informed consent is provided, complete the *"Questions for Collaborator/Purchaser"* form.
    a. Use the COPA survey tool to record temp (°C) and humidity (% RH).
    b. Make sure to correspond the code on the sampling list to the code that you write on the POD pack.
5. If the participant has elected to participate in the survey, hand them the *"Question for the POD Participant"* questionnaire for completion at that time.
    a. Write the POD ID code on both questionnaire forms (Purchaser and Seller).
6. Proceed with the purchase of one full treatment course of antimalarial medicine. It is important that the medicines must contain the APIs, Artemether and Lumefantrine. Brand or strength is not important.
7. Return all items regarding the sampled POD into the coded resealable bag. Items include; *"Participant Consent (Seller of Medication)", "Question for the POD Participant",* and *"Questions for Collaborator/Purchaser"* documents, and the purchased antimalarial medicines.
8. Also ask about non-registered medicine providers in the vicinity to identify geographically comparable non-registered PODs.
9. At the end of every visit record the global positioning system (GPS) location of POD on the *"Questions for Collaborator/Purchaser"* form and on the POD pack label. One longitudinal and latitudinal reading is to be taken. Ensure that the POD code is on the required pages.
10. Move onto the next provider on the sampling list and repeat steps 1 – 9 for all registered private pharmacies and elected non-registered sellers.
    a. When attending non-registered sellers within 1 km of registered private pharmacies, use the identical POD ID code as the registered private pharmacy when completing the survey or using the survey tool. Identify the POD as non-registered seller through marking an asterisk (*) next to the code on all pages.

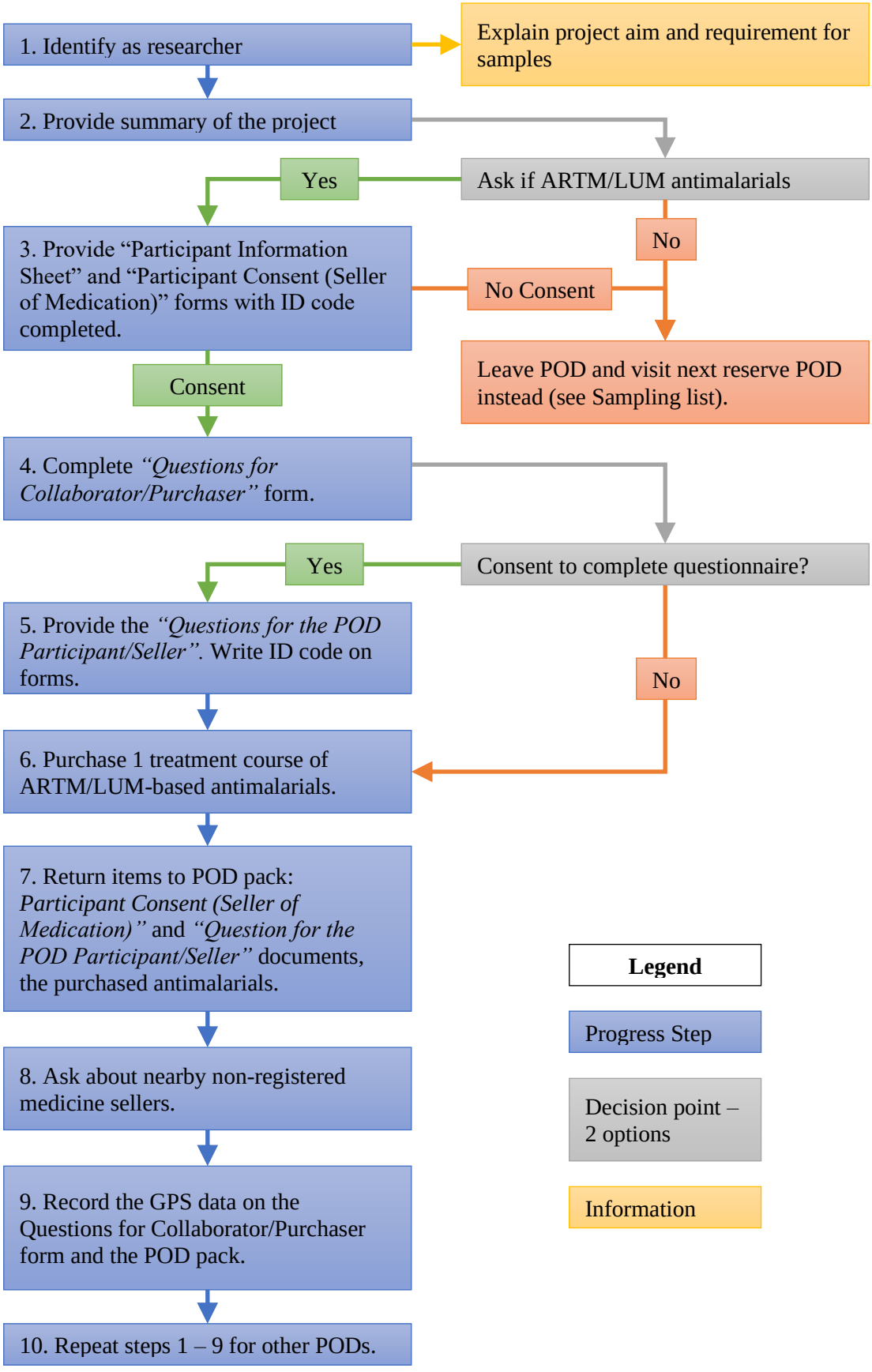


Figure 5.15. Sampling SOP decision chart.

UNIVERSITY OF CANBERRA

ID Code (For Collaborator Only)

Appendix 5.B. The COPA research participation consent forms.

Participant Consent (Seller of Medication)

Project Title

Characterisation of Antimalarial Medicine Quality (COPA)

Consent Statement

- I have read and understood the 'Participant Information Sheet' for this project.
- I acknowledge that the nature, purpose, and considered effects of the project so far as it affects me, and I understand that my consent is given voluntarily.
- I agree to participate in this project by undertaking the following procedure(s):
    - The sale of antimalarial medication ☐ Yes ☐ No
    - Completion of accompanying survey ☐ Yes ☐ No
- I agree that research data gathered from me for the study may be published provided that I cannot be publicly identified as a participant.
- I understand that all research data will be securely stored on the University of Canberra premises for at least five years, and will then be destroyed.
- I have been given the opportunity to ask questions about my participation and any questions I have asked have been answered to my satisfaction.
- I agree to participate in this project and understand that I may withdraw at any time without any consequences.
- I understand that the project has received ethical approval from the University of Canberra Human Research Ethics Committee. I have been provided with adequate contact details if I wish to express any concerns of an ethical nature or complaints about the manner in which the project is conducted.
- I am over the age of 18 years.

Name: _____

Signature: _____

Date: _____

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ID Code (For Collaborator Only)

Participant Consent (Purchaser of Medication)

Project Title

Characterisation of Antimalarial Medicine Quality

Consent Statement

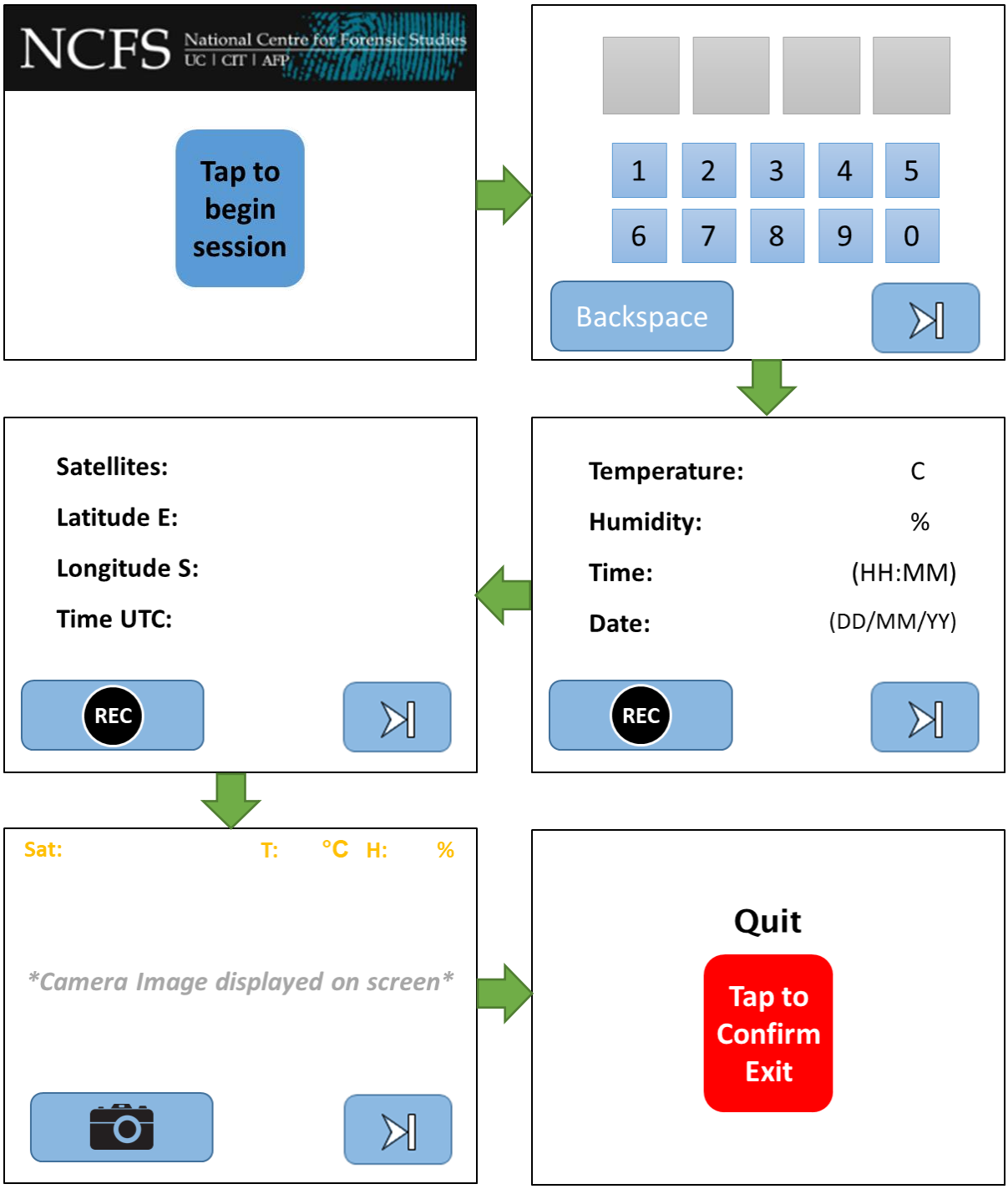
- I have read and understood the information about this project.
- I acknowledge that the nature, purpose, and considered effects of the project so far as it affects me, and I understand that my consent is given voluntarily.
- I agree to participate in this project by undertaking the following tasks:
    - The purchase of antimalarial medication ☐ Yes ☐ No
    - Distribution of accompanying survey ☐ Yes ☐ No
- I agree to act in a manner in accordance with the ethical regulations of the University of Canberra throughout all aspects of the research project.
- I understand that all research data will be securely stored on the University of Canberra premises for at least five years, and will then be destroyed.
- I have been given the opportunity to ask questions about my participation and any questions I have asked have been answered to my satisfaction.
- I agree to participate in this project and understand that I may withdraw at any time without any consequences.
- I understand that the project has received ethical approval from the University of Canberra Human Research Ethics Committee. I have been provided with adequate contact details if I wish to express any concerns of an ethical nature or complaints about the manner in which the project is conducted.
- I am over the age of 18 years.

