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FORENSIC PHARMACEUTICAL ANALYSIS OF COUNTERFEIT MEDICINES

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy (Ph.D) in Forensic Pharmaceutical Analysis

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DEDICATION

This thesis is dedicated to God Almighty for the strength and grace to go through the process.

ABSTRACT

The World Health Organisation suggests that falsified and substandard medicines (FSMs) constitute approximately 10% of medicines globally with higher figures expected in low and middle income countries (LMICs). To combat the proliferation of FSMs this study is aimed at developing simple and rapid instrumental methods for the identification and quantification of these medicines. Attenuated Total Reflection-Fourier Transform Infrared (ATR-FTIR) spectroscopy, Raman spectroscopy and two probe Mass Spectrometry (MS) methods were assessed for the rapid screening of tablet dosage forms. These systems were chosen because NO solvent extraction of the sample was required. Comparison with analyses of the tablets by accepted but more time consuming methods (UV-Vis and LC-MS) assessed the quality of the data obtained. Analgesic/antipyretic and antimalarial medicines tablet dosage forms are commonly falsified and for this study tablets were obtained opportunistically from different countries around the world. Reference spectra of appropriate active pharmaceutical ingredients (APIs) and excipients were created, for each method, as part of the identification process. Currently only Raman and ATR-FTIR delivered quantitative results which were based on automated multivariate analysis.

For tablets with a single API, Raman and ATR-FTIR provided the simplest route to API confirmation and for tablets with multiple APIs or APIs present at <10%w/w, in the tablet, probe MS methods were superior. Quantitative screening using ATR-FTIR required the samples to be weighed and crushed to produce reproducible data. Comparison of API confirmation tests between trial methods and LC-MS showed complete agreement and the quantitative results were within ±15% of the UV-Vis data.

Each of the new tests can be completed in under five minutes and a survey of 69 paracetamol tablets, from around the world, showed that 10% were suspect. Subsequent probe MS showed the presence of a second undeclared API in different samples. More complex tablet formulations, for example the antimalarials were difficult to quantify rapidly. Raman and PCA methods provide a rapid approach to tablet identification within a limited range of possibilities. Factors that may affect Raman spectra of tablets include the expected API, the API levels, different excipients, colours or surface coatings for the tablets.

The simplicity, speed and cost effectiveness of the proposed analytical methods make them suitable for use in LMICs. The potential use of these simple analytical methods in addition to already established pharmacopoeia approved (solvent extraction) techniques could help provide more comprehensive data about FSMs globally.

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PUBLICATIONS

A list of publications from this PhD

Lawson, G., **Ogwu, J.** and Tanna, S. (2018) Quantitative screening of the pharmaceutical ingredient for the rapid identification of substandard and falsified medicines using reflectance infrared spectroscopy. *PLoS ONE*, 13 (8): e0202059.

Lawson, G., **Ogwu, J.** and Tanna, S. (2014) Counterfeit Tablet Investigations: Can ATR FT/IR Provide Rapid Targeted Quantitative Analyses? *Journal of Analytical & Bioanalytical Techniques*, 5: 214.

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ABBREVIATIONS

ACMD   Advisory Council on the Misuse of Drugs

API   Active Pharmaceutical Ingredient

ASAP-MS   Atmospheric Pressure Solids Analysis Probe-Mass Spectrometry

ATR-FTIR   Attenuated Total Reflectance- Fourier Transform Infrared

DART   Direct Analysis in Real Time

DESI   Desorption Electrospray Ionisation

DIP-MS   Direct Insertion Probe-Mass Spectrometry

EMVS   European Medicines Verification System

EU   European Union

FDA   Food and Drug Administration

FSM   Falsified and Substandard Medicine

GPHF   Global Pharma Health Fund

GPS   Global Positioning System

GSMS   Global Surveillance and Monitoring System

HPLC   High Performance Liquid Chromatography

IP   Intellectual Property

IR   Infrared

KBr   Potassium Bromide

LC-MS   Liquid Chromatography-Mass Spectrometry

LMICs   Low and Middle Income Countries

MIR   Mid Infrared

MCC   Microcrystalline Cellulose

NIR   Near Infrared

NHS   National Health Service

NMR	Nuclear Magnetic Resonance
NMRA	National Medicines Regulatory Authority
OOS	Out of Specification
OTC	Over the Counter
PCA	Principal Component Analysis
PLS	Partial Least Squares
PDA	Photodiode Array
PSI	Pharmaceutical Security Institute
RFID	Radio Frequency Identification
TLC	Thin Layer Chromatography
SSFFC	Substandard/ Spurious/ Falsely-labelled/ Falsified/ Counterfeit
UV-Vis	Ultraviolet Visible
WHO	World Health Organisation
WTO	World Trade Organisation

CHAPTER ONE

Introduction and Aim of study

1.1 Introduction

Medicines, particularly tablet formulations, may not correspond to the description on the packaging. In the literature, such tablets are variously described as: counterfeit, falsified or substandard. The currently appropriate choice of descriptions are discussed in Chapter 2.

Falsification of medicines is a growing global problem and a serious crime considering the threat they pose to human health. The United Nations Sustainable Development Goals for 2030 also includes access to "safe, effective, quality and affordable essential medicines" acknowledging the severity of the falsified medicines situation (Petersen et al, 2017). The difficulty in controlling the falsified medicines market can be attributed in no small measure to the complex medicines distribution network with numerous actors involved at different stages of the medicine distribution chain (Martino et al, 2010; Dégardin et al, 2014). The huge profits that can be made by the falsifiers of medicines could be a driving force for this phenomenon (Martino et al, 2010; Rebiere et al, 2017). Counterfeiting of medicines is an age old practice which dates back to the 4th century BC with Pedanius Dioscorides being the first to mention counterfeit medicines (Newton et al, 2006). However, regulations on the trade of the medicines did not include the control of counterfeits until 1988 when resolutions against counterfeit medicines were adopted (Newton et al, 2006).

Over time, falsification of medicines has been on the rise with current estimates indicating that about 10% of the medicines sold worldwide are falsified or substandard (Dégardin et al, 2014). Industrialisation, globalisation and the increasing access to medicines via the internet makes the situation even more alarming. Reports suggest that over 50% of the medicines sold via the internet may be counterfeit even in developed countries like the United States of America (USA) and United Kingdom

(Martino et al, 2010; Jackson et al, 2012). This is alarming and calls for urgent action even more so because children may have access to and abuse prescription medicines exposing them to these falsified medicines. Falsification of medicines has also been identified as a branch of organised crime (Dégardin et al, 2015).

Medicines presented to a patient are usually a combination of the active ingredient with several non-active materials called excipients. The excipients generally have no bioactivity but are present to protect, bulk out or make the active pharmaceutical ingredient (API) more readily available for bio assimilation.

Whilst medication formulations may be liquids or solids, there is an industry move towards tablet formulations so that the patient can take responsibility for adherence to the prescribed regimen. API doses can range from 1-1000mg whilst typical tablet overall masses range from 100-1100mg. The API level ranges from < 1%, for some tablets, to approximately 90% for paracetamol tablets. The aim of the counterfeiter therefore is to produce medicines that would pass as or are similar to the genuine formulation even though it is falsified.

In this thesis, the expression falsified/substandard medicines (FSMs) will be the term used for all suspect/ questionable medicines. "Falsified" and "counterfeit" will be used interchangeably when referring to medicines that are fraudulently mislabelled or misrepresented with respect to their identity or source. This is because they refer to the same group of medicines as mentioned earlier. Falsified and substandard medicines range from those with incorrect amounts of the Active Pharmaceutical Ingredient (API) to those with no API(s) present (Davison, 2011). Unlike the counterfeiting of other goods, counterfeit medicines could be potentially life threatening and as such, require urgent attention (Mackey and Liang, 2011). More sophisticated techniques are now

employed in the manufacture and packaging of falsified and substandard medicines making it increasingly difficult to detect them (Kovacs et al, 2014).

Trade in falsified and substandard medicines depends on profit margins like any other commerce and so will thrive where demand is high and there is shortage of supply. It is important to note that even cheap medicines are attractive to falsifiers of medicines provided the potential volume of sales is high enough. In addition, trade in falsified and substandard medicines is facilitated by the ignorant, the careless, the unscrupulous and the criminal and so thrives in regions where the risk of detection is low and the technical capacity is weak (WHO, 2017). Limited access to quality medicines that are both safe and affordable is another huge challenge especially in low and middle-income countries. The cost pressures and inadequate supply of medicines at any point in the medicines supply chain allows counterfeiters to fill in or take care of the deficit with falsified/substandard medicines. There is also the lack of legislation prohibiting the manufacture or sale of FSMs in many countries and corruption among officials, which allows counterfeiters of medicines to thrive. Penalties for manufacturing FSMs in many other cases are weak and therefore encourage the counterfeiters to continue with this crime of medicine falsification with unfavourable implications on the legal manufacturer and the consumers of the falsified medicines (Dégardin et al, 2015).

Falsified medicines (whether with incorrect amounts of API(s) or no API(s) present) can lead to therapeutic failure. Therapeutic failure due to falsified or substandard medicines implies that the patient does not get better, the patient's condition worsens or ultimately the patient/consumer dies. For instance, incorrect amount of API(s) could lead to drug resistance reducing the bioactivity of the medicine as well as providing no relief for the patient even after administration of the required dose. Drug resistance is a serious global challenge especially with antimalarial medicine and antibiotics costing the pharmaceutical industry millions of pounds every year. Generally, drug resistance is

linked to abuse of medication but falsified and substandard medicines might be responsible for even more cases of drug resistance than have been reported. This is because drug resistance is identified, only in retrospect, when the patient shows no positive response to the prescribed dosage of the medication administered. It is therefore difficult to say conclusively whether one or a combination of factors is responsible for drug resistance in each case reported. This implies that several cases of drug resistance caused by falsified and substandard medicines may be going unnoticed and attributed to other factors unless measures are put in place to assess medicines from manufacturing level to the point of sale to the final consumer. Besides the health risk associated with therapeutic failure from falsified or substandard medicines, there is the additional costs to healthcare suppliers who have to spend more funds to provide treatment for the patients in cases where medication did not work.

Irrespective of whether people live in low and middle income or developed countries, they can be affected by the sale/distribution of falsified and substandard medicines (Dégardin et al, 2015). A typical example would be patients suffering from tuberculosis, who have been administered falsified anti-tuberculosis medicines. The patients taking falsified anti-tuberculosis medicines could pose a huge health risk to all those they come in contact with including tourists from different countries. Falsification of medicines also leads to economic losses for both the legal manufacturer of the medicine and the consumer/patient purchasing the medication. Due to low cost of production, falsified medicines are generally cheaper and more accessible to consumers. It is more likely that the demand for the cheaper and more affordable falsified medicines will be higher than for the more expensive genuine product. Declining demand for genuine medicines would invariably lead to losses for the legal manufacturers of the medicine. For the patient/consumer on the other hand, money

spent on the falsified medicine would be lost since the falsified/substandard medicine does not provide any relief from the debilitating ailment (Deisingh, 2005; Dégardin et al, 2014). It is therefore important that there are quality checks and assessments for medicines at different points throughout the medicines distribution chain.

Current techniques used in the detection of falsified and substandard medicines involve lengthy procedures for analysis and the techniques are very expensive making these analyses less feasible in low and middle-income countries where resources are limited (Kovacs et al, 2014). Therefore, a simple, rapid and cost effective detection system for falsified and substandard medicines would help in addressing this challenge of medicine falsification. Different approaches have been employed in the detection of counterfeit medicines, the first being visual inspection or physical examination of the packaging and then the medicines (Davison, 2011).

Visual inspection/physical examination of medicines starts with the packaging; are there any unique features, which could be covert or overt such as logos, watermarks or radio-frequency identification tags? For example, absence of leaflets and/or inconsistencies in labelling on packaging compared to authentic tablets could help identify falsified medication. Newton et al. (2011) identified falsified antimalarial medicines based on observed differences in their packaging. Tablet inspection could include noting the length, width, thickness, mass, shape, colour and markings/inscriptions on the tablet (Kovacs et al, 2014). Custers et al (2016), in their study showed measurements of tablets length and mass helped discriminate genuine Viagra® tablets from falsified ones.

Nowadays, simple visual inspection of the medicines no longer suffices for the screening of medicines since the falsifiers of medicines now use more sophisticated approaches (Martino et al, 2010; Dégardin et al, 2014). Consequently, more advanced analytical techniques are required to detect suspect medicines as fast as possible, to

get them out of the market to help curb the threat falsified and substandard medicines pose to public health.

Some simple and easy analytical methods used for screening of medicines include, colorimetry, thin-layer chromatography (TLC) and melting point determination (Dégardin et al, 2014). These simple analytical methods easily identify the presence or absence of the API and/or excipient in the medication, hence, provide quick screening of samples. The absence of API(s)/excipients declared on the packaging or the presence of API(s)/excipients not declared suggests the medicine might be counterfeit and needs further analysis. The drawback with the simpler colorimetric and TLC based methods for quick screening of medicines mentioned earlier is that they are qualitative based techniques and so will not be able to detect counterfeits where incorrect amounts of APIs are present but rather identify the presence or absence of the API concerned. Furthermore, some of these simple qualitative methods are specific for particular medicines such as the paper test cards for assessing antimalarial tablets (Weaver and Lieberman, 2015) implying that different methods will be developed for different medicines making the process laborious (Martino et al, 2010). Nevertheless, the simple qualitative methods are useful as fast preliminary checks for medicines.

Other more advanced separation techniques that could be used for further quantification of APIs or excipients in medicines are gas chromatography (GC), high performance liquid chromatography (HPLC), Ultraviolet Visible (UV-vis) spectroscopy, nuclear magnetic resonance (NMR) and mass spectrometry (MS) (Görög, 2015). Hyphenated mass spectrometry techniques such as GC-MS and LC-MS have also been investigated in the quantification of APIs. Despite the fact that these techniques are more robust, much longer sample preparation times are required since APIs and/or excipients have to be solvent extracted before analysis. These more advanced

techniques are also expensive and require skilled personnel for both analysis and interpretation of results (Kovacs et al, 2014).

Simple techniques requiring little or no sample preparation prior to analysis help to speed up analysis time and thus would be apt for the first-line analysis of falsified medicines (Kovacs et al, 2014). Cost-effectiveness and portability are also important features to consider when choosing methods for screening falsified and substandard medicines. Cheaper (cost of production and maintenance) methods will make the techniques more accessible to wide range of users globally and therefore facilitate quicker analysis at different points in the medicines distribution chain. Portability of the equipment is also important in screening for falsified medicines in order to ensure ease of use on the field or at the point of sale of the medicines. The speed of analysis, cheaper costs, portability and simplicity of the technique employed in the screening of medicines will go a long way in facilitating in-field analysis of medicines especially in low and middle-income countries (Höllein et al, 2016).

Taking into account the important features for techniques employed in screening falsified medicines (highlighted in the previous paragraph), vibrational spectroscopic techniques would be well suited for screening medicines since minimal sample preparation is required prior to analysis. With spectroscopic-based methods, the excitation of fundamental molecular rotations or vibrations gives rise to unique spectra for the individual samples (Been, 2011). Generally, spectroscopic techniques can assess both solid and liquid samples and require minimal amount of sample (just enough to cover the sampling window) because they are surface based techniques. Although the IR system was initially a transmission system, current modifications of the system allows the excitation of fundamental vibrations only few micrometres beyond the sample surface. Very small amounts of sample needed will in turn reduce exposure of the analyst to harmful chemicals or substances in the medicines investigated.

Raman and Near Infrared (NIR) spectroscopy have been extensively used for the analysis of pharmaceutical tablet (solid dosage) formulations though most investigations were qualitative, focusing on identifying the sources of the medicines (de Veij et al, 2007; Moffat et al, 2010; Mbinze et al, 2015; Dégardin et al, 2016). Raman spectroscopy involves heating the test tablet sample via intense laser radiation that could in turn destroy the sample or cover the Raman spectrum. Although no sample preparation is needed with Raman spectroscopy and samples can be analysed through packaging, there might be challenges obtaining good data/ Raman spectra from solid formulations which are poorly homogenised or medicines that fluoresce. Poor Raman spectra obtained could be because of the weak Raman effect (scattering the inelastic part of light by a sample irradiated by a laser). As such, Raman instrumentation has to be highly optimised in order to obtain valuable spectra (Deisingh, 2005). The analyst may also be exposed to strong laser radiation which is a health risk. NIR on the other hand produces spectra with broad peaks which are less specific and are rather indicative of vibrational combination and overtones of oxygen-hydrogen (-OH), nitrogen-hydrogen (-NH) and carbon-hydrogen (-CH), not particular functional group vibrations or rotations (Moffat et al, 2010; Dégardin et al, 2016). Fourier transform infrared (FTIR) has been investigated in the analysis of falsified medicines as it eliminates the need for solvent extraction of the APIs/excipients. However, skilled personnel are needed for sample preparation using transmission FTIR as this involves dispersing about 0.1- 2% w/w of the sample in a KBr matrix and pressing it into a disc before analysis. The strength of the IR spectra is dependent on the amount and homogeneity of the powder sample dispersed in the matrix. Therefore, ensuring the right amount of sample in the matrix is crucial in order to obtain good and well-defined FTIR spectra. Sample preparation involving dispersion in a matrix in turn lengthens time for analysis (Mallah et al, 2015).

The use of Attenuated Total Reflection- Fourier Transform Infrared (ATR-FTIR) spectroscopy can address the problems identified with Raman and NIR spectroscopy (Custers et al, 2011). The spectral peaks with ATR-FTIR spectroscopy are also more specific addressing the challenges of poor specificity with NIR spectroscopy. In addition, minimal sample preparation is needed, solid pharmaceutical formulations are only crushed before analysis. For a tablet, just enough powder to cover the tip of a micro spatula is needed for analysis. The ATR-FTIR spectrum is obtained within a few seconds. So far, this technique has been used primarily for qualitative analysis of medicines (Ortiz et al, 2013; Custers et al, 2015).

Apart from the spectroscopic techniques, mass spectrometry (MS) was investigated as a technique for the rapid authentication of medicines based on its high accuracy, selectivity and quantitative abilities. While the spectroscopic techniques are relatively low cost, rapid and are able to detect the presence of the expected API(s), they do not provide detailed compositional information pertaining to contaminants or wrong APIs. MS is able to detect molecules based on the ionisation of the analyte and sorts the ions according to their mass-to-charge ratios (*m/z*) thereby providing a valuable tool for the characterisation of individual components in medicines. The ability of MS to characterise individual components in medicines will facilitate the detection of falsified medicines that might contain other APIs not declared on the packaging or contaminants. Detailed MS data could also aid forensic investigations in linking/tracing falsified medicines to their source or countries of origin. On the other hand, MS is only capable of detecting analytes that are converted to gas phase ions. Liquid and gas chromatography coupled to mass spectrometry (LC-MS and GC-MS respectively) are the most commonly used MS techniques by regulatory agencies and laboratories in pharmaceutical companies in developed countries (Culzoni et al, 2014). However, LC-MS and GC-MS are expensive techniques and have limited sample throughput

resulting from extensive sample preparation requirements before analysis and actual duration of the chromatographic runs. The high cost and limited sample throughput using LC-MS and GC-MS implies that these techniques (LC-MS and GC-MS) will not be easily accessible in LMICs. More cost-effective complementary techniques with increased sample throughput that can provide reliable answers rapidly are highly desirable especially in LMICs.

Improving sensitivity has been the focus of recent efforts towards miniaturization of mass spectrometers to make them more portable (Kumano et al, 2015; Snyder et al, 2015). Optimisation of these portable mass spectrometry (MS) based techniques could be important in the efforts towards detecting falsified and substandard medicines. Kumano et al, (2015) recently developed a probe heating method for a portable mass spectrometer, for the analysis of solid samples. This method was used in the detection of illicit drugs but there was no mention of the use of the probe heating MS method as a technique for detection of falsified medicines. Probe heating MS method otherwise known as Direct Insertion Probe- Mass Spectrometry (DIP-MS) could occur by electron ionisation (conventional hard ionisation) or by ambient (soft) ionisation. Minute amount of sample is also required for DIP-MS (just a flick of TLC spotter dipped in sample). The conditions influencing fragmentation pattern of samples being analysed include the ionisation environment (atmospheric or vacuum) within which ionisation takes place as well as the energy (soft or hard) employed during ionisation of the samples.

MS methods such as Direct Analysis in Real Time (DART) and Desorption Electrospray Ionisation (DESI) have been identified as suitable for assessing medicines since minimal sample preparation is needed before analysis (Culzoni et al, 2014; Rebiere et al, 2017; Zou et al, 2017). DART and DESI-MS are ambient ionisation MS methods meaning ionisation occurs in open air and outside of the vacuum environment of the mass spectrometer and they generate ions softly (Monge et al, 2013). The high

throughput capabilities of DART and DESI-MS make them valuable methods for quick and reliable screening of large numbers of both solid and liquid pharmaceutical formulations (Culzoni et al, 2014; Bernier et al, 2016). Culzoni et al, (2014) identifies DART and DESI as solid-liquid extraction based MS techniques suggesting solid samples are dissolved in a solvent prior to analysis. Another, ambient ionisation technique used for analysis in recent times is the direct analysis probe also known as Atmospheric Solid Analysis Probe (ASAP) but as mentioned earlier (with DIP-MS) its potential in the screening of medicines has scarcely been explored. ASAP-MS require soft ionisation, allows fast analysis of both solid and liquid samples with quality data available within seconds and minimal sample preparation is needed prior to analysis as with DART and DESI. In addition to the advantages ASAP-MS shares with other ambient MS techniques like DART and DESI, it is also a low cost alternative to the other direct analysis MS techniques. Furthermore, ASAP-MS provides a replacement for the conventional electron ionisation (EI) vacuum solids probe which involves longer sampling time and requires hard ionisation. Therefore, ASAP-MS could potentially be a valuable option for the investigation of medicines.

1.2 Aim of Study

The purpose of this study was to research into the development of rapid instrumental methods addressing both the qualitative and the quantitative analysis of medicines since many counterfeit medicines in the international market also contain incorrect amounts of the API(s). It is therefore important to go beyond confirming the presence or absence of the APIs/ excipients to determine if the medicine contains the APIs in the right amounts. The following techniques were investigated: Attenuated Total Reflection Fourier Transform Infrared (ATR/FTIR) Spectroscopy, Raman Spectroscopy, Atmospheric Solid Analysis Probe-Mass Spectrometry (ASAP-MS), Direct Insertion Probe Mass Spectrometry (DIP MS) by electron ionisation (EI), UV Vis spectroscopy

and Liquid Chromatography- Mass Spectrometry (LC-MS). Direct Insertion Probe Mass Spectrometry by EI and ambient ionisation (ASAP-MS), UV Vis spectroscopy and LC-MS were used to validate the results based on the ATR/FTIR and Raman spectroscopy. In summary, this research proposes:

- Simple, rapid and robust instrumental methods for the authentication of solid pharmaceutical formulations/ medicines
- The use of data obtained based on developed methods to facilitate forensic investigations in tracing falsified medicines to their origin or source.

Optimised methods were employed in the analysis of analgesic/antipyretic and antimalarial tablets available to patients in the UK and worldwide. The results show the potential of the spectroscopic and DIP-MS techniques and it is hoped that this will help facilitate both qualitative and quantitative detection of falsified medicines (especially in the field) and in turn forensic investigations of falsified and substandard medicines even in LMICs.

1.3 Thesis Outline

This thesis focuses on the investigation of simple and rapid instrumental methods for qualitative and quantitative analysis of falsified medicines as mentioned in 1.2. The first chapter of this thesis gives an overview and general background of the research project including the aim of the study. Chapter 2 provides a theoretical background with current literature in the subject highlighting the extent of the problem and analytical techniques commonly used for investigating counterfeit medicines.

Methods based on different analytical techniques were assessed for their potential for the rapid detection of counterfeit pharmaceutical tablet formulations. The next three chapters focus on rapid qualitative and quantitative analysis of counterfeit medicines. Chapter 3 covers the development of novel screening methods for counterfeit

medicines based on ATR-FTIR. Chapters 4 and 5 describe methods for the analysis of tablet medication using Raman spectroscopy and DIP-MS respectively. DIP-MS by EI and ambient ionisation (ASAP-MS) were considered for their potential in the detection of counterfeit medicines with very low API concentrations which were difficult to detect or not well-resolved with the spectroscopic techniques (ATR/FTIR and Raman).