Name: _____

Signature: _____

Date: _____

Appendix 5.C. Design of Survey Tool User-Interface During Collection.



Appendix 5.D. Detailed lists of the costs associated with the project.

Table 5.6 details the cost of each component of the COPA survey tool. The device was constructed for less than $ 250 USD.

Table 5.6. List of the custom survey tool components with prices.

Item	Cost ($USD)
PITFT Plus Assembled 320x240 2.8" TFT + Resistive Touchscreen	34.95
Lithium-Ion Battery Pack - 3.7V 6600mah	29.50
Adafruit Ultimate GPS Breakout - 66 Channel W/10 Hz Update	39.95
Powerboost Charger - Rechargeable 5V Lipo USB Boost @ 1A - 1000C	19.95
AM2302 (Wired DHT22) Temperature-Humidity Sensor	15.00
Raspberry Pi Zero Budget Pack - Includes Pi Zero	29.50
8MP Raspberry Pi Camera Board	30.13
2nd Gen - Stackable USB Hub for Raspberry Pi Zero	7.47
2 x SanDisk 16 GB Micro SD Card	24.13
In-car USB Charging Unit	10.00
Total	**240.58**

Table 5.7 details the costs associated with performing the Ugandan the field survey. The costs are included to increase transparency of health surveys (Visser *et al.* 2014). In doing so, increased transparency demonstrates that the benefits and capabilities that a field survey can provide.

Table 5.7. The summary of research costs of the Ugandan field survey.

Item	Quantity	Cost in $ USD	% of Total Cost
Antimalarial Medicines	182	1,402.28	24.6
Travel Expenses, including; - *Fuel* - *Accommodation* - *Other*	NA	1,444.71	25.3
Custom Survey Tool	1	240.80	4.2
Shipping	NA	495.00	8.7
HPLC consumables (approx. $8.50 USD each tablet)	250	2,125.00	37.2
Total Costs		5707.79	100.0

Appendix 5.E. Questionnaire for the local research collaborator and the attendee of the point of distribution.

Question for the POD Participant

1. How often do you procure antimalarial medication?

☐ < 3 months ☐ 3 – 6 months ☐ 6 – 9 months

☐ 9 – 12 months ☐ 12 – 18 months ☐ > 18 months

2. How many treatment courses do you typically procure each time? Please list the number.

_____

_____

3. How confident are you that the antimalarial medicine you procure are of high-quality?

☐ Highly Confident ☐ Confident ☐ Somewhat Confident ☐ Not Confident

4. Do you dispose of expired antimalarial medicine stock? ☐ Yes ☐ No

If yes: a) Please estimate the number of treatment courses disposed of per year. _____

b) What is the method of disposal? _____

5. What is the typical on-site storage time of the medicine prior to sale?

☐ < 3 months ☐ 3 – 6 months ☐ 6 – 9 months

☐ 9 – 12 months ☐ 12 – 18 months ☐ > 18 months

6. Is the temperature within the location regulated or controlled? ☐ Yes ☐ No

If yes: a) What temperature? _____°C_

7. What is the highest qualification of the person in charge during dispense?

☐ Secondary School ☐ Bachelor Degree ☐ Master's Degree ☐ Doctoral Degree

Discipline: _____

8. How this survey easy to understand?

☐ Very Easy ☐ Easy ☐ Difficult ☐ I did not understand

Questions for Collaborator/Purchaser

Approximate time: _____ Date: _____/_____/_____

Name of POD: _____,

Name of Pharmacist: _____

Address (including district and province): _____

_____

GPS Coordinates: Latitude: _____ Longitude: _____

Is the participant willing to complete the questionnaire? ☐ Yes ☐ No

Has the participant read and understood the survey information and provided informed consent? ☐ Yes ☐ No

Does the participant wish to be informed of any failed results from their POD? ☐ Yes ☐ No

If yes; Email/Ph:

_____

1. What was the antimalarial purchased?

Brand Name	**Strength**	**Cost**

2. Please choose the closest description of the POD.

☐ Market Stall ☐ Non-specialised general store

☐ Independent dispensary ☐ Commercial dispensary (Chain)

☐ Small scale retailer ☐ Large scale retailer (pharmacy)

☐ Wholesaler

3. What is the current temperature and humidity at the POD? _____°C _____% RH
4. Does the POD appear to have temperature control? ☐ Yes ☐ No

   If yes; a) How?

_____

5. Is a pharmacist present on site? ☐ Yes ☐ No
6. Is there is information available regarding falsified medicines? ☐ Yes ☐ No
7. Did the participant ask for a prescription? ☐ Yes ☐ No

Appendix 5.F. Details of the 125 PODs selected for content analysis.

Table 5.8 lists the source and analysis information for all the Ugandan samples that were analysed for content. Expiry dates denoted with '*' were expired at the time of analysis. Bold samples were identified as poor-quality, either falsified or were outside the ± 15 % quality standards.

Table 5.8. Batch-specific information of the Ugandan samples that were analysed for content, including the regulation status of the POD.

District	Status	Brand	Strength	Batch No.	Exp. Date	Content (%)	
						ATM	LUM
Hoima	Reg	Combiart	20/120	7227575	Jun-18	90	96
Hoima	Reg	Lonart	20/120	F1AFJ018	Mar-18	97	90
Hoima	Reg	Lumartem	20/120	ID63262	Aug-19	99	92
Hoima	Unreg	Artefan	20/120	P07915I	08/2017*	96	106
Hoima	Unreg	Lumartem	20/120	Q60042	Dec-17	94	93
Hoima	Unreg	Lumartem	20/120	Q60042	Dec-17	90	95
Hoima	Unreg	Lumartem	20/120	ID62356	May-19	88	98
Jinja	Reg	Artefan	20/120	P06515D	Mar-17	99	106
Jinja	Reg	Artefan	20/120	P08816B	Jan-18	103	102
Jinja	Reg	Artefan	20/120	P18316B	Jun-18	106	100
Jinja	Reg	Artefan	20/120	P00816B	Jan-18	95	97
Jinja	Reg	Artefan	20/120	P15016A	Dec-17	88	103
Jinja	Reg	Combiart	20/120	7226859	Mar-18	99	95
Jinja	Reg	Combiart	20/120	7225503	Sep-17	94	99
Jinja	Reg	Combiart	20/120	7225503	09/2017*	102	100
Jinja	Reg	Lonart	20/120	E1AFJ059	06/2017*	96	95
Jinja	Reg	Lonart	20/120	E1AFJ090	08/2017*	95	99
Jinja	Reg	Lonart	20/120	E1AFJ049	06/2017*	94	91
Jinja	Reg	Lonart	20/120	E1AFJ092	08/2017*	102	101
Jinja	Reg	Lonart	20/120	F1AFJ020	Apr-18	102	86
Jinja	Reg	Lumartem	20/120	ID62397	May-19	97	95
Jinja	Reg	Lumartem	20/120	Q60042	Dec-17	93	104
Jinja	Reg	Lumartem	20/120	Q60796	Aug-19	91	95
Jinja	Reg	Lumartem	20/120	ID62408	Jun-19	90	107
Jinja	Unreg	Artefan	20/120	P11815E	04/2017*	97	96
Jinja	Unreg	Combiart	20/120	7227191	Apr-18	100	99
Jinja	Unreg	Combiart	20/120	7227191	Apr-18	104	98
Jinja	Unreg	Combiart	20/120	7227451	May-18	97	105
Jinja	Unreg	Lumartem	20/120	ID62338	May-19	92	98

Table 5.8. Batch-specific information of the Ugandan samples that were analysed for content, including the regulation status of the POD.

District	Status	Brand	Strength	Batch No.	Exp. Date	Content (%)	
						ATM	LUM
Jinja	Unreg	Lumartem	20/120	Q60372	May-18	105	93
Kampala	Reg	Artefan	20/120	P08316B	Jan-18	93	111
Kampala	Reg	Artefan	20/120	P07915J	Sep-17	102	95
Kampala	Reg	Artefan	20/120	P16515J	Sep-17	100	96
Kampala	Reg	Artefan	20/120	P00816B	Jan-18	101	97
Kampala	Reg	Artefan	20/120	P11915E	Apr-17	93	98
Kampala	Reg	Artefan	20/120	P00816B	Jan-18	102	90
Kampala	Reg	Artefan	20/120	P00816B	Jan-18	91	97
Kampala	Reg	Artefan	20/120	P15116A	Dec-17	111	96
Kampala	Reg	Artefan	20/120	P02315F	05/2017*	103	105
Kampala	Reg	Artefan	20/120	P08316B	Jan-18	102	93
Kampala	Reg	Artefan	20/120	P00816B	Jan-18	101	90
Kampala	Reg	Cach-Art	20/120	CHRT/6001E	Jun-18	94	108
Kampala	Reg	Coartem	20/120	K0160	Nov-17	104	95
Kampala	Reg	Coartem	20/120	K0041	07/2017*	103	105
Kampala	Reg	Coartem	20/120	K0160	11/2017*	98	111
Kampala	Reg	Coartem	20/120	K0159	Nov-17	104	95
Kampala	Reg	Combiart	20/120	7227184	Apr-18	91	106
Kampala	Reg	Lonart	20/120	E1AFJ070	Jul-17	95	95
Kampala	Reg	Lonart	20/120	F1AFJ020	Apr-18	98	97
Kampala	Reg	Lonart	20/120	E1AFJ045	Jun-17	94	99
Kampala	Reg	Lonart	80/480	F1AFM012	Feb-18	105	90
Kampala	**Reg**	**Lonart**	**20/120**	**Falsified**	**06/2017***	**96**	**98**
Kampala	Reg	Lonart	20/120	F1A4J024	Apr-18	104	98
Kampala	Reg	Lonart	80/480	E1AFM213	Nov-17	93	94
Kampala	Reg	Lonart	40/240	E1AFL038	10/2017*	100	86
Kampala	Reg	Lonart	20/120	E1AFJ096	09/2017*	95	102
Kampala	Reg	Lonart	40/240	F1AFL017	Jun-18	100	94
Kampala	Reg	Lonart	20/120	F1AFJ019	Mar-18	98	103
Kampala	Reg	Lonart	40/240	F1AFL017	Jun-18	111	103
Kampala	Reg	Lonart	20/120	F1AFJ019	Mar-18	105	94
Kampala	Reg	Lumartem	20/120	Q60755	Aug-19	93	90
Kampala	Reg	Lumartem	20/120	Q60347	Apr-18	93	97
Kampala	Reg	Lumartem	20/120	ID62426	May-19	93	108
Kampala	Reg	Lumartem	20/120	Q61008	Oct-18	95	98
Kampala	Reg	Lumartem	20/120	ID62426	May-19	95	94
Kampala	Reg	Lumartem	20/120	Q60405	May-18	98	87
Kampala	Reg	Lumartem	20/120	Q60749	Jul-19	94	105

Table 5.8. Batch-specific information of the Ugandan samples that were analysed for content, including the regulation status of the POD.

District	Status	Brand	Strength	Batch No.	Exp. Date	Content (%)	
						ATM	LUM
Kampala	Reg	Lumartem	20/120	Q60749	Jul-19	94	103
Kampala	Reg	Lumartem	20/120	ID62085	May-19	103	90
Kampala	Reg	Lumartem	20/120	ID62230	May-19	97	90
Kampala	Reg	Lumartem	20/120	ID62397	May-19	102	96
Kampala	Reg	Lumartem	20/120	Q60781	Aug-19	97	96
Kampala	Reg	Lumartem	20/120	ID62355	May-19	89	97
Kampala	Reg	Lumartem	20/120	Q60405	May-18	93	107
Kampala	**Reg**	**Lumartem**	**20/120**	**Q60755**	**Aug-19**	**103**	**79**
Kampala	Reg	Lumartem	20/120	Q60368	May-18	95	88
Kampala	Reg	Lumartem	20/120	ID63255	Aug-19	96	95
Kampala	Reg	Lumartem	20/120	ID62408	May-19	94	97
Kampala	Reg	Lumartem	20/120	Q60807	Jun-19	93	95
Kampala	Reg	Lumartem	20/120	ID62353	May-19	92	99
Kampala	Reg	Lumartem	20/120	Q60755	Aug-19	95	101
Kampala	Reg	Lumartem	20/120	Q60053	Dec-17	90	96
Kampala	Reg	Lumartem	20/120	ID60509	Dec-17	88	100
Kampala	Reg	Lumartem	20/120	ID62353	May-19	89	97
Kampala	Reg	Lumartem	20/120	Q60037	Dec-17	93	91
Kampala	Reg	Lumartem	20/120	Q60125	Jan-18	96	96
Kampala	Reg	Lumartem	20/120	ID62353	May-19	88	90
Kampala	Reg	Lumartem	20/120	Q60347	Apr-18	96	86
Kampala	Unreg	Artefan	20/120	P00816B	Jan-18	101	98
Kampala	Unreg	Combiart	20/120	7226858	Mar-18	97	87
Kampala	Unreg	Komefan	40/240	3043055	06/2017*	100	87
Kampala	Unreg	Lumartem	20/120	Q50621	Oct-17	89	97
Kampala	Unreg	Lumartem	20/120	Q60749	Jul-19	89	103
Kampala	Unreg	Lumartem	20/120	Q60373	May-18	96	98
Kampala	Unreg	Lumartem	20/120	Q60123	Jan-18	104	87
Kampala	Unreg	Lumartem	20/120	Q60121	Jan-18	108	88
Kampala	Unreg	Lumartem	20/120	ID60582	Dec-17	88	109
Kampala	Unreg	Lumartem	20/120	ID62425	May-19	88	95
Kampala	Unreg	Lumartem	20/120	ID60515	Dec-17	101	98
Kampala	Unreg	Lumartem	20/120	ID62401	May-19	88	96
Kampala	Unreg	Lumartem	20/120	Q60053	Dec-17	103	95
Luwero	Reg	Coartem	20/120	K0159	Nov-17	92	91
Luwero	Reg	Laritem	80/480	DGX5004	08/2017*	97	109
Luwero	Reg	Lumartem	20/120	Q60741	Aug-19	95	103
Luwero	Reg	Lumartem	20/120	ID62401	May-19	95	96

Table 5.8. Batch-specific information of the Ugandan samples that were analysed for content, including the regulation status of the POD.

District	Status	Brand	Strength	Batch No.	Exp. Date	Content (%)	
						ATM	LUM
Luwero	Reg	Lumartem	20/120	ID60719	11/2017*	92	95
Luwero	Reg	Lumiter	20/120	16TAI1231A	Jul-19	110	106
Luwero	Unreg	Artefan	20/120	P08816B	Jan-18	98	94
Luwero	Unreg	Combiart	20/120	7227190	Apr-18	95	107
Luwero	Unreg	Combiart	20/120	7227502	May-18	104	105
Luwero	Unreg	Lumartem	20/120	P00816B	Jan-18	95	94
Luwero	Unreg	Lumartem	20/120	Q60328	Apr-18	92	101
Luwero	Unreg	Lumartem	20/120	ID62354	May-19	88	95
Mukono	Reg	Artefan	20/120	P00816B	Jan-18	94	109
Mukono	Reg	Artefan	20/120	P20316F	May-18	100	101
Mukono	Reg	Coartem	20/120	K0160	Nov-17	99	106
Mukono	Reg	Lonart	20/120	E1AFJ060	06/2017*	98	98
Mukono	Reg	Lonart	20/120	E1AFJ092	08/2017*	105	103
Mukono	Reg	Lumartem	20/120	Q60755	Aug-19	103	85
Mukono	Reg	Lumartem	20/120	ID60509	Dec-17	87	110
Mukono	Reg	Lumartem	20/120	ID62408	May-19	90	87
Mukono	Unreg	Lonart	20/120	E1AFJ070	07/2017*	101	110
Mukono	Unreg	Lonart	80/480	F1AFM012	Feb-18	105	103
Mukono	Unreg	Lumartem	20/120	ID62401	May-19	92	97
Mukono	Unreg	Lumartem	20/120	Q60755	Aug-19	93	89

Chapter 6. Identification of Antimalarial Degradants.

6.1. Background.

From the outset, the characterisation of poor-quality antimalarials (COPA) protocol has set out to detect degraded medicines. To achieve this, the identity of the degradation peaks must be known. In Chapter 2, the method development described the difficulties in applying a liquid chromatography-tandem mass spectrometry (LC-MS/MS) due to in-source fragmentation when using an Agilent 6410 triple quadrupole instrument. For this reason, a HPLC-PDA method was developed, validated, and implemented in Chapters 3 through 5. In those chapters, potential degradation products were detected but were not able to be confirmed due to the limitations of the HPLC-PDA technique. However, the future adaptation of the COPA protocol for LC-MS/MS was considered during HPLC development by using LC-MS/MS compatible solvents (e.g. malonic acid) and fine solvent filtration processes.

Experimentation in the previous chapters established the RTs and UV spectra of the known APIs, ATM and LUM, and detected the presence of three potential degradant products.

The aim of the following chapter is to implement the use of high-resolution mass spectrometry (HRMS) to confirm the presence of the API and identify the three potential degradant products. HRMS was not available at the University of Canberra at the time of this research and therefore, adequate access was not possible without a large associated cost.

6.2. Methods and Materials.

6.2.1. Instrumental Analysis.

Separation was achieved using an Agilent Technologies Zorbax SB-C18 Rapid Resolution HT 2.1 x 50 mm (1.8-micron, 82770-902) analytical column. The chromatography conditions were identical to the previously reported HPLC conditions (see Section 2.3.6): The gradient elution program was initiated at 43 %B, increasing to 75 % B at eight minutes over

two minutes, holding for four minutes before re-equilibrating to 43 % B over three minutes and holding for four minutes.