Since the optimised rapid methods based on the techniques in Chapters 3-5 were able to detect the presence or absence of the APIs, Chapters 6 and 7 outline conventional/pharmacopoeia approved solvent extraction methods for quantitative determination of tablet medications using UV-Vis spectroscopy and LC-MS respectively. These conventional methods were applied in the analysis of the tablet medication in order to assess the YES and NO of the data based on the rapid methods and to ascertain the potential of the rapid methods in the screening of falsified medicines. Chapter 8 compares the different techniques for assessing medicines investigated based on data obtained. Finally, the main conclusions and results of this research project including future directions of the project are presented in the last chapter (Chapter 9).

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CHAPTER TWO

Falsified and Substandard Medicines Overview

2.1 What are falsified and substandard medicines?

The difficulty in reaching an agreement on a clear-cut, globally accepted definition for falsified medicines (or counterfeit medicines as they were called) led to several consultations by the World Health Organisation (WHO) (Attaran et al, 2011; Newton et al, 2011; Dégardin et al, 2015). Initial amendments by the World Health Organisation (WHO) led to the use of the ambiguous term "substandard/ spurious/ falsely-labelled/ falsified/ counterfeit (SSFFC) medicines" (WHO, 2016). In defining SSFFC medicines, the WHO held on to the public health meaning and the life-threatening potential of counterfeit medicines. These amendments of the definition for counterfeit medicines helped clarify the difference between counterfeit and substandard medicines. Prior to the WHO (2016) definition of counterfeit medicines, Reggi (2007) suggested that all counterfeit medicines are substandard medicines but substandard medicines may not be termed counterfeits if there is no fraudulent intention. The WHO (2016) defines counterfeit and substandard medicines thus:

"A counterfeit medicine is one which is deliberately and fraudulently mislabelled with respect to identity and/or source. Counterfeiting can apply to both branded and generic products and counterfeit products may include products with the correct ingredients or with the wrong ingredients, without active ingredients, with insufficient (inadequate quantities of ingredient(s) or with fake packaging."

"Substandard medicines (also called out of specification (OOS) products) are genuine medicines produced by manufacturers authorized by the National Medicines Regulatory Authority (NMRA) which do not meet quality specifications set for them by National standards" (WHO, 2016).

There was also the argument that it is difficult to distinguish a counterfeit medicine from a substandard one since the only major difference between the counterfeit and

substandard medicine is the intent to deceive by the manufacturer (Chika et al, 2011). Either way, both counterfeit and substandard medicines are not what they say on the packet so would pose a threat to public health.

On the other hand, counterfeiting of medicines can legally be described as a violation of intellectual property (IP). The Agreement On Trade-Related Aspects Of Intellectual Property Rights (TRIPS), defines "counterfeit trademark goods" as;

 "*any goods, including packaging, bearing without authorization a trademark which is identical to the trademark validly registered in respect of such goods, or which cannot be distinguished in its essential aspects from such a trademark, and which thereby infringes the rights of the owner of the trademark in question under the law of the country of importation*" (WTO, 2016).

Intellectual property (IP) violation is punishable by law. This definition of counterfeit or falsified medicines based on IP violation enables the manufacturers of branded medicines to have a certain level of protection over their products. However, punishments may not be commensurate to the crime, since the public health threat posed by counterfeit and substandard medicines is neglected. Moreover, the intellectual property approach to defining counterfeit medicines does not take account of generic medicines (Shepherd, 2010; Attaran et al, 2011).

In a bid to highlight more clearly the public health threat posed by these SSFFC medicines, the WHO have recently adopted the term "substandard and falsified medical products" highlighting the public health threat posed by these medicines at the seventieth World Health Assembly (WHO, 2017). The adopted definitions are outlined as follows:

Substandard medical products or out of specification medical products "are authorised medical products that fail to meet either their quality standards or their specifications, or both".

Falsified medical products refer to "medical products that deliberately/fraudulently misrepresent their identity, composition or source"

Unregistered or unlicensed medical products are those "that have not undergone evaluation and/or approval by the national or regulatory authority for the market in which they are marketed/distributed or used, subject to permitted conditions under national or regional regulation and legislation" (WHO, 2017).

Although the latest definitions by the WHO help to clarify what a falsified medicine really is, there is still no consensus among countries on what constitutes a falsified or substandard medicine. Consequently, there is a disparity in the legislation addressing falsified and substandard between countries, making a general overview difficult. The absence of an international legal framework is indeed a major challenge in curbing the proliferation of falsified medicines (Attaran et al, 2011, WHO, 2017).

2.1.2 Types of falsified and substandard medicines

Falsified medicines differ in type and quality and this depends mostly on their destination or point of purchase. Copies of a pharmaceutical formulation often have the same API(s) as the genuine product, and in some cases API(s) are present in the same proportion making it more difficult to distinguish between the genuine and falsified copies of the pharmaceutical formulation (Seiter, 2009; Dégardin et al, 2014). Other falsified medicines contain wrong amounts of APIs, no APIs or even other APIs in some cases. Wrong APIs with chemical structures similar to the genuine ones could be used (Newton et al., 2008; Rebiere et al, 2017). Other falsified medicines contain a wrong API that gives the patient a false sense of relief by dealing with or addressing a

symptom rather than the ailment itself. A typical example is falsified antimalarial medicines that reduce the fever in patients with malaria but fail to cure the disease itself (Davison, 2011). In some other cases, genuine medicines that are expired or degraded are repacked and resold, making them difficult to identify (Mukhopadhyay, 2007; Davison, 2011). There are also those commonly referred to as "placebo counterfeits" made up of just excipients (inactive additives) which may not be harmful but do not deliver the desired therapeutic effect. Beyond the medicines themselves, falsifiers of medicines also often target the packaging and documentation (Vanderdonck, 2007; Dégardin et al, 2014). It can be argued that it is the medicine that the patient ingests that puts the individual at risk and not the packaging. In other words, fake packaging never killed anyone but taking the wrong medication might. However, fake packaging can provide a good opportunity for falsifiers of medicine to "dilute" a consignment of genuine medicines obtained illegally or stolen in order to double unit volume. For instance, blister-packed medicines can be taken out of their secondary packaging and the blisters (which have not been tampered with) are repacked into fake boxes while the genuine boxes are repacked with blisters of the falsified medicine. Considering this approach of repacking medicine boxes, the falsifier of medicines ensure that unit volume is increased and all consignments also contain both genuine and falsified medicines. Authentication of medicines where both genuine and falsified medicines are present in the same consignment is then a complex situation where there is a possibility of the falsified medicines being overlooked or not even noticed due to the presence of genuine medicines in the same lot. Medicines can be falsified in different ways depending on the individual(s) and the manufacturing steps involved. These manufacturing steps for falsified medicines could be carried out manually or in a pharmaceutical plant on an industrial scale. Therefore, falsification of medicines is not a uniform activity and results in products that are very variable and can take different forms. In 2012, the WHO considered reported cases of falsified and substandard

medicines and their magnitude and grouped them into different categories as shown in Table 2.1 below:

Table 2. 1 Types of falsified medicines and their relative levels based on reported cases

Products	Percentage (%)
Without active ingredients (APIs)	32
Wrong ingredients	22
Incorrect quantities of APIs	20
Falsified packaging	16
Impurities and contaminants	9
Copies of original product	1

(Source: WHO, 2012)

Although the data presented in Table 2.1 provides some information about the types of falsified medicines reported to the WHO at the time, the WHO acknowledges the data is not a clear indicator of the levels of these falsified medicines already in circulation in the legal pharmaceutical supply chain (WHO, 2017). Since falsification of medicines is usually carried out in hiding, available data about the types and levels of falsified medicines would be dependent on how efficient the medicines regulatory agencies are in not only detecting but also reporting cases of medicine falsification.

The oral route of administration of medicines is identified as the most preferred making oral dosage forms a prime target for falsifiers of medication (Mackey et al, 2015; Qui et al, 2016; Mc Gillicuddy et al, 2017). Mc Gillicuddy et al (2017), in their report of data for falsified medicines obtained from the Pharmaceutical Security Institute Counterfeit Incident System (PSI CIS) suggest that over 75% of the falsified medicines reported were oral dosage formulations with injectable biologic drugs identified as the second most reported (about 15%) route of administration for medicines. Therefore, falsifiers can target all medicines regardless of the route of administration / delivery.

Earlier reports indicated that life-saving or therapeutic medicines like those for malaria, tuberculosis, microbial infections and human immunodeficiency virus/acquired

immunodeficiency syndrome (HIV/AIDS) were the main targets for medicine falsifiers in developing countries (Zou et al, 2017). On the other hand, lifestyle medicines such as sexual enhancement supplements and medicines used for weight management and hair loss were the more likely targets for the industrialized countries (Almuzaini et al, 2013). With diversification in falsification of medicines, the falsifiers are now targeting other sought-after products such as medicines for cardiovascular diseases and even expensive cancer medicines (Dégardin et al, 2014; Antignac et al, 2017). Low cost medicines are also included in the falsified medicines market because the demand exceeds supply in most regions. In recent years, reports of falsified medicines received by the WHO Global Surveillance and Monitoring System (GSMS) suggest falsifiers of medicines now target almost all therapeutic fields and not just expensive or popular brand names (Figure 2.1) (WHO, 2017).

Percentage of all products reported to database

- Diabetes Medicines 1%
- Vaccines 2%
- Contraception and Fertility Treatments 2%
- HIV/Hepatitis Medicines 3%
- Mental Health Medicines 3%
- Cardiovascular Medicines 5%
- Cancer Medicines 7%
- Other therapeutic Classes 22%
- Malaria Medicines 20%
- Antibiotics 17%
- Anaesthetics and Painkillers 9%
- Lifestyle Products 9%
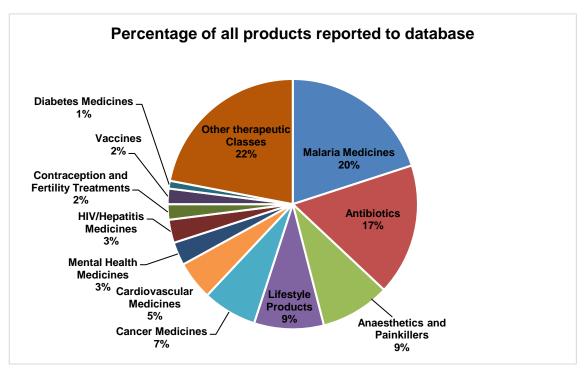
Figure 2. 1 Reports of falsified medicines by therapeutic class received by the WHO GSMS (2013–2017) (Adapted from WHO, 2017).

The data from the WHO GSMS in Figure 2.1 highlights antimalarial medicines and antibiotics as the most frequently reported falsified and substandard medicines globally

(Bassat et al, 2016; Krakowska et al, 2016; WHO, 2017). In addition, Figure 2.1 suggests high demand over-the-counter (OTC) medicines for pain relief have also been the target of counterfeiters (Newton et al, 2010; Davison, 2011; WHO, 2017). The 2016 Advisory Council on the Misuse of Drugs (ACMD) report on diversion and illicit supply of medicines identifies painkillers as one of the main group of medicines being supplied through illicit means in the UK (ACMD, 2016). One API commonly used as a painkiller and for reducing fever is paracetamol. Paracetamol, also known as acetaminophen (4-acetamidophenol, *N*-acetyl-*p*-phenacetin), (Bosch et al, 2006) has been identified as the second most widely used API after acetylsalicylic acid and the most commonly used medicine in children (Star and Choonara, 2014). Falsifiers have therefore targeted paracetamol containing OTC medicines (Davison, 2011, WHO, 2017). An example is a case of suspect paracetamol tablets in the Democratic Republic of Congo in 2014, reported to the WHO (WHO, 2017). Patients taking the paracetamol tablets were complaining of lethargy and were also found to have low blood pressure. Low blood pressure due to the paracetamol tablets resulted in slower heartbeat in the foetus of pregnant women, potentially hampering its growth. Preliminary testing performed locally by the national pharmacovigilance programme confirmed the presence of paracetamol in the tablets. However, further tests by WHO in a European laboratory showed that although the tablets contained the API paracetamol, it was present in wildly different doses ranging from the expected amount of 500mg to as low as 100mg. In addition, the tablets contained other ingredients not declared on the packaging such as the barbiturate phenobarbital a common treatment for epilepsy, which helps to slow down heart rate and breathing. Even more intriguing was the fact that the very inconsistent paracetamol tablet formulations all bore the same batch number – a clear indication that good manufacturing practices were not followed.