The mobile phase consisted of 0.1 % *v/v* malonic acid with 0.01 % *v/v* TEA in Millipore water (solvent A) and LiChrosolv® ACN (solvent B). The pH of mobile phase solvent A was adjusted to pH 3.0 with 10 % *w/v* ammonium hydroxide. All mobile phase solvents were filtered through 0.22 µm nylon discs under vacuum prior to use.

Mass information was collected using a Q-Exactive Plus mass spectrometer manufactured by ThermoFisher Scientific (Massachusetts, United States). Data processing and analysis was performed on the XCalibur™ software (ThermoFisher Scientific).

6.2.2. Sample Selection.

A total of twelve samples were selected based on previous content analysis results, comprised of three tablets from each of the accelerated, cyclic, Darwin, and Ugandan studies. The blister packs that contained tablets that reported the highest DPR for Degradant Peak 1 were identified and another tablet from within those blister packs was randomly selected. The samples consisted of: two Artrin tablets (manufactured by Medreich Limited) and one Lumiter tablet (manufactured by Macleods Pharmaceuticals Limited) after accelerated stability testing, three Lumiter tablets that underwent cyclic stability testing, three Lumiter tablets that had been stored in Darwin, and one Artefan tablet and two Combiart tablets that had been purchased in Uganda. The extraction solvent solely consisted of LiChrosolv® ACN with the extract being further diluted with ACN, either with or without the addition of dodecylamine (DDA).

6.2.3. Sample Preparation.

The samples were extracted from the powered tablets using the process to obtain ATM, with ACN as the extraction solvent (Chapter 2). While LUM extraction is aided with an acid modifier (e.g. 0.1 % *v/v* malonic acid), the substance is still very slightly soluble in ACN (Kotila

et al. 2013). Therefore, it was anticipated the ATM extract solution would still contain enough LUM to qualitatively detect the substance.

The tablet extracts were identically diluted as previous Chapters to give an estimated concentration of ATM at 2000 µg/mL. Two dilution solutions were then prepared, pure ACN, as used in previous content analysis, and ACN containing 100 µM DDA, as attempted in previous LC-MS/MS analysis (see Section 2.2), producing two sample extracts for each tablet. The purpose for adding DDA was an attempt to stabilise the ATM derivatives during the ionisation process and avoid in-source fragmentation (Keoluangkhot *et al.* 2008).

6.3. Results and Discussion.

Since the chromatographic conditions were identical to the previous experimentation, it was expected that the RTs and order would closely reflect those that were previously collected. Figure 6.1 presents a chromatogram segment of ATM/LUM tablet extract after the powdered tablet was exposed to a harsh degradation study of storage at 90 °C for 120 minutes. The chromatogram identified the three potential degradant products in red and shows ATM and its IS, ATS. For this LC-HRMS qualitative experimentation, no IS, ATS nor LOS, were added to the samples.

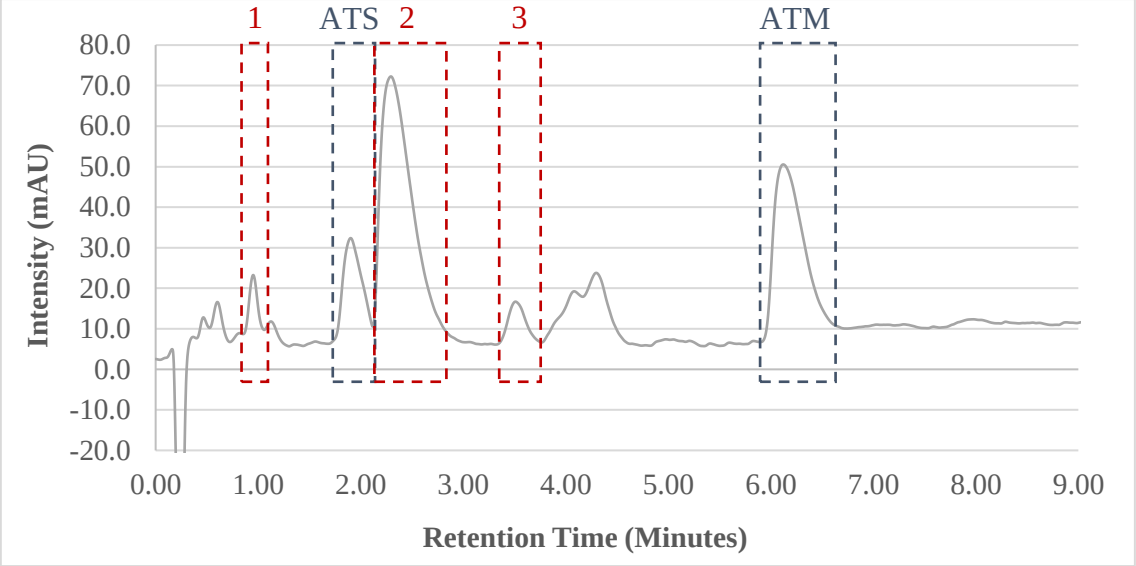


Figure 6.1. Chromatogram (200 nm) of ATM/LUM extract following harsh degradation (90 °C for 120 minutes). Refer to Figure 2.7 for chromatographic conditions.

6.3.1. System Interferences.

With the addition of TEA in the mobile phase or DDA in the samples as a stabilising agent, it is expected that indicators of these compounds can influence the mass spectrum of analysed samples. The following information details the mass information of TEA and DDA, as the presence of these compounds is observed in the later identification of the API and degradant products.

DDA was used in Keoluangkhot *et al.* (2008) as a stabilising agent for ATM derivatives through the formation of DDA-bound adducts. However, this study expected there would be excess DDA present in the samples. The chemical formula of unbound DDA is $CH_3(CH_2)_{11}NH_2$ with the molecular mass of 185.214 amu.

TEA is a common reagent used in chromatography to reduce the tailing of basic compounds such as LUM. The theoretical *m/z* ratio for protonated TEA ($[M+H]^+$) is 102.19 amu. When establishing the system peaks, *m/z* 102 was observed at a high intensity that overloaded the detector, suppressing the detection of other ions. For this reason, the full-scan MS range was set to *m/z* 110 – 1000 to avoid the interference of TEA in the collection of the target sample data.

6.3.2. HRMS Confirmation of Active Pharmaceutical Ingredients.

The two APIs in the tablets had been previously confirmed through the comparison against known reference materials using chromatographic RTs and matching UV spectra. However, for both API, it was important to collect the mass information to that of reference articles to confirm the starting point of any degradation products (Verbeken *et al.* 2011; Vandercruyssen *et al.* 2014). Furthermore, for ATM it was important to observe the parent API to determine whether in-source fragmentation was still occurring with the addition of DDA.

6.3.2.1. *Artemether.*

The structure of ATM is presented in Figure 6.2.

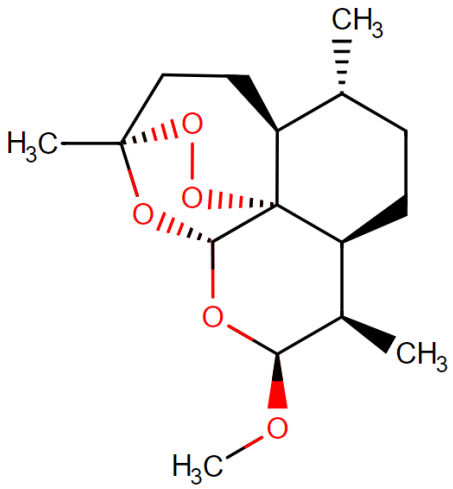


Figure 6.2. Chemical structure of ATM (Vandercruyssen *et al.* 2014)

Based on the chemical formula, $C_{16}H_{26}O_5$, the molecular mass of the parent compound is expected to be 298.374 amu. Although, as presented in Chapter 2, if in-source fragmentation occurs, the fragments at the *m/z* 267.160 and 221.154 are also expected (Figure 6.3).

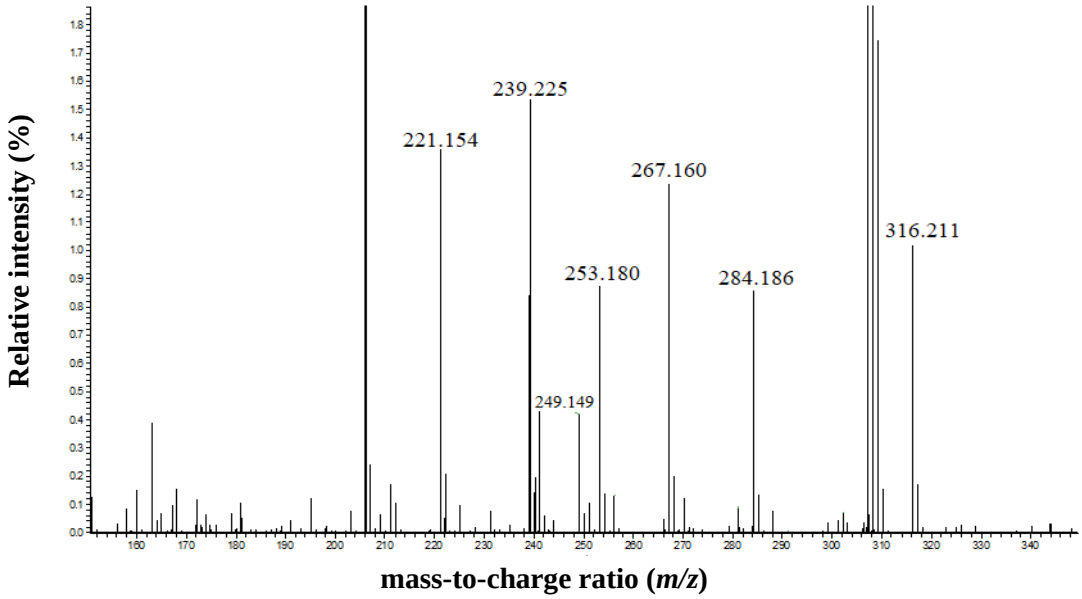


Figure 6.3. Typical HRMS spectrum of ATM, collected from Sample ACLU001B16, acquired between 5.50 to 6.50 minutes.

The larger peaks at *m/z* 206 and between 307 to 311 are unidentified systems contaminants that remain at a consistent abundance throughout the analysis. The *m/z* 316.211 represents the ammonium (NH_4^+) adduct of ATM+17.03 ($[M+NH_4]^+$). The known fragments of ATM are also present at *m/z* 267.338, 221.318, and 163.239. (Santos *et al.* 2012; Vandercruyssen *et al.* 2014). The fragments were also present in the samples that contained the DDA stabilising agent with no observable increase in $[M+H]^+$ with *m/z* 299 nor $[M+DDA+H]^+$ at *m/z* 484 . The presence of the ammonium adduct of ATM (0.001 amu difference from theoretical) and the typical ATM fragments at the expected RT of ATM confirms the presence of the API.

6.3.2.2. *Lumefantrine.*

The chemical structure of LUM is illustrated in Figure 6.4. The molecular formula for LUM is $C_{30}H_{32}Cl_3NO$.

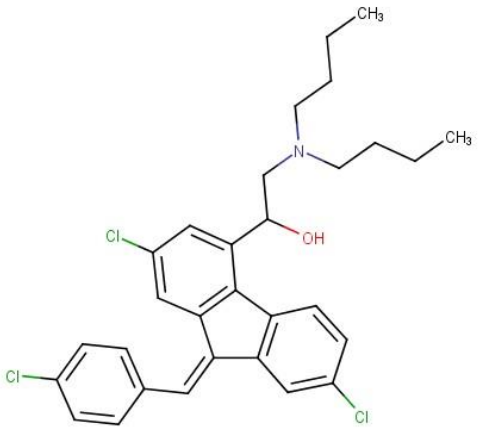


Figure 6.4. The chemical structure of LUM (Verbeken *et al.* 2011).

Based on the chemical structure, the theoretical mass of LUM is calculated to be 527.155 amu. However, due to the presence of chlorine in the structure it was expected that there would be naturally occurring isotopes of the compound. The presence of chlorine results in a typical isotope profile of ^{35}Cl as the major isotope and ^{37}Cl as a minor, naturally occurring isotope (Coplen *et al.* 2002). This characteristic MS profile is useful when confirming that any degradant products contains a chlorine and therefore are related to LUM. Figure 6.5 presents two spectra, one collected at the expected RT of LUM from a sample stored in Darwin, and another of the theoretical spectrum of the LUM's molecular formula.

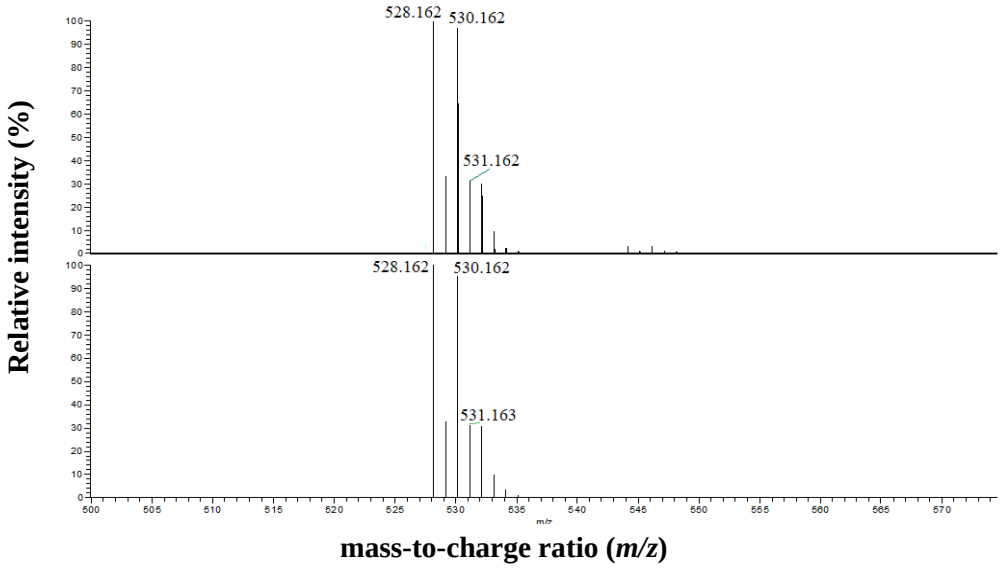


Figure 6.5. Typical HRMS spectrum of LUM, collected from Sample DALU001A12, acquired between 10.25 to 11.25 minutes (top). Theoretical HRMS spectrum of LUM ($[M+H]^+$ adduct) from the formula, $C_{30}H_{32}Cl_3NO$ (bottom).

In both spectra, the influence of the chlorine isotope is observed with a 2.00 m/z difference between the two major peaks. The surrounding smaller peaks are considered to be produced by ^{13}C isotopes. The presence of the LUM mass spectra at the expected RT confirms the presence of LUM in the tablet extracts.

6.3.3. Degradant Products.

6.3.3.1. *Degradant Peak 1.*

From previous experimentation, the peak for Degradant Peak 1 was expected between 0.50 – 1.50 minutes. Hence, the MS information during this period was captured (Figure 6.6).

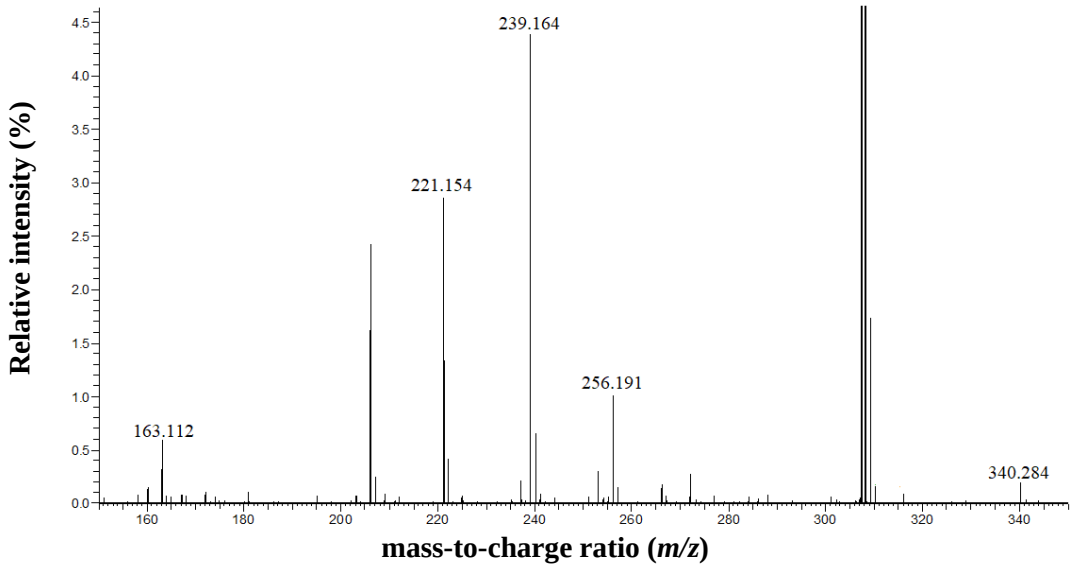


Figure 6.6. Typical HRMS spectrum of Degradant Peak 1, collected from Sample DILU001C08, acquired between 0.50 to 1.50 minutes.

The five major spectrum peaks detected between 0.50 – 1.50 minutes possessed *m/z* 239.164, 221.154, 256.191, 163.112, and 340.284. Once again, the peaks at 206 and between 307 to 311 are unidentified systems contaminants that remain at a consistent abundance throughout the analysis. Based on the presence of these peaks, it was concluded that the parent ion was *m/z* 239.164. The reasons for this conclusion was the presence of two adducts with that

m/z; $[M+H+NH_3]^+$ at 256.191 amu (239.16 + 17.03), and $[M+H+TEA]^+$ at 340.284 amu (239.16 + 101.19). The presence of the previously published ATM derivative fragments at *m/z* 221.154 and *m/z* 163.112 is also noted.

Based on this evidence, the mass of Degradant Peak 1 was concluded to be 238.164 g/mol. Vandercruyssen *et al.* (2014) has previously published a list of compounds related to β-ATM that included their relative RTs using HPLC. Diketo-aldehyde (DKA, Figure 6.7) was identified and indicated to have the lowest relative RT, like Degradant Peak 1.

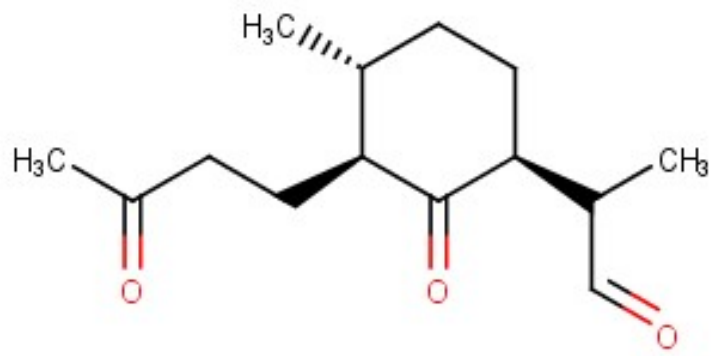


Figure 6.7. The chemical structure of diketo-aldehyde (DKA) (Vandercruyssen *et al.* 2014).