Recently, the Philippine President Rodrigo Duterte took a stand against falsified OTC medicines in a bid to curb the spread of falsified paracetamol by ordering the arrest of those responsible by the police (Reuters News, 2018).

Furthermore, paracetamol was detected in falsified medicines where the anticipated API was missing (de Veij et al, 2007; Khuluza et al, 2016). Paracetamol can be a very dangerous chemical if taken incorrectly and unexpected overdoses can occur as a result of miss-labelled or substandard (too much API) medicines, leading to toxic effects including renal failure and possible death (Star and Choonara, 2014; Buckley et al, 2016). A report of a situation in the USA, where 500mg paracetamol tablets were actually labelled as 325mg, suggest that people in both industrialised and Low and Middle Income Countries (LMICs) are exposed to miss-labelled (falsified) medication (Barry, 2015).

2.1.3 Extent of the falsified/substandard medicines problem worldwide

In a bid to tackle falsified and substandard medicines, some important questions need to be addressed such as: "how many are there and where can they be found?" These questions are disappointingly difficult to answer due to the clandestine nature of the falsified and substandard medicines market. Reiterating earlier comments made in 2.1.2, available information regarding FSMs, based on those discovered and reported, implies that a lot more might not be accounted for or documented. Data from the Pharmaceutical Security Institute (PSI) show that reported incidents of medicine falsification from 127 countries around the world increased from 2018 incidents in 2012 to 3147 in 2016 (PSI, 2018). These PSI data for reported incidents of medicine falsification highlighted in Fig 2.2 were obtained from seven regions of the world indicating that falsification of medicines is not just peculiar to certain regions of the world but is a global problem. Consequently, anyone anywhere in the world can be exposed to falsified medicines. The data from PSI in 2016 also indicates a 5% increase

in reports of falsified and substandard medicines from the previous year (2015) worldwide.
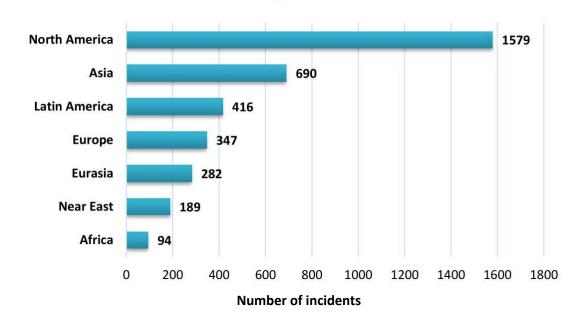
Figure 2. 2 Incidents of falsification of medicines in different regions of the world in 2016. (PSI, 2016).

The PSI suggests that the data obtained may indicate increased awareness on the part of both the public and law enforcement authorities in regions where higher incidents of medicine falsification were reported (PSI, 2018). In other words, reports of higher incidents of medicine falsification in a particular region does not necessarily translate to poor regulations, weak enforcement programs and a high prevalence of FSMs in that region. Rather, it might be an indicator of more efficient regulatory practices within those regions.

Considering reports that falsified medicines occur more frequently in low and middle-income regions, like Africa and Latin America, more reports of medicine falsification would be expected from these regions. This is not necessarily the case, as indicated in Fig 2.2 since the data suggests fewer reports coming from Africa. This might be due to several issues such as; lack of awareness of FSMs, inadequate records of FSMs, poor regulations or simply a case of lack of appropriate equipment to facilitate detection of

FSMs. Some other governments or regulatory agencies would rather not report cases of FSMs because there is the assumption that it gives their countries a bad reputation (WHO, 2017). Limited resources and lack of necessary facilities for the authentication of medicines in LMICs implies that fewer cases will be reported in these regions. Most reports will only be made after there is an adverse reaction to the falsified or substandard medicine. Generally, for falsified medicines and their prevalence, it is more a case of "the more you look, the more you will find." Therefore, the figures reported for incidents of medicine falsification might be very different and much lower than the real figures of falsified medicines in circulation (Mukhopadhyay, 2007; Roudaut, 2011). Therefore, it is important that issues regarding the falsified and substandard medicines market be understood and the facts noted because they further complicate region-to-region comparison for incidents of falsification of medicines (Dégardin et al, 2014).

Legend:
- Countries in which substandard and falsified medical products have been discovered and reported to the WHO GSMS, 2013–2017.
- Not applicable
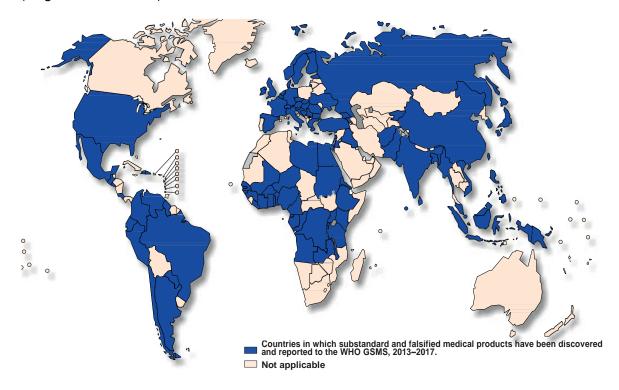
Figure 2. 3 Countries where reports of substandard and falsified medical products were received by the WHO GSMS (2013–2017) (Adapted from WHO, 2017).

Current estimations of the falsified medicines problem are nevertheless disturbing and it is clearly a worldwide problem (Sammons and Choonara, 2017; WHO, 2017). Incidents of medicine falsification reported to the WHO from around the world (Fig 2.3) further accentuates the global nature of this public heath challenge. It is also important to note that, as with the PSI data, unshaded countries in Fig 2.3 do not necessarily imply that no FSMs exist in those countries but rather that no reports of FSMs were made within the duration for which the WHO compiled the data. As mentioned earlier, the number of reports of FSMs is dependent on who is looking out for the products, whether they have the appropriate facilities to spot the FSMs, whether they understand how to report incidents of FSMs, and whether those reports are documented and forwarded to the appropriate regulatory body (WHO, 2017). Some reported cases of falsified and substandard medicines are outlined below:

- United States of America- In 2018 (Los Angeles), thousands of misbranded and falsified medicines including Viagra and Diprospan (an injectable anti-inflammatory medicine) were found to have been sold to the public via unlicensed vendors. Among these vendors, selling falsified medicines was a storefront supposedly selling candy and piñatas (Macias Jr, 2018).

- United Kingdom- In 2017, antimalarial medicines (Artemether-Lumefantrine) used to treat four patients who had visited Africa did not work suggesting resistance of the parasite to the medication or falsification of the medicines administered (Gallagher, 2017).

- Niger- In 2017, a batch of falsified meningitis vaccine was discovered before it reached the market. Packaging was different from original and the Brazilian manufacturer indicated on the pack does not manufacture the vaccine in question (Anderson, 2017).

- Malawi - In 2016, tablets containing a mixture of paracetamol and co-trimoxazole were repackaged and labelled as a brand of the antimalarial medicine sufadoxine/pyrimethamine for resale (Khuluza et al, 2016).

- Democratic Republic of Congo- In 2014, over 1000 people were admitted to clinics with symptoms of meningitis neck stiffness. Investigations revealed patients had taken tablets thought to be diazepam but actually contained haloperidol (an antipsychotic medicine) which can cause involuntary action in the arms, neck and face (Baggaley, 2017).

- Republic of Tanzania- In 2013, Halfan tablets expected to contain halofantrine hydrochloride was confirmed to contain no API (Höllein et al, 2016).

- United States of America- In 2012, falsified Avastin for cancer lacking active ingredient affected 19 medical practices in the USA (WHO, 2012).

- Pakistan- In 2012, over 200 patients died and about 1000 hospitalised after taking medicine used to prevent angina attacks (Isotab- containing isosorbide mononitrate 20mg) which also contained toxic amounts of the antimalarial API pyrimethamine (Arie, 2012; WHO, 2017).

The reported cases of FSMs above are a pointer to the global dimension of FSMs challenge and that medicine falsifiers can target any medicine. A proper understanding of the marketing and distribution of medicines worldwide will provide more information about the loop holes within the system that allows medicine falsifiers carry out their activities unnoticed. Section 2.1.4 provides more detail about the falsified and substandard medicines market globally.

2.1.4 Overview of the falsified and substandard medicines market
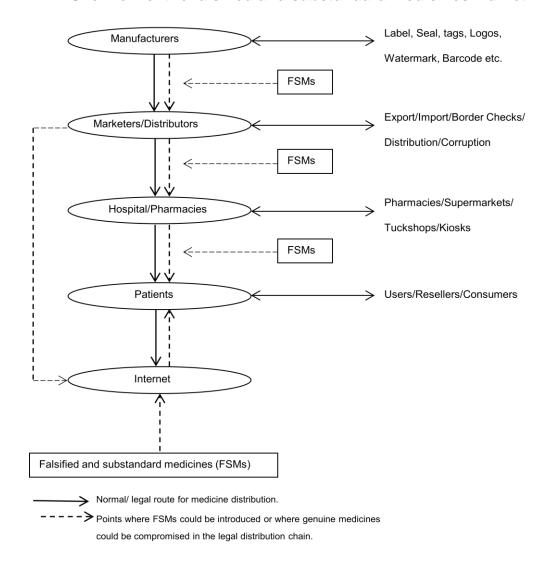
Figure 2. 4 Diagram showing how the falsified and substandard medicines (FSMs) market operates within the legal medicines distribution chain.

Falsification of medicines has evolved into an organised industry with some stakeholders (manufacturers, wholesalers, distributors and local retailers or vendors) across all levels of the medicine distribution chain involved as highlighted in Fig 2.4. At the manufacturing phase, security features on medicines like the label, seal and logo of the manufacturer can be copied for use on falsified versions of the same medicine to enable preliminary authentication of the product. Some corrupt marketers and distributors of medicines are also involved in this syndicate (medicine counterfeiters) where they interfere with checks for medicines whether across the border or by the

national regulatory agencies. These marketers and distributors of medicines interfere with the process by diverting medicines from their normal route. Diversion of medicines makes it easier to smuggle medicines into countries avoiding the necessary security checks. The hospitals, pharmacies or medicine vendors are normally the last point of contact in the medicines distribution chain since they are involved in administering the medicines required or prescribed to the patients. Again, Fig 2.4 indicates that falsifiers of medicines are still able to carry out their activities through unscrupulous individuals working in the hospitals, pharmacies or as medicine vendors especially in regions where regulations are lax.

Falsifiers of medicines strive to complicate the market so that the traffic of FSMs goes unnoticed (Roudaut, 2011). Townsend (2009), reports of falsified medicines (including anti-psychotic and anti-cancer medicines) that found their way into the NHS, UK which were discovered to be manufactured in China with labelling in French and shipped to Singapore from where the medicines got to Liverpool and were then sold to the NHS. The report by Townsend (2009) clearly highlights the complex nature of the falsified and substandard medicines market. This implies that falsification of medicines can occur at any point in the medicines distribution chain but must be such that the identity of the medicine falsifiers is not revealed. For instance, discarded packaging for genuine medicines can be retrieved and reused as packaging for falsified copies of the same product while expired or withdrawn stock can be relabelled as safe and within its shelf life (Davison, 2011). Reusing genuine packaging of medicines obtained will provide the counterfeiters with batch numbers and any other identification data that will enable them pass the FSMs as the genuine product during the initial screening of the medicines.

It is also difficult to establish distribution of FSMs as the information available mostly depends on where they are discovered which might be completely different from the

original point of manufacture. There is also the issue of underreporting by WHO member states (as mentioned earlier) and the lack of supplementary data for incidents of falsified and substandard medicines from other organisations.

With increasing awareness about the threat to public health posed by FSMs, it is expected that there will be more reports and more WHO member states will be involved in the fight against FSMs but currently, it is not certain if this is the case. Figure 2.5 gives a picture of the global distribution of FSMs across the medicine distribution chain based on reports available at the time.
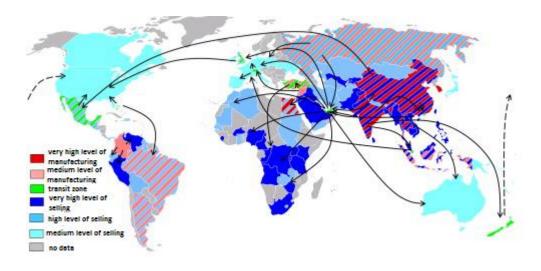
Figure 2. 5 Global distribution of medicine falsification- from production to point of sale (Adapted from Dégardin et al, 2014).