The structural formula for DKA is $C_{14}H_{22}O_3$ with an exact molecular mass of 238.156 amu. Figure 6.8 presents the extracted ion chromatograms (XICs) for the three adducts of Degradant Peak 1 (DKA), $[M+H]^+$, $[M+H+NH_3]^+$, and $[M+H+TEA]^+$, observed between 0.50 and 1.50 minutes.

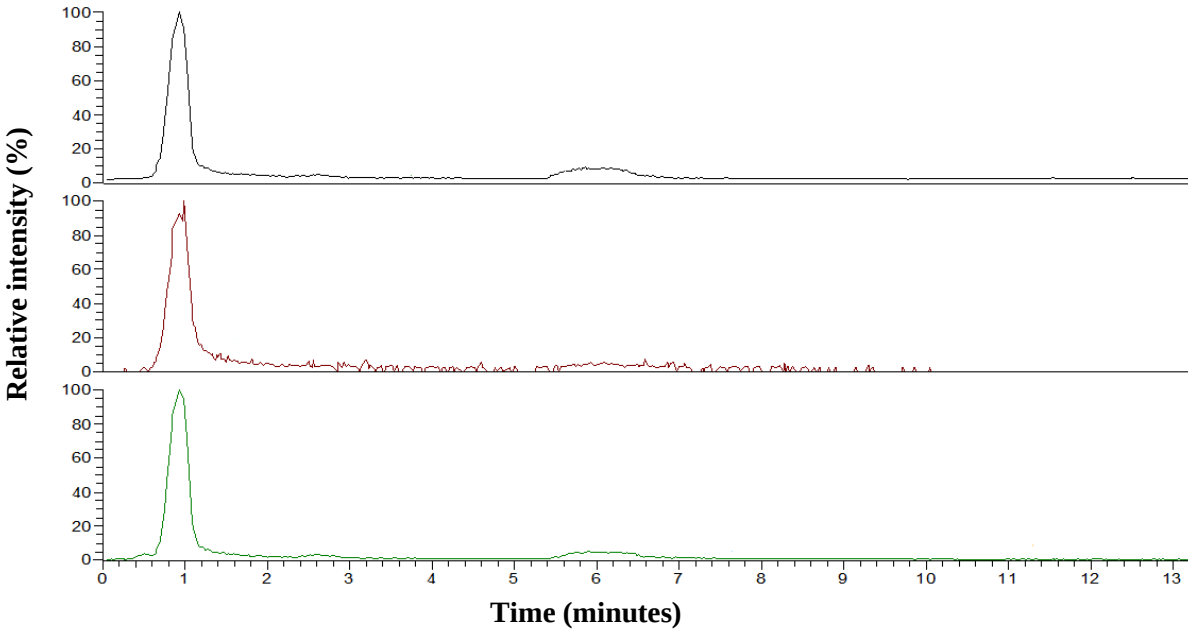


Figure 6.8. Extracted ion chromatograms (XIC) collected from Sample DILU001C08. XIC *m/z* 239.16 (top), XIC *m/z* 340.28 (middle), XIC *m/z* 256.19 (bottom).

The additional peak observed between 5.50 and 6.50 minutes for the *m/z* 239.16 and *m/z* 256.19 suggest that an isobaric compound of DKA is also present, but at a much lower intensity. However, the $[M+H+TEA]^+$ adduct (*m/z* 340.28) is only discernible from the baseline noise during the 0.50 – 1.50 minute time-period. The presence of three adducts of DKA and that one of the adducts is only present during the expected RT provides adequate justification on the confirmation of the identity of Degradant Peak 1 as DKA.

DKA was detected in all 12 test samples except for UGCB173B12, which did not demonstrate an adequate *S/N*. The DKA peaks demonstrated better peak resolution in the laboratory-based stability tests, accelerated and cyclic, than in the Darwin and Ugandan samples. The improved resolution is considered to be associated with an increased concentration of DKA in the sample.

6.3.3.2. *Degradant Peak 2.*

Based on previous experimentation, the peak for Degradant Peak 2 was expected between 2.10 – 3.10 minutes. Therefore, the MS information during this period was captured (Figure 6.9). The identity of Degradant Peak 2 has been reported in previous chapters with the

presumptive identity as DBK based on a similar UV-spectra to that reported in Verbeken *et al.* (2011). Verbeken *et al.* (2011) also reported on the toxicity of DBK after an in-silico investigation using ToxTree® and Derek®, in which it was determine that DBK has a similar toxicity to LUM. The theoretical spectrum of DBK is also presented in Figure 6.9.

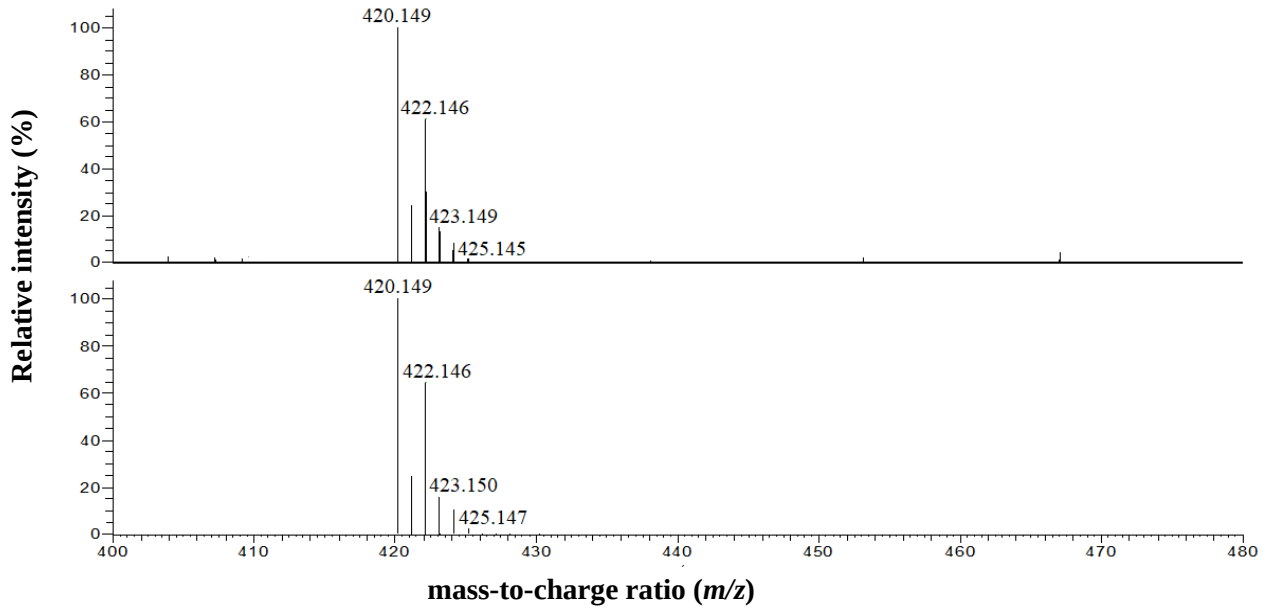


Figure 6.9. Typical HRMS spectrum of Degradant Peak 2, collected from Sample DILU001C08, acquired between 2.10 to 3.10 minutes (top). Theoretical spectrum of DBK ($[M+H]^+$ adduct) from the formula, $C_{23}H_{27}Cl_2NO_2$ (bottom).

The two major peak spectrum peaks are at *m/z* 420.149 and 422.146. The ratio of the major spectrum peaks is like that demonstrated in the LUM spectrum with ^{37}Cl isotope generating the second highest *m/z* peak. Figure 6.10 shows the chemical structure of DBK, with the presence of the two chlorine halogens.

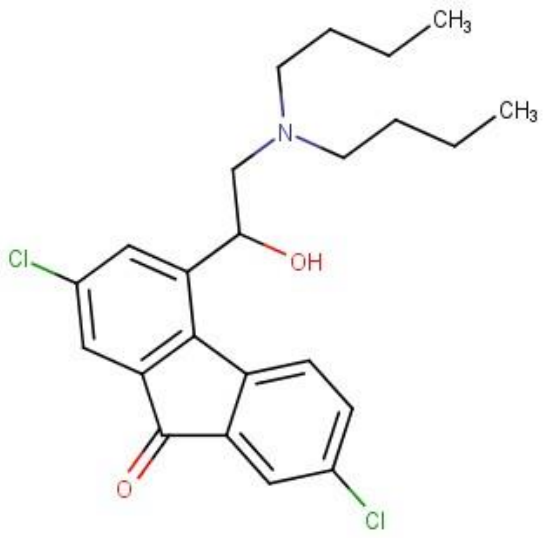


Figure 6.10. The chemical structure of DBK (Verbeken *et al.* 2011).

The structural formula for DBK is $C_{23}H_{27}Cl_2NO_2$ with an exact molecular mass of 419.14 g/mol. Figure 6.11 demonstrated the XIC for the *m/z* range between 419.80 – 420.20. The RT period of Degradant Peak 2, between 2.50 – 3.50 minutes, is the period for the highest intensity of the selected *m/z* range. There is also a very small (< 5 % relative intensity) peak for the *m/z* range at approximately 10.50 minutes, the RT in which LUM is expected. This indicates that LUM may also be subject to in-source fragmentation but at a very low level.

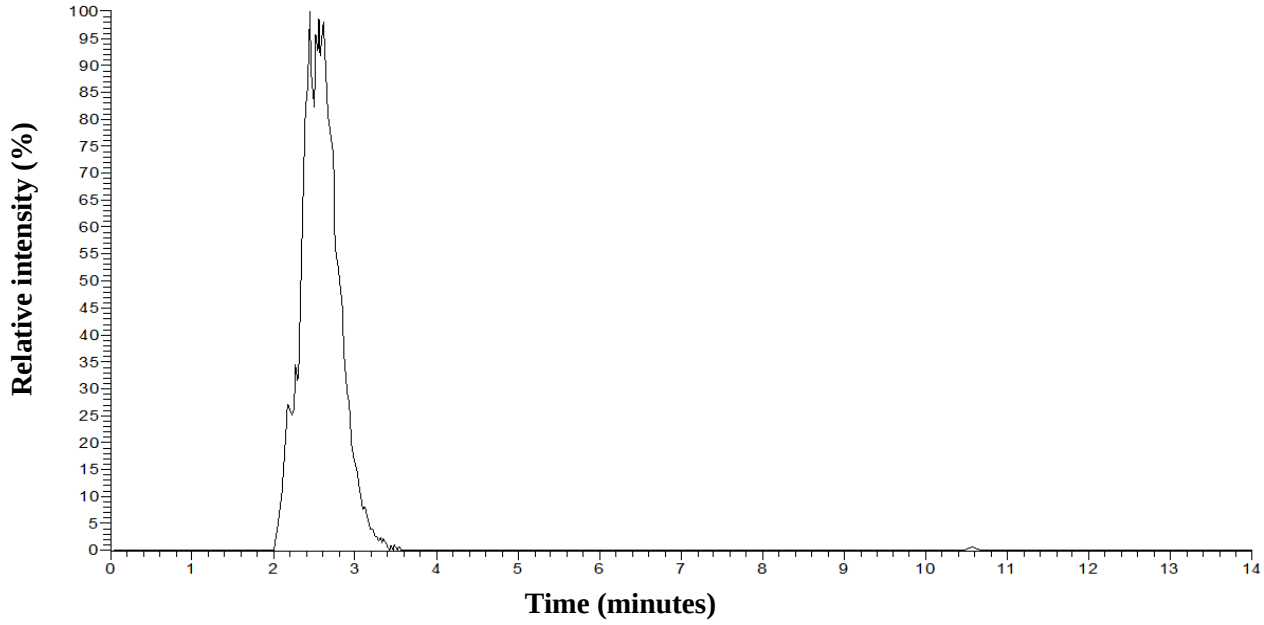


Figure 6.11. Typical HRMS extracted ion chromatogram (XIC) for *m/z* 419.80 – 420.20 range. Collected from the Sample DILU001C08.

Degradant Peak 2 was preliminarily identified earlier through the comparison of the UV spectrum against previously published data by Verbeken *et al.* (2011). The collected mass information supports the preliminary identification, confirming the presence of DBK. In the 12 samples that were tested, DBK was detected in all.

6.3.3.3. *Degradant Peak 3.*

Three degradant peaks had been identified based on previous HPLC analysis. However, the number of samples that the peaks were detected varied, with Degradant Peak 3 being detected in only 41 % of samples in the six storage condition data sets. The 12 samples selected for MS analysis were done so based on their reported DPR for Degradant Peak 1 as that peak was more common and had a stronger correlation to the API loss. An unfortunate consequence of this decision is that of the 12 samples, Degradant Peak 3 was not detected in any of the samples and therefore, could not be identified.

6.4. Conclusions.

The purpose of the LC-HRMS analysis was to confirm the mass information of the two API and to identify the common degradant peaks. Two of the three previously detected degradant peaks were successfully identified as diketo-aldehyde, a degradant of ATM, and desbenzylketo-lumefantrine, a degradant of LUM. Unfortunately, in the subsample of tablets selected for LC-HRMS analysis, none contained Degradant Peak 3. Therefore, the peak remains unidentified.

The experimentation was able to demonstrate that the COPA protocol is largely applicable to LC-HRMS analysis; however, the adaptation still has its shortcomings. As demonstrated above, the issue of in-source fragmentation is still present. As such, the complete conversion to LC-HRMS is still not yet practicable.

The identification of a degradant of both API is highly beneficial as now the HPLC-PDA method can differentiate whether poor-quality tablets are substandard or degraded for

both API individually, not just with the tablet as a whole. The HPLC-PDA method can now be confidently used to characterise a PQM as substandard or degraded.

Chapter 7. General Discussion, Future Directions, and Conclusion.

7.1. General Discussion of the COPA Protocol.

The "Characterisation of Poor-Quality Antimalarials" (COPA) protocol sought to accurately characterise poor-quality medicines as falsified, substandard or degraded using a series of assessment techniques, both analytical and non-analytical. Falsified medicines possess misinformation on their packaging with respect to identity or source and are therefore characterised based on critically assessing all the packaging of the products. Substandard medicines are made by legitimate manufacturers but whether intentional or not, do not possess the required amount of active pharmaceutical ingredients (API) and are characterised based on the percent content of the API contained in the tablet and the absence of degradation products. Degraded medicines are made by legitimate manufacturers but due to poor supply chain management have been exposed to temperature and humidity conditions that have led to the API physically changing to a degree in which the medicine is no longer effective. Degraded medicines are also characterised based on the percent content of the API in addition to the presence of degradation products that indicate the medicine has degraded since manufacture.

The COPA protocol combines the assessment of quality indicators, including specific packaging criteria and a high-performance liquid chromatography photodiode array (HPLC-PDA) method to characterise poor-quality medicines into the three categories: falsified, substandard, or degraded.

7.1.1. Packaging Analysis.

Packaging analysis is a critical component in characterising medicines as falsified (World Health Organisation 2017). The COPA research project combined packaging assessment criteria applied previously to generate a series of questions to determine the

authenticity of the packaging (World Health Professions Alliance 2007; Newton *et al.* 2011a). Packaging analysis includes assessing information such as: correct spelling, appropriateness of the active ingredients and trade name, and anti-counterfeiting technology (e.g. hologram). Using this approach, the COPA project was able to identify a falsified medicine during the Ugandan field survey (Chapter 5).

7.1.2. Physical Measurements.

The Lumiter tablets appeared to be more susceptible to physical changes after storage in heightened conditions. In both the laboratory-based (Chapter 3) and atypical (Chapter 4) stability tests, more Lumiter tablets were found to be statistically different to the baseline than both Coartem and Artrin tablets. This observation does not malign Lumiter tablets, as the conditions to which the medicines were stored were purposefully heightened and therefore changes were expected. However, based on this observation there is evidence that medicines can present different physical changes after experiencing heightened temperature and humidity conditions. Therefore, there is no single indication to medicine degradation based on physical measurements alone, content analysis using an analytical instrumentation is required.

7.1.3. Content Analysis.

The primary outcome of the COPA project was to develop a protocol that would deliver practical guidance to analyse and report medicines as falsified, substandard, or degraded. A major component of this was achieved when a HPLC-PDA method was developed and validated that could quantitatively determine artemether (ATM) and lumefantrine (LUM) content and detect the presence of degradation markers (Chapter 2). The significance of the HPLC-PDA method was that the literature review that shaped the COPA project identified most of the field surveys reviewed had access to a HPLC analytical instrument (Grech *et al.* 2017). However, even as recently as 2018, research groups are still reporting the lack of a capability to characterise medicines as degraded while possessing the analytical instrument

required to do so (Schiavetti *et al.* 2018). This occurrence demonstrates that international need for practical guidance on the analysis of poor-quality antimalarials.

Furthermore, the secondary outcome of the COPA project was achieved when the COPA protocol was implemented during a field survey of five administrative districts across Uganda, Africa (Chapter 5). Through implementing the protocol, one falsified medicine and one degraded medicine were identified. The results indicates two positives: 1) the COPA protocol was successfully applied, and 2) the percentage of poor-quality antimalarials in Uganda in the current field survey are lower than the average of previous field survey performed world-wide (Grech *et al.* 2017). However, it is important to consider that because the samples were collected using an overt sample collection method that the POD participant had the ability to provide samples known to be of higher quality or samples that had not been at the POD for a long period, therefore, reducing the chance of degradation and/or falsified observations. Therefore, comparison of the results obtained in the current field survey against a covert sampling method would provide further insight.

7.2. Real-world Applicability of Laboratory-based Stability Testing.

A novel approach into cyclic stability testing was also investigated (Chapter 3). The study simulated heightened daily temperature and humidity cycles of Kampala, Uganda, in a controlled climate chamber. The purpose of which was to bridge the gap between laboratory-based stability testing and the real-world conditions medicines may be exposed to in a supply chain with limited climate regulation. Hall *et al.* (2016) recently acknowledged the importance of recreating the conditions at the end of the supply chain. However, unlike Hall *et al.* (2016), the COPA project followed the temperature and humidity conditions of the target region and also the cyclic nature in which the conditions fluctuated.

Furthermore, the COPA project successfully completed an atypical stability study, storing medicines in an environment exposed to natural climate variations of Perth and Darwin,

where the storage conditions were monitored but not controlled (Chapter 4). Importantly, Darwin is classified in the same Köppen-Geiger climate classification as Uganda, the target country in Africa. In 2015, Phommasone *et al.* (2015) performed a similar atypical stability study on rapid diagnostic tests. However, this appears to be the first instance of an atypical stability study on antimalarial medicines.

Through comparing the degradation patterns of the cyclic stability test and the Darwin atypical stability test, the COPA research demonstrated similar degradation patterns, inferring a relationship between the cyclic laboratory-based stability test and the conditions in a climate region like Darwin and Uganda. There was insufficient collection of degraded medicines during the Ugandan field study to confidently draw definite correlations between the laboratory-based, cyclic stability testing and the Ugandan samples, e.g. that the degradation patterns for the Ugandan samples reflected the cyclic testing samples. However, with the widened implementation of the COPA protocol, standardised and consistent analyses of degradants of a region would slowly form a profile for that region. With such data, the connection between the cyclic laboratory testing and real-world stability can be drawn. Two important actions need to occur for this connection to be adequately drawn. First, that the outcomes of field surveys be combined as described above. Secondly, that the type of cyclic stability testing described in the COPA research be further adapted to a wider range of medicines, as the results from the COPA project indicate that the cyclic stability testing may be a closer representative laboratory-based stability test than accelerated testing.