Manufacturing of FSMs seems to be concentrated in Asia and particularly in India and China as illustrated in Fig 2.5 (Chika et al, 2011; Dégardin et al, 2014). This can be attributed to the abundant and cheap workforce in the region. Some parts of Latin America are also involved in the manufacture of FSMs (Roudaut, 2011). The Middle East (United Arab Emirates) has been identified as a major transit point for the distribution of medicines and therefore FSMs as indicated in Fig 2.5. This allows for the manufacturers of falsified medicines to conceal their identity and the true origin of the product. It is not clear why the United Arab Emirates is highlighted as a major transit

point for FSMs but the free trade zones and trade regulations in the region that allow movement of such falsified medicines among other products might be the reason for using that route as a transit point by falsifiers of medicines. On the other hand, sale of falsified medicines is huge in Africa and South-East Asia and also high in Latin America (Almuzaini et al, 2013) (Figure 2.5). Most medicines in LMICs in regions like Africa and Latin America are imported thereby exposing these regions to the falsifiers of medicines. Developed countries like the UK where medicines are imported are also at risk.

With reference to Fig 4.2, specific issues regarding surveillance of medicines in the LMICs include lack of security across the medicines distribution chain and poor storage conditions. There are not enough facilities to carry out routine checks at borders and specialists with proper understanding of security features on medicines are also lacking. Furthermore, most medicines are to be stored below 25°C but the average temperature in most LMICs (in the tropics) is well above 25°C which could lead to degradation of the medicines. As a result, medicine storage will be a challenge these countries especially where facilities are not readily available.

In many LMICs, medicine falsifiers take advantage of the fact that medicines can be sold in street markets. In some cases, medicines sold in these street markets are not stored under appropriate conditions and medicines are taken out of the original packaging before selling to the patients. For instance, tablet medicines in blister packs can be taken out of the secondary packaging and sold in plastic zip lock bags so patients who are not able to buy the full pack of the medicine can have access to the smaller amounts of the medicine they can afford. Removal of medicines from packaging leaves the patient with little or no documentation and no information leaflet accompanying the medicine which in turn provides a conducive environment for the proliferation of FSMs. Falsifiers of medicines are able to take advantage of situations

like this to introduce falsified medicines into the system (Wall, 2016). Medicine falsifiers also target the mainstream distribution chain of medicines even in industrialised countries though the approach might be different, more subtle and sophisticated since the market is highly regulated in these countries.

Manufacturers of FSMs medicines might opt for sale of these medicines on the internet using websites that do not clearly provide the contact details of the seller (Orizio et al, 2009). The patient therefore stands a huge risk of buying falsified medicines on the internet. The anonymity of these websites make it more difficult to identify the perpetuators of the crime since it is difficult to track their exact location. A study considering the hidden parts of the internet suggests that about 12% of the illegal products sold online are pharmaceutical products (Megget, 2016). These websites provide unregulated access to prescription medications as well as supplements. Reports suggest around 50% of the pharmaceutical products available online could be falsified (Dégardin et al, 2014; Lavorgna, 2015; Lee et al, 2017). In the United States of America, the number of individuals buying medicines off the internet has grown by 400% in the last decade bringing with it an increased exposure to FSMs. The survey also suggests that in the USA alone, between 19 and 26 million people buy medicines online and this figure is on the rise (WHO, 2017). Buying medicines over the internet is becoming increasingly popular in LMICs too. An in-depth understanding of the different factors that allow falsifiers of medicines to perpetuate the crime is crucial in order to address the problem.

2.1.5 Falsified and substandard medicines: the causes

Falsification of medicines is a lucrative business and largely risk-free for the falsifiers of medicines since they operate within the legal medicine distribution network unlike with those involved in the distribution of drugs of abuse/ controlled substances. It is therefore more difficult to identify falsifiers of medicine than traffickers of illicit

medicines (Dégardin et al, 2014). Reports from the international police organisation (INTERPOL) suggests that organised criminal networks formerly involved in the illicit drugs market now target legal medicines because of the high profits, low risks of detection and prosecution and the fact that the penalties when prosecuted for falsifying medicines are much less severe than those incurred for trafficking illicit drugs (Dégardin et al, 2014).

There are a number of reasons for the boom of the falsified medicine market; foremost among which is the deficiencies in legislation and enforcement. Deficiencies in legislation and enforcement would range from poor ethical practices to greed and corruption at all levels of the medicine distribution chain. Countries where regulation and enforcement is limited and weak are therefore possible breeding grounds for medicine falsifiers (Dégardin et al, 2014). Furthermore, the absence of an international legal framework has not helped in addressing the FSMs problem (Martino et al, 2010; Rebiere et al, 2017). Corruption is widespread in many LMICs with medicines being frequently diverted and pilfered at different levels of the medicines distribution chain. The approval of questionable medicines is secured with bribes at profitable prices (Berman and Swani, 2010; Mori et al, 2018). Although corruption is rampant in LMICs, it is important to note that it also exists in developed countries. There have been calls for doctors in the UK to declare their earnings with reports of a significant increase in the cash and hospitality given to doctors by pharmaceutical companies and the refusal by a third of the doctors to declare their earnings making it suspicious (Bodkin, 2017). Other reports in the USA suggest opioid manufacturing pharmaceutical companies offered more money to doctors who wrote the most opioid prescriptions; almost implying that the doctors were being bribed to help the sale of their medicines (Kessler et al, 2018). The aforementioned cases in the UK and USA might not necessarily

confirm clear cases of corruption in the countries but are pointers to the fact that these developed nations are not immune to corruption.

Limited access to affordable, safe and effective medicines and poor technical capacity/tools for ensuring good manufacturing practices, quality control and distribution of medicines are some other factors that would allow FSM manufacturers to thrive in a region. Some medicines are prime targets for falsifiers because of the value attached to the products and their corresponding high prices. The selected targets are either high added-value medicines such as cancer and antiretroviral medicines aimed at narrow markets (smaller/specific population) or cheaper medicines such as pain killers and antimalarial medicines aimed at broader markets (wider population) (Reggi, 2007; Degardin et al, 2014). In other words, high cost or high demand medicines are the most targeted by falsifiers of medicines. This makes them even more profitable for the falsifiers of those medicines whose production costs is nothing compared to that of the legal manufacturers, with nothing spent on research and development or licensing (Reggi, 2007; WHO, 2017). Even if the falsifiers of medicine use the right ingredients, their quality standards will be much lower. For most medicines, the APIs account for bulk of the production costs. For generic medicines, APIs generally make up four-fifth of the production cost. Use of lower amounts of the API(s) by medicine falsifiers will therefore help to maximise their profit while using just enough API for the medicines to pass initial screening tests for the presence of the expected API (Reynolds and Mckee, 2010; WHO, 2017).

The high costs of some prescription medicines means they might not be affordable for LMICs unless they are able to access donated medicines. Patients (with no insurance or in countries where cost of medication is borne by the people) are forced to seek out cheaper alternatives because of the high costs of genuine medicines, which are often burdened by import duty. This in turn encourages street-market (in LMICs) or internet

distribution and the spread of non-regulated outlet production of falsified medicines (Wertheimer and Norris, 2009). Cost pressures also have an implication on the production and supply chain for medicines. In a bid to cut costs and maximise profit, some manufacturers of medicines go for cheaper alternatives to the actual APIs and excipients used in the genuine medicine. As a result, the quality of medicines reaching the patient might be compromised.

Availability or access to medicines is becoming more of a problem in the developed countries and patients can be affected by the high cost of medicines especially if they have to pay for them. Moon (2017), reports that the outrage over the high cost of medicines was identified as one of the very few issues bringing voters together during the 2016 elections in the United States of America (USA). The trade-off of affordability versus quality of medicines by the patient is largely based on necessity and not on choice because people would generally purchase the best medicines they can afford. Some FSMs are also sold for the same price as the genuine medicine from the legal manufacturer to avoid suspicions as to their origin (Shepherd, 2010). Consequently, the cost of medicines does not guarantee authenticity because both cheap and expensive copies of genuine medicines abound in the market.

When the demand for medicines exceeds the supply, it creates an opportunity for medicine falsifiers to provide products to address the deficit. There are several situations where demand could exceed supply such as when there is outbreak of disease, war/conflict, economic crisis and natural disasters. Access to medicines is limited as demand increases in these situations providing a conducive environment for falsifiers of medicines to thrive. An example that readily comes to mind is recent plans by the UK government to stockpile medicines just in case the UK leaves the EU with no deal (Buchan, 2018). Stockpiling of medicines would mean increase in demand for

such medicines, which the falsifiers of medicine could readily take advantage of to infiltrate the market.

Rapid communication systems and technological advancement have also boosted links between medicine falsifiers at different points in the medicine distribution chain, especially across international borders (Reynolds and Mckee, 2010). For instance, medicine falsifiers in one country can now provide real time updates to others in different countries about the movement of the falsified pharmaceutical products and falsified medicines are also accessible to people in parts of the world where the facilities are not available to produce them (LMICs). Globalisation of the financial markets has aided illegal trade where international transactions can be made in real time between counterfeiters of medicines and is now widespread in some countries (Reynolds and Mckee, 2010). Industrialised countries have more control measures for goods produced within their country than for goods to be exported to other countries. Falsified medicines are often exported via free trade zones, with their origin concealed or changed with fresh labels (Degardin et al, 2014). The issue is more political in some countries where it is perceived there is lack of willingness to tackle the problems (Roudaut, 2011). In some parts of Europe, medicines can also be sold at low prices and then resold elsewhere at a higher price. This phenomenon, identified as "pharmaceutical parallel trade," leaves intermediaries/middlemen with great profit (Orizio et al, 2009). The United Kingdom is the largest import market in Europe with 70% of the parallel trade. Falsifiers of medicine across Europe and around the world, seeking to make more profit can also take advantage of this market by again repacking medicines to be resold to countries like the UK where cost of medicine obtained via the NHS are higher (Degardin et al, 2014).

Just as parallel trade is considered Europe's problem, America's main problem is reimportation. This means that medicines that were previously exported to other

countries are brought back to America. This practice allows for the importation of medicines that the regulatory authorities can hardly control (Palumbo et al, 2007). Producers normally sell the medicines to wholesalers who in turn send them directly to hospitals or pharmacies. However, between the producer and the patient, so many intermediaries could manipulate medicines (Vanderdonck, 2007). Although the legal medicine trade cannot be blamed for the issue of falsification of medicines, it is important to note that its globalisation and the complications surrounding the distribution of medicines worldwide aid the activities of medicine falsifiers (Shepherd, 2010).

2.1.6 Falsified and substandard medicines: the consequences

The proliferation of falsified and substandard medicines could have far-reaching effects not only on the patient taking the medicine but also on the licensed manufacturers and the government. Non-compliance with good manufacturing practices allows falsifiers of medicine to cut production costs to maximise profit while manufacturing medicines that could potentially cause harm to patients (Vanderdonck, 2007; Seiter, 2009). Since falsifiers of medicine operate in an unregulated environment, manufacturing FSMs might not require much workspace and can often be carried out in small workshops usually by unskilled workers. Falsifiers of medicine could also use bigger manufacturing plants for large-scale production (Dégardin et al, 2014).

2.1.6.1 Wrong level of APIs

If the FSM contains more than the stated amount of the expected API, too high doses will usually be toxic or harmful with adverse reactions that could result in the death of the patient. When the API is absent or present in amounts lower than expected, the medicine does not offer any therapeutic benefit. This could lead to the deterioration of the patient's condition or could ultimately lead to death in certain disease conditions. Again, when lower than expected amounts of API are present in FSMs, this could lead

to drug resistance, which will weaken the patient's response to future medication (Newton et al, 2010; Kelesidis and Falagas, 2015). A typical example is the case in the UK, mentioned in 2.1.3 where patients being treated for malaria did not respond to the antimalarial medicine (Artemether-Lumefantrine) administered (Gallagher, 2017). The reason for the patients' non-response to the antimalarial medicine is not yet known but could be as a result of absence of the API or a case of resistance to the medicine. The adverse effects of FSMs can be fatal if other APIs not declared on the packaging are present (Vanderdonck, 2007).

2.1.6.2 Lack of patient information

Some FSMs are sold without patient-information leaflets making it easy for patients to self-medicate or abuse the medicines. Self-medication with FSMs could be very dangerous especially when the falsified medicines within a pack contain varying amounts of the expected API. No therapeutic benefit observed after taking medicine with lower than expected API might lead to increasing the dosage. However, increasing dosage with medicine from the same pack, which contains higher amounts of API than the one previously administered, might result in toxicity due to higher than expected levels of API in the patient (Degardin et al, 2014).

No one will want to continue taking a particular medicine if it does not work or has adverse effects. This could lead to fear and lack of trust in the medical system which goes beyond fear of the medicine itself to lack of trust in those involved in the prescription of such medications like the doctors, pharmacists and nurses (Martino et al, 2010; Davison, 2011; Degardin et al, 2014).

2.1.6.3 Economic consequences

Although the health repercussions on the consumer are the most important, the economic impact of falsification of medicines must not be ignored. The European Union

Intellectual Property Office (EUIPO) reports that FSMs cost the European Union pharmaceutical sector about 10.2 billion every year with about 37,700 jobs lost (EUIPO, 2016). Previous reports from the Centre for Medicines in the Public Interest estimated the worldwide trade of FSMs to be worth around $75 billion by 2010, exceeding the illicit drug market, valued to be $50 billion at the time (Vanderdonck, 2007). Falsification of medicines engenders costs for society as a whole, especially in the developing world where the resources are not readily available (Seiter, 2009; Mori et al, 2018).

Economic losses due to FSMs affect not only the pharmaceutical industry and the government but also the patients who suffer losses in income if cost of medicine is borne by them. The patient taking these FSMs also loses the money spent in purchase costs since the medicines do not work and further help or medical advice is required. Falsifiers of medicines tarnish the image of the licensed pharmaceutical industries, and so, there is the constant burden of protecting their products against the falsifiers (Jack, 2007; Berman and Swani, 2010). This burden is in turn transferred to the patients buying these medicines as prices are hiked so as to account for the extra costs involved in the production and protection of the product. Generally, healthcare systems are also affected since there is an increase in social and healthcare costs even in countries with health insurance cover. Recent reports by the UK National Health Service (NHS) suggest that prescribing common painkillers like paracetamol costs about four times the cost of the same medicines over the counter (NHS News, 2017). This has led to on-going deliberations about removing such medicines that can be purchased over-the-counter, at a lower cost, off the NHS prescription list. This would save money and free up more funds for frontline care in the NHS. This will in turn transfer the cost of the medicines to the patients and could expose them to falsified medicines especially over the internet where there are fewer restrictions. With the

steady rise in the number of medicine counterfeiting cases, specialists have even warned of a macroeconomic pandemic (downturn in the economies of many nations) if effective measures are not taken (Dégardin et al, 2014). According to Wertheimer and Norris (2009) and Van Baelen et al (2017), the health and economic challenges due to falsification of medicines would be so disastrous that the resources in the health sector would be completely overwhelmed if nothing were done to curb the situation.

2.1.7 Tackling the challenges posed by falsified and substandard medicines

The WHO Programme for International Drug Monitoring (WHO PIDM) refers to "*the science and activities relating to the detection, assessment, understanding and prevention of adverse effects or any other drug-related problem*" as Pharmacovigilance- PV (WHO PIDM, 2016). Considering the public health risk associated with falsified medicines, it is important that response to this phenomenon be rapid and globalised in order to get them out of the market and the global supply chain within the shortest possible time (Dégardin et al, 2014). To achieve this, the National agencies involved in the regulation and control of medicines need to work towards developing easier methods for detection of FSMs especially on the field- hence the need for this research. Various information dissemination systems should create awareness about FSMs among all stakeholders (from manufacturers to patients) involved in the pharmaceutical market. Television, radio, newspaper adverts and even social media platforms could provide accurate information on the risks of FSMs and even more information on how to detect them, how to avoid them and how to report suspect cases of medicine falsification. Consumers should learn what features to look out for when purchasing medicines and the dangers of purchasing medicines from informal markets. While education and awareness is crucial in the fight against falsified medicines, the amount of information available to the public should be just enough for

basic first-line screening or visual inspection of medicines in order not to provide falsifiers of medicines with enough information to circumvent the system.

Davison (2011) and Dégardin et al (2014), propose multi-layered protection as an effective approach towards safeguarding medicines since they will be more expensive and hence, difficult for counterfeiters to replicate. This will involve simple and easily detectable features, such as the shape, embossing (raised features), or debossing (sunken/lowered features) on the medicine or labels, logos, barcodes and seals on its packaging.

The more overt security features on the medicine and the packaging can be supplemented with other covert features known to a select few involved in the regulatory process (Fig. 2.6). Figure 2.6 illustrates the "authentication pyramid" with the vertical dimension indicating the number of individuals participating at various security levels in the authentication of medicines. From the base of the pyramid to the apex, a gradation of simple to more complicated authentication techniques/methods are employed. Consequently, the number of individuals involved in the authentication process decreases going up the pyramid from the base where the general public are involved in simple visual inspection tests to the apex where one or two highly skilled individuals are involved in more complex authentication forensic investigation.

It is also evident from the authentication pyramid that pharmacovigilance is improved when consumers and those closest to them in the medicines distribution chain (such as pharmacists) are provided with simple tools or information for spotting falsified and substandard medicines.
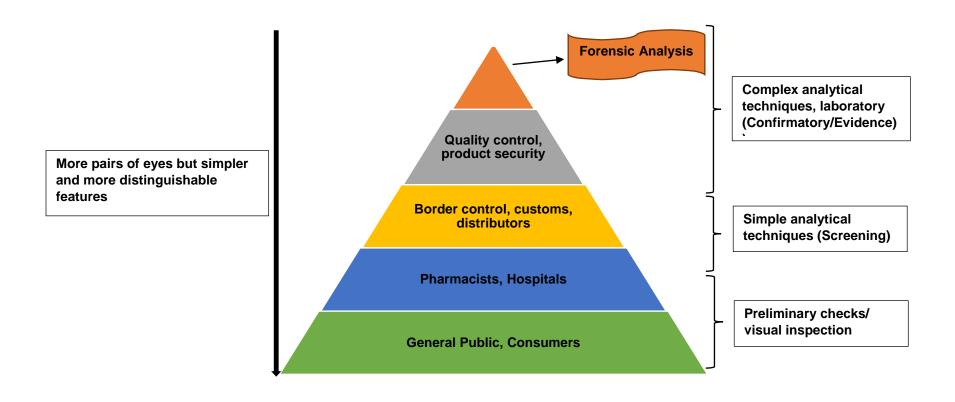
Figure 2. 6 The Authentication pyramid. More overt security features towards the base of the pyramid allows more individuals to be involved in the validation of medicines (Adapted from Davison, 2011).

Consumers will therefore be more alert with more information about the medicine being administered to them. On the other hand, there is the conflicting factor that wider dissemination of security features on products will also make them more vulnerable to falsifiers as highlighted earlier. Therefore, a combination of techniques and security features is necessary to provide individuals at all levels of the pyramid with various ways of medicine authentication.

Collaboration between government agencies is necessary for effective results since the distribution channels for these medicines are very complicated. International cooperation between National regulatory agencies will also help in effective border control and monitoring which will in turn help in curbing the menace caused by falsified and substandard medicines. To this effect, the WHO launched the Global Surveillance and Monitoring System (WHO GSMS) in 2013 with the number of participating countries currently put at 113 (WHO, 2016). The WHO GSMS was supposed to help in linking information on incidents of medicine counterfeiting between countries as well as provide more data to enable a better understanding of the extent of the problem worldwide. Collaboration between countries could also facilitate prompt arrest and prosecution of the counterfeiters of medicine. An example of collaboration between countries or regional collaboration is the European Union (EU) medicines verification system (EMVS) expected to take effect by February 2019, which will facilitate verification of medicines throughout the medicines distribution chain in the region until the time the medicine is dispensed to the patient (EU, 2016).

As is the case with drugs of abuse, legislation with stiff penalties for counterfeiters of medicines should be enforced by the National regulatory agencies to serve as a deterrent to others. Considering the number of individuals exposed to falsified and substandard medicines, it can be argued that counterfeiters of medicines should face even stiffer penalties than those involved in drugs of abuse because the drugs of abuse

market is smaller with fewer individuals exposed to these addictive substances. The FSMs market is much bigger because it affects both therapeutic and lifestyle medicines with anyone around the world being susceptible to these medicines. In addition, most individuals at the receiving end (consumers) in the drugs of abuse/ legal highs market are in it as a matter of choice even though the substances are addictive. Consumers of FSMs on the other hand do not have a choice because it is the intent of the counterfeiter to deceive the consumers of such medicines being well aware that no one will take medicine knowing it is not what it says on the box.

Efforts towards reducing the cost of production and by extension the burden transferred to the consumer in terms of the cost of the medicines should be further explored. Import duty on medicines could be subsidised by individual countries. This will help in reducing the costs of medicines and making them more affordable.

Finally, further development of suitable analytical methods for the detection of counterfeit medicines within the quickest time possible and closest to the final consumers of the medicines (where most counterfeiting would most likely occur) will go a long way in addressing this problem. This is because public safety with reference to FSMs is dependent on how fast the suspect medicines are detected and taken off the market. It is in this regard that this research considers more effective, efficient and robust methods for the authentication of FSMs both in-field and in laboratories and also in different regions of the globe. Referring back to the authentication pyramid (Fig 2.6), we established that at different levels of the medicine distribution chain, the analytical method required for screening of medicines are different. The analytical method of choice for screening medicines will be dependent on a number of factors such as the amount and type of information needed ranging from a quick "YES" or "NO" check to comprehensive forensic analysis. Analytical methods for screening FSMs will be the focus in the next section.

2.2 Analytical methods for screening falsified and substandard medicines

The fight against the falsification of medicines has led to the investigation of several analytical methods for the detection of FSMs, which include both spectrometric and separation techniques as discussed in detail in successive subsections (Deisingh, 2005; Martino et al, 2010; Anzanello et al, 2014; Dégardin et al, 2014). As falsifiers of medicines get more sophisticated in the act, the pharmaceutical industries and agencies have to keep improving their analytical methods with more reliable and up-to-date techniques in order to detect their activities (Deconinck et al, 2013; Dégardin et al, 2014).

Recently, research by Armitage (2018), applied a collection of techniques in the assessment of cardiovascular tablet medicines. Rebiere et al (2017), identifies sampling of suspect medicines as a vital step because it should give information of a representative portion of the test sample analysed. Information obtained from physical and chemical study of medicines could provide a better understanding of medicine counterfeiting at different levels. Marini (2010) and Dégardin et al (2014) suggest that to initiate prompt action of pharmaceutical companies and other relevant regulatory authorities involved in curbing medicine falsification, analytical methods for authentication of medicines must first be able to identify or distinguish genuine and counterfeit pharmaceutical products. The analytical method for authentication must also be able to do so as fast as possible as mentioned earlier in 2.1.7.

2.2.1 Preliminary Screening for falsified and substandard medicines

The screening process for falsified and substandard medicines begins with visual inspection of the medicines. A detailed study of the label, package and content of the suspected medicine yields data that are then compared with that of the genuine product. Visual inspection of packaging items, like boxes, leaflet inserts, blister packs,

and vials, entails the study of features like holograms, logos, taggants and printing (Deisingh, 2005; Rodomonte et al, 2010; Shah et al, 2010; Ortiz et al, 2012). Electronic tracking systems using Radio Frequency Identification (RFID) or the Global Positioning System (GPS) can be used to track genuine medicines and isolate counterfeit medicines (Catarinucci et al, 2012). The RFID is based on a two-way radio communication system between an RFID tag on an item being tracked and a receiver within range of the tracked item. They can be employed for spot checks by the police or border control agencies but the short range of the signal implies they can only be used for screening medicines in-field and not for remote tracking (Davison, 2011). On the other hand, the GPS tracking system is similar to the satellite navigation systems used in cars and phones where satellite signals received by small devices hidden in the cargo, truck or the medicine boxes are transmitted to a control centre via cellular phone networks. The information received by the GPS system is used to ascertain the position or location of the item being tracked in real time. Real-time monitoring of the movement of medicines will help provide alerts to regulatory agencies when there is a deviation or unplanned stop while medicines are transported along the expected route of the medicines distribution chain. Although GPS can function remotely, it is also dependent on the strength of the satellite signal, which can be interrupted if the signal is impeded along its path. In criminal cases, the system could be jammed so the control centre is not able to receive positional information. Tracking systems are more suited for monitoring bulk items in transit due to the high cost of production of tracking devices. It is therefore important to consider screening methods for small quantities of medicines, within the medicines distribution chain from manufacturer to consumer (Davison, 2011).

Physical characteristics of the medicine dosage form, like their colour, weight or shape, can be evaluated and compared with reference samples of the medicine though this is

not always possible. Nevertheless, physical inspection is not sufficient to identify counterfeit and authentic medicines due to increased sophistication by the counterfeiters who use technologically advanced equipment to produce almost identical copies of genuine medicines (Rodomonte et al, 2010; WHO, 2017). Simple tests, consisting for instance in disintegration tests (Martino et al, 2010; Kaale et al, 2016) and simple density or viscosity measurements (Dégardin et al, 2014) are cheap and quick methods that sometimes prove very effective for the initial screening of medicines. Furthermore, with advancement in technology and mobile connectivity, unique mobile phone apps are now available for the authentication of medicines by scanning the medicines or packaging for particular security features, in order to distinguish between genuine and falsified medicines (Steinhubl et al, 2015; Yu et al, 2016).

Apart from the various visual inspection methods for screening FSMs, other simple analytical techniques have been employed. Table 2.2 highlights techniques like colorimetry (Green et al, 2000; Rodomonte et al, 2010; Chikowe et al, 2015), and in recent times, the Global Pharma Health Fund (GPHF) Minilab™ (Petersen et al, 2017). The GPHF Minilab™ combines thin layer chromatography (TLC) and colorimetric tests which has been proven a useful low cost semi quantitative analytical method for an in-field detection of counterfeit medicines. Due to the ease of use and the inexpensive test kits, the GPHF has been the technique of choice for screening falsified and substandard medicines in LMICs (Kovacs et al, 2014; Petersen et al, 2017). However, Kovacs et al (2014), suggests that the GPHF-Minilab is only able to identify grossly substandard medicines containing less than 80% of the expected API. Several studies have considered more economical, rapid and efficient methods of screening counterfeit medicines for use most especially in LMICs and also at the point of care (Hoellein and Holzgrabe, 2014; Koesdjojo et al, 2014; Yemoa et al, 2017). The paper device or paper

chromatography cards for detecting antimalarial medicines (Koesdjojo et al, 2014), the counterfeit detection device (CD-3) by the FDA (Ranieri et al, 2014; Platek et al, 2016) and PharmaCheck (Amifar, 2016) are some of the portable devices developed recently for on-site monitoring of FSMs. However, even though these simple tests based on the physical or chemical properties of the medicine enable quick preliminary assessment of medicines and are inexpensive (with prices ranging from about £1 per test with paper test cards to £4000 per unit of the GPHF- Minilab), they are mostly qualitative (Kovacs et al, 2014). In addition, they often suffer from a lack of specificity and do not provide much information about either the identity or the amount of the API in the medicines (Martino et al, 2010; Deconinck et al, 2013; Venhuis et al, 2014). Simple analytical methods that are more robust providing both qualitative and quantitative forensic information would therefore be valuable for efficient first line screening of medicines.

Table 2. 2 Comparison of devices for detecting falsified and substandard medicines

Technique	Use/Purpose	Performance	Needs sample preparation	Speed	Training required	Cost*	Facility requirements	Reference(s)
Colorimetry	Preliminary classification/assessment	High specificity (0.94-1.00)	Yes	Fast	Lab. technician	Low	Portable	Green et al, 2000; Chikowe et al, 2015
Paper chromatography cards	Preliminary classification/assessment	High sensitivity (0.92-1) and specificity (0.88-1)	Yes	Fast	Lab technician	Low	Portable	Koesdjojo et al, 2014
Counterfeit Device #3	Chemical profiling with visual inspection	Unknown	No	Fast	Minimal training	Low	Portable	Batson et al, 2016; Platek et al, 2016
PharmaCheck	Chemical separation, identification and quantification	High specificity with dissolution component detected within 3%	Yes	Fast	Lab technician	Low	Portable	Amirfar, 2016
TLC-GPHF-MiniLab	Chemical separation, identification and quantification	Low as it is only able to detect wide variation/disparities in the expected amounts of API I the medicine	Yes	Fast	Lab technician	Low	Portable	Risha et al, 2008; Kaale et al, 2011; Petersen et al, 2017
Near Infrared Spectroscopy	Chemical profiling	Medium	No	Fast	Lab technician	Medium	Portable	De Peinder, 2008; Dégardin, 2016; Rodionova et al, 2018
Raman Spectroscopy	Chemical profiling	Medium	No	Fast	Lab technician	Medium	Portable	De Peinder, 2008, Hajjou et al, 2013; Rebiere et al, 2017
Fourier Transform Infrared (FTIR) spectroscopy	Chemical profiling	Moderate as it analyses only the surface of the sample	No but samples is dispersed in a matrix	Fast	Lab technician	Medium	Portable	Farouk et al, 2011
Attenuated Total Reflection-Fourier Transform Infrared (ATR-FTIR) spectroscopy	Chemical profiling with quantification of APIs	Medium and simpler than FTIR	No	Fast	Lab technician	Medium	Portable	Custers et al, 2015; Lawson et al, 2018

Table 2.2 (continued)

Technique	Use/Purpose	Performance	Needs sample preparation	Speed	Training required	Cost*	Facility requirements	Reference(s)
Direct Analysis in Real Time (DART)	Identification and quantification of APIs	Moderate but not as sensitive as LC-MS or GC-MS	No	Fast	Highly trained chemist	High	Research laboratory	Chernetsova and Morlock, 2011; Bernier et al, 2016
Atmospheric Pressure Solids Analysis Probe (ASAP)	Identification and quantification of APIs	Moderate and similar to DART	No	Fast	Chemist	Medium	Research laboratory	Twohig et al, 2010
Direct Insertion Probe (DIP)	Identification and quantification of APIs	High sensitivity and specificity. Similar to ASAP	No	Moderate	Chemist	Medium	Research laboratory	Kumano et al, 2015
NMR spectroscopy	Chemical profiling	High sensitivity and specificity	Yes	Slow	Chemist	High	Research laboratory	Holzgrabe and Malet-Martino, 2011
UV-Vis Spectroscopy	Identification and quantification od APIs	High sensitivity and specificity.	Yes	Slow	Lab technician	Medium	Research laboratory	Behera et al, 2012
Gas Chromatography-Flame Ionisation Detector (FID)	Chemical separation, identification and quantification	High though performs lower than GC-MS	No	Slow	Highly trained lab technician	Medium	Research laboratory	Deisingh, 2005
Headspace (sampler) gas chromatography	Chemical separation, identification and quantification	High sensitivity and specificity	No	Slow	Highly trained lab technician	Medium	Research laboratory	Custers et al, 2014
Gas Chromatography – Mass Spectrometry	Identification and quantification of APIs	Gold standard	Yes	Slow	Chemist	High	Research laboratory	Deisingh, 2005; Zou et al, 2017
High performance liquid chromatography (HPLC)	Chemical separation, identification and quantification	Gold standard	Yes	Slow	Highly trained lab technician	Medium	Research laboratory	Hoellein, 2014
Liquid Chromatography-Mass Spectrometry	Identification and quantification of APIs	Gold standard	Yes	Slow	Highly trained chemist	High	Research laboratory	Fiori and Andrisano, 2014

* Low cost= <£10,000; Medium cost= £10,000- £100,000; High cost= > £100,000 (Kovacs et al., 2014; Rebiere et al, 2017; Zou et al, 2017).

2.2.2 Authentication Techniques for Falsified and Substandard Medicines

The portable technologies mentioned in 2.2.1 like the GPHF- Minilab, PharmaCheck and paper chromatography test cards all require sample preparation before analysis thereby increasing time of analysis. It is therefore important that techniques that combine both speed of analysis with sensitivity and specificity be considered for the authentication of medicines. Many analytical tools have proved effective for the identification of counterfeit medicines (Talati, 2011; Zou et al, 2017). Appreciating the fact that time is of essence in the detection of counterfeit medicines, there has been increased use of vibrational spectroscopy for the analysis of suspect materials in recent times (Custers et al, 2016; Höllein et al, 2016; Rebiere et al, 2017; Zou et al, 2017).

Spectroscopic techniques are often preferred for the assessment of falsified and substandard medicines because they are fast and require little or no sample preparation (Sacré et al, 2010; Kovacs et al, 2014). Although these spectroscopic techniques are more expensive than the preliminary screening techniques mentioned in 2.2.1 (circa £50,000), they are faster, more robust, sensitive and portable and so can be used in the field for analysis. Mid-Infrared (MIR) more commonly known as Fourier-Transform Infrared (FTIR) Spectroscopy (Farouk et al, 2011), Near Infrared (NIR) (Moffat et al, 2010; Assi et al, 2011) and Raman spectroscopy (Dégardin et al, 2011) are all individually capable of producing measurements and an interpretation of the results within minutes. Direct observation of overlaid spectra or mathematical models (chemometrics) can be used for the identification of suspect counterfeit medicines. A limitation with these spectroscopic techniques is the dependence on the use of reference libraries for the individual APIs and excipients. This implies that the reference libraries have to be routinely updated when there are new compounds in the market and this could limit their feasibility (Kovacs et al, 2014).

ATR-FTIR involves direct contact of the sample with the ATR crystal meaning samples must be taken out of their packages for analysis. Raman spectroscopy is a scattering technique whereas FTIR spectroscopy and NIR spectroscopy are both absorption techniques (Martino et al, 2010; Zou et al, 2017). Also NIR imaging data (Puchert et al, 2010) and Raman microscopy (Kwok and Taylor, 2012) provide direct and simple information about the genuineness of the medicine. Field-portable, hand-held instruments, which are mostly Raman and NIR spectrometers, can be used for screening of the authenticity of the content of the dosage form (Ricci et al, 2008; Hajjou et al, 2013). The sensitivity of these hand-held devices has been questioned (Hajjou et al, 2013).

These simple techniques as shown in Table 2.2 help ascertain the quality of the pharmaceutical product, with respect to both packaging and dosage form (Martino et al, 2010; Kovacs et al, 2014). In other words, they provide initial "YES" or "NO" checks on medicines. The case is closed if the medicine in question is confirmed to be genuine otherwise further chemical analyses have to be performed.

2.2.3 Confirmatory Techniques for Falsified and Substandard Medicines

In the event that data for medicines based on the assessment techniques identified in 2.2.2 are inconclusive, several analytical methods can provide the chemical composition of the sample to confirm if it is indeed falsified and not just a false positive result. For counterfeits of pharmaceutical solid dosage forms, they can first be identified by the measured spectra which is compared to the spectra of the reference tablet sample during the detection/authentication step as mentioned in section 2.2.2 (Lanzarotta et al, 2011; Matousek et al, 2011). Confirmatory techniques are highly sensitive but also quite expensive and so are more suited for national regulatory laboratories where suspect FSMs identified in the field are taken for confirmation. As highlighted in Table 2.2, these confirmatory techniques range from those with medium

costs (£10,000-£100,000) like direct insertion probe mass spectrometry (DIP-MS) and Ultraviolet Visible (UV-vis) spectroscopy to those with high costs (about £100,000 and above) like liquid chromatography and gas chromatography. Mass spectrometry (MS) is useful for confirmatory testing of medicines by identification of APIs, excipients as well as contaminants. Although the MS techniques are expensive, they can also be used in tandem with other techniques. Direct Analysis in Real Time (DART) (Likar et al, 2011; Chernetsova and Morlock, 2011; Bernier et al, 2016) and Desorption Electrospray Ionization (DESI) MS (Fernández, 2006; Culzoni et al, 2014) are ion sources that can be used in tandem with MS techniques for the direct analysis of solid pharmaceutical dosage forms (Culzoni et al, 2014; Bernier et al, 2016). Like DART and DESI, direct insertion probe mass spectrometry (DIP-MS) is another important technique that allows the direct analysis of samples without solvent extraction or sample preparation. DIP-MS could either be by hard ionisation (electron ionisation) in a vacuum or soft ionisation under atmospheric (ambient) conditions (also known as atmospheric solids analysis probe mass spectrometry (ASAP- MS)). The application of DIP-MS as a fast confirmatory technique in the screening of medicines is discussed in detail in chapter 5.

Chromatographic techniques are also well known for confirmatory assessment of medicines and like the other confirmatory techniques, they are expensive and much slower than the preliminary screening and authentication techniques with analysis time stretching to several hours per sample (Mwalwisi, 2016). Commonly used chromatographic techniques range from the cheap and simple thin layer chromatography –TLC used as an authentication technique (Sherma, 2007; Yemoa et al 2017) to the more sophisticated and expensive ones like high-performance liquid chromatography (HPLC). The chromatographic techniques can be used to confirm or validate the results of authentication tests like the spectroscopic techniques. Methods

like liquid and gas chromatography coupled with Mass Spectrometry (MS) (Deconinck et al, 2012; Custers et al, 2014) might then help in the quantification of active pharmaceutical ingredients (APIs) and provide information about possible impurities. HPLC has been identified as the most popular instrumental technique for pharmaceutical analysis and is noted as a gold standard for the quantitative analysis of pharmaceutical formulation contents (Martino et al, 2010; Chikowe et al, 2015; Yemoa et al, 2017). Ultraviolet Visible (UV-vis) spectroscopy is another solvent extraction based confirmatory technique used mainly for the quantitative determination/ analysis of different analytes identified in a particular medicine. Further details of the use of UV-vis spectroscopy as a confirmatory technique in the assessment of medicines are outlined in chapter 6.

One of the advantages of HPLC over the other techniques is that several detectors can be coupled with it. These include ultraviolet (UV), electrochemical, photodiode array (PDA) and MS detectors making it more versatile in its ability to detect the contents of different pharmaceutical formulations (Cai et al, 2010; Martino et al, 2010). Nuclear Magnetic Resonance (NMR) is also increasingly used for screening counterfeit medicines, especially for the identification of high molecular APIs (Holzgrabe and Malet-Martino, 2011; Barras, 2013). Chromatographic or NMR methods are necessary to determine the composition of liquid products like protein based medicines.

 The analytical data obtained using these more sophisticated confirmatory methods could provide forensic intelligence information about the criminal activities of medicine counterfeiters. The analytical profile also provides valuable information about the spread and globalisation of medicine counterfeiting and its link to organised crime. As a result, a trend can be established by tracing and comparing new falsified medicines with former cases. This will help in characterising falsified medicines with respect to source/origin (Deconinck et al, 2013; Dégardin et al, 2014) as was the case in

September, 2013 when falsified cough medicines found in Paraguay where identified to be similar to those detected in Pakistan as mentioned earlier in section 2.1.3 (WHO, 2017). Assessment of the suspect cough medicines in Paraguay was therefore quicker because forensic information was available to the WHO from a similar case of medicine falsification in Pakistan.

All the analytical techniques employed in the analysis of falsified and substandard medicines have been proven to enable the detection of these medicines (Table 2.2) but no individual technique can be identified as the "magic bullet" to FSMs detection (Martino et al, 2010, p.91; Rebiere et al, 2017). Therefore, the choice of the most suitable technique to be employed at any given time will depend on the following (Martino et al, 2010; Dégardin et al, 2014):

- Expertise of the operator or analyst

- Site where analysis is to be performed (developed or low income countries; forensic laboratories or field).

- Time limit for the screening exercise

- Funds available for the analysis

- Invasive or non-invasive method (Tampering or not tampering with sealed packaging)

- Purpose of testing/ required information (screening or confirmatory; Qualitative, semi-quantitative or quantitative).

2.3 Conclusion

Despite all the efforts to address the issue, counterfeit medicines are still a challenge globally. This is because the counterfeit medicine market thrives by taking advantage of the loopholes in the regulatory system or breaching established guidelines locally or internationally. Therefore, safeguarding of medicines or pharmacovigilance remains

continually relevant and crucial in order to ensure the safety of the medicines available to the general public. Also, because these medicines are a potential public health risk, the ease and speed of their detection will be a point to note if this phenomenon is to be addressed. With studies showing that more counterfeit medicines are found further away from the manufacturer in the distribution chain but closer to the final consumer, it is important that more simple, cost effective and quick analytical screening methods be developed so as to facilitate the process of authenticating medicines on the field.

This thesis will investigate the use of ATR-FTIR spectroscopy, Raman spectroscopy and DIP-MS. The rationale for these choices is that they are readily available, low cost, simple, rapid and can analyse solid mixtures. Although ATR-FTIR spectroscopy and Raman spectroscopy have previously been used for screening medicines, this research considers novel applications of these techniques in quantifying APIs and discrimination of samples based on their constituents. On the other hand DIP-MS has not been explored as a screening method for counterfeit solid pharmaceutical formulations. Further details about the use of ATR-FTIR spectroscopy, Raman spectroscopy and DIP-MS in the investigation of solid pharmaceutical formulations are presented in chapters 3, 4 and 5 respectively.

Chapter 6 and 7 will cover quantitative analysis of counterfeit medicines based on UV-vis spectroscopy and LC-MS respectively. These pharmacopoeia approved methods (UV-vis and LC-MS) are used for validation of results obtained using the rapid methods in earlier chapters. Chapter 8 is an assessment of the various techniques employed in this study based on speed of analysis, cost, portability, sensitivity and specificity for their feasibility in different settings or regions globally.

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CHAPTER THREE

Rapid identification of falsified and substandard medicines: Reflectance infrared spectroscopy for quantitative screening of the pharmaceutical ingredient

3.1 Introduction

With the growing prevalence of falsified and substandard medicines globally, the provision of simple, fast and cost effective methods of analysis for screening these medications would be beneficial especially in low and middle income countries (LMICs) (Glass, 2014; Hoellein and Holzgrabe, 2014; Koesdjojo et al, 2014; Mackey et al, 2015; Otte et al, 2015). Vibrational spectroscopic techniques are widely-documented as being suitable analytical methods for the authentication of pharmaceuticals (Sacré et al, 2010; Ortiz et al, 2013; Custers et al, 2015; Neuberger and Neusüß, 2015; Hoellein et al, 2016). There has also been increased interest in the use of handheld devices that combine spectroscopic and chemical analyses for screening medicines in the field (Kwok and Taylor, 2012; Kovacs et al, 2014; Ranieri et al, 2014; Sukkar, 2014). Raman spectroscopy in particular has been applied extensively in the characterization and identification of suspected medicines (De Veij et al, 2008; Dégardin et al, 2011; Neuberger and Neusüß, 2015) and also in quantification of paracetamol (Lyndgaard et al, 2013). Furthermore, Near Infrared spectroscopy (NIR) in combination with Raman spectroscopy, has been used for authentication of counterfeit medicines (Vredenbregt et al, 2006; Sacré et al, 2010). However, Neuberger and Neusüß (2015) identifies the limitations of Raman spectroscopy in its inability to discriminate between variations in mixing quality. Fourier Transform Infrared spectroscopy (FTIR) addresses this limitation as it is sensitive to mixing quality and granulation (Farouk et al, 2011).

There is also the issue of chemical peaks not being well defined or separated (poor chemical peak specificity) in NIR spectroscopy making spectra difficult to interpret (Roggo et al, 2007; Kovacs et al, 2014). Interest in this area is increasing by the day with the development of new equipment such as the

combination of optical microscopy with sensitive Raman analysis for forensic and analytical testing (Horiba, 2016).

Most applications of FTIR to pharmaceuticals like other spectroscopic techniques include characterisation and identifying the presence or absence of APIs and excipients (Sacré et al, 2010; Custers et al, 2015). Conventional FTIR with multivariate analysis has also been used for the quantification of APIs in antidiabetic drugs (Farouk et al, 2011) and paracetamol (Mallah et al, 2015) in solid pharmaceutical dosage forms. This approach requires skilled personnel since samples must be prepared as crushed powders finely dispersed in a KBr matrix which is then compressed into discs before analysis. This is both tedious and time consuming.

Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR) has revolutionized conventional FTIR by eliminating the main challenges in the analysis of pharmaceutical solid dosage forms notably the time spent in sample preparation: sample extraction or KBr disc preparation and the lack of spectral reproducibility. ATR-FTIR is quicker than the pharmacopoeia approved methods and conventional FTIR since the samples only need to be powdered before analysis (Lawson et al, 2018). The ATR accessory measures the changes that occur in a totally internally reflected infrared beam when the beam comes into contact with a sample (Fig. 3.1). The spectrum obtained is a fingerprint of the analyzed sample indicative of its unique features such as the functional groups and the chemical bonds in the sample (PerkinElmer, 2005).
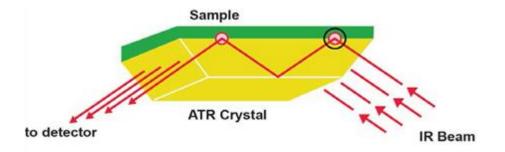
Figure 3. 1 Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) Schematic (Adapted from Lawson et al, 2014)

The aim of this study was to develop a simple fast and cost effective method using ATR- FTIR to distinguish between genuine and falsified or substandard tablet medication by identifying and quantifying the stated API in the presence of excipients. Tablet dosage forms generally consist of the therapeutic part (API) and the inactive part (excipients). The study also aimed at developing methods based on portable techniques that could potentially be used in the field for authenticating medicines especially in the LMICs. Due to their widespread use paracetamol/acetaminophen and chloroquine in tablet dosage forms, collected from different countries, were the primary test samples chosen for analysis. As stated earlier in Chapter 2, the increase in demand for a particular medicine increases the likelihood of it being counterfeited. Paracetamol is a common painkiller used worldwide making it a target for counterfeiters. Chloroquine, a typical antimalarial medicine, was chosen because of the high prevalence of counterfeit antimalarial in regions which are mostly LMICs (Ansari et al, 2013). The WHO World Malaria Report 2017 indicates that 90% of the malaria cases in 2016 occurred in the WHO African region further highlighting the magnitude of the problem in these LMICs (WHO, 2017). If validated rapid screening methods for medicines will enable withdrawal of counterfeit tablet medication from the market within the shortest possible time, thus reducing the risk to public health.

3.2. Materials and Methods

3.2.1 Reference Chemicals

Analytical grade APIs (analgesics and antimalarials) were obtained from Sigma Aldrich, Dorset, UK. Excipients were obtained from Fisher Scientific Ltd Loughborough, UK. The APIs and excipients for analysis are outlined in Table 3.1.

Table 3. 1 Active Pharmaceutical Ingredients and Excipients for ATR-FTIR analysis

Active Pharmaceutical Ingredients (APIs)	Excipients
Paracetamol	Calcium stearate
Chloroquine phosphate	Carboxymethyl cellulose
Aspirin	Maize starch
Amodiaquine dihydrochloride	Microcrystalline cellulose
Artemether	Magnesium stearate
Artesunate	Methylcellulose
Pyrimethamine	Potato starch
Sulfadoxine	Sodium starch glycolate
Caffeine	Zinc stearate
	Mannitol
	Crosscarmellose sodium
	Sodium carbonate
	Sodium starch glycolate

3.2.2 Test tablet samples

Single and multiple API tablet samples containing paracetamol were obtained opportunistically for analysis from a range of sources available to tourists in Europe, Asia, Africa and the Caribbean Islands. Sources from which tablet samples were obtained include pharmacies, hospitals, tuckshops, supermarkets, patent medicine stores and street vendors. Likewise tablet samples containing chloroquine from Europe, Asia and Africa were obtained for analysis. Paracetamol and chloroquine were the tablet samples of choice because of their common use globally as a painkiller/antipyretic and antimalarial medicine respectively (as mentioned earlier). Tables 3.2, 3.3 and 3.4 give a summary of the country of origin and the expected dose of the paracetamol and chloroquine tablets.

Table 3. 2 Single API paracetamol tablets analysed, their countries of origin and expected amount

Country (Number of Tablets)	Tablets Analysed*		Paracetamol Tablet Labelled as	Expected Amount (mg)
UK (n=2)	UK P1T1	UK P1T2	Paracetamol	
Cyprus (n=2)	Cyp P1T1	Cyp P1T2	Remedol	500
Switzerland (n=2)	Swz P1T1	Swz P1T2	Dafalgan	
Spain (n=6)	Spn P1T1	Spn P1T2	Panadol	
	Spn P2T1	Spn P2T2	Paracetamol Teva	650
	Spn P3T1	Spn P3T2	Paracetamol Teva	1000
Belgium (n=2)	Bel P1T1	Bel P1T2	Paracetamol EG	
India (n=19)	Ind P1T1	Ind P1T2	P1- Crocin Advance	500
	Ind P2T1	Ind P2T2	P2- G-mol	
	Ind P3T1	Ind P3T2	P3- Paracin	
	Ind P3T3	Ind P4T1	P4- Doliprane	
	Ind