7.3. Public Health Implications and the COPA Project

Based on the outcomes of the field survey of Ugandan PODs, consumers of antimalarials in the region can be relatively confident in the quality of their medicine, as only a low percent of poor-quality medicines were found (approximately 2 %) compared with the results of the systemic review reported in Chapter 1 (18 %). However, there are two important

limitations associated with the conclusion of the Ugandan field survey. The results only represent AMs collected from private PODs that were collected by an overt sampling method. There is a risk that the medicine collected were selected by the POD participant because they were known to be of authentic source and probably high quality. Therefore, it is suggested that the COPA protocol be implemented in a field survey that investigates antimalarials collected from public PODs using a covert sample collection method for comparison. The recent article addressing the ethical considerations of covert collection will improve the possibility of covert collections, which was not available to this project (Tabernero *et al.* 2016). Acknowledging that the covert sample collection method does not allow the researcher to collect information relating to pharmacy practices via a survey, the survey could be requested at a time after the samples have been collected.

It is important to recognise that the COPA protocol does not solve poor-quality medicines, nor does it act as a countermeasure to do so. What the COPA protocol does, is act as a tool for future research to achieve these goals. There are significant public health implications of poor-quality medicines that can be addressed through implementing the COPA protocol.

Hamilton *et al.* (2016) identified pharmacist practitioner training in recognising and reporting falsified medicines as an effective countermeasure to the problem. The Ugandan field survey questionnaire (Section 2.3.3.3) identified that most pharmacists have adequate academic education to understand the importance of identifying falsified medicines. Therefore, what is required is targeted education packages informing the pharmacists of the checklist applied by the COPA project and the World Health Professions Alliance (WHPA), which has been proven capable of identifying falsified medicines (World Health Professions Alliance 2007). An example of these educational packages are the information videos provided by the WHPA, which are readily available on the internet.

It is the joint responsibility of researchers, pharmaceutical companies, and policy-makers to improve medicine quality. India is a major supplier of generic medicines, worldwide, while consequently also being a major source of substandard medicines (Khan and Khar 2015). For this reason, India has attempted to implement regulatory countermeasures by amending the Drug and Cosmetic Act in 2008 to include penal provisions "relating to manufacturing of spurious or adulterated drugs" (Kumar *et al.* 2017). With the epicentre of malaria infections located in Africa, it is not entirely unreasonable to assume that there will be a demand for locally-sourced AM medicines in an effort to reduce prices. Therefore, if the pharmaceutical and healthcare system in Africa are to develop as described by Mackintosh *et al.* (2017), then there is a responsibility for the local and regional governments to introduce legislation similar to that introduced in India in the last decade. However, it is ultimately the responsibility of the pharmaceutical companies to then abide by those regulations and manufacturing processes to improve medicine quality.

7.4. Future Directions.

The COPA research project was successful in achieving the intended outcomes of the project. However, through the research process, the following matters were either deemed to be outside the scope of the COPA project or would be beneficial to future medicine quality research.

- Quality assessment procedures such as the COPA protocol would be greatly aided by the assistance of pharmaceutical companies in providing reference packaging material. The COPA project experienced limited response to the pharmaceutical companies that were approached, as only three participated in providing samples and/or reference packaging materials. However, it is important to note that requests to the pharmaceutical companies need to be specific. For example, pharmaceutical companies would likely possess multiple varieties of a medicines packaging based on the location

and language of the final product (Batson *et al.* 2016). Therefore, if reference packaging for a field survey is sought then it is important to reference the location of the field survey.

- Attempts were made during the COPA research to develop a network in which medicines could be sampled and analysed at each stage in the supply chain, from release at the manufacturer to the time of sale at the POD. Unfortunately, the contacts and logistics to conduct such research could not be finalised in addition to the field survey described in Chapter 5. Supply chain survey research would be critical in determining the points throughout the supply chain where the medicines are most at risk of a temperature or humidity excursion and possessing the medicine quality data to determine the impact of the excursion. Ultimately, the outcomes could help identify and mitigate situations that lead to the degradation of high-quality medicines. It is recommended that this type of medicine quality investigation occur in the future if possible.
- The COPA protocol was successful in characterising poor-quality antimalarials using HPLC-PDA. Major shortcomings of HPLC-PDA analysis are that the technique is destructive, i.e. the tablet is consumed in the analysis, and that the technique is not field-portable. The characterisation of poor-quality medicines would be greatly aided in the identification and field-validation of a technique that is non-destructive and field-portable. While devices such as the TruScan™ handheld Raman analyser, manufactured by Thermo Scientific™, has demonstrated the ability to identify falsified medicines, it still lacks the ability to characterise substandard and degraded medicines (Batson *et al.* 2016). With increasing sensitivity and improved in-system chemometrics, it is hoped that a future Raman analyser will be capable of characterising all types of poor-quality antimalarials (Vickers *et al.* 2018).

- Further analytical progress would be centred around the analysis of ATM and its derivatives using mass spectrometry. The COPA protocol successfully completed a proof-of-concept application of high-resolution mass spectrometry (HRMS) in elucidating the structure of ATM and its degradant, diketo-aldehyde. However, the application of HRMS still encountered the issue of in-source fragmentation of the two analytes. Future research investigating mass information of artemisinin-derived combination therapies (ACTs) can continue in two ways: 1) the development of softer ionisation techniques or the introduction of new adduct combinations to maintain the integrity of the parent ion, or 2) exploring alternate techniques such as electron-ionisation liquid chromatography-mass spectrometry (Palma *et al.* 2011). The intended outcome would be identical; the ability to collect mass information from all the medicine's components with enough accuracy and detail so that structural elucidation is possible. Achieving that capability may lead to the identification of additional ATM degradants and improve the characterisation of ACTs.

7.5. Conclusion.

The COPA project identified the need for practical guidance to improve the characterisation of results from medicine quality field surveys (Chapter 1). To provide this guidance through the creation of the COPA protocol, two main project aims were identified: to develop and validate an analytical method capable of quantifying ATM, LUM, and their degradation products, and to demonstrate the applicability of the protocol through its successful implementation in a field survey (Chapters 2 and 6). To be confident that the protocol was capable of quantifying degradant products that were expected in real-world samples, a series of stability tests were performed to generate any potential degradant products (Chapters 3 and 4). Completing those two tasks achieved the requirements for the first project aim (Figure 7.1).

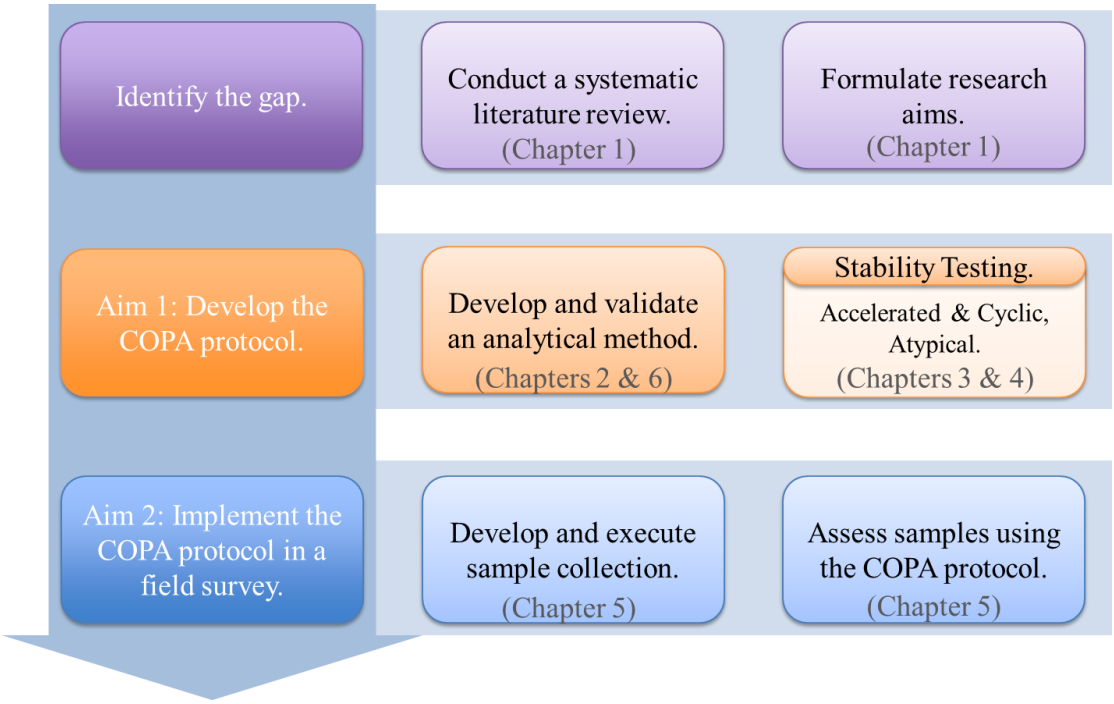


Figure 7.1. The structure of the COPA project.

To implement the COPA protocol, a stratified random sampling method was applied to the administrative districts of Uganda based on climate information. Five districts were selected for sampling and the collection was successfully executed. Samples were transported to the University of Canberra where they were assessed using the COPA protocol. From this assessment, a single falsified and a single degraded medicine were identified. Completing the collection and assessment of the samples from Uganda, the second project aim was achieved.

The additional aim for the project was to observe a connection between the laboratory-based stability testing and the conditions to which medicine are exposed to when stored in uncontrolled environments during transport and at the point of distribution. Similarities in the loss of API and the degradant profiles for the samples that underwent the cyclic laboratory-based stability test and those that were stored in the shipping container in Darwin lends support to the potential for cyclic stability testing to be further examined as a standard stability test for medicine development. However, with minimal degradation witnessed in the Ugandan samples, the final step in finding a link between the laboratory, the atypical tests, and the field survey requires further assessment.

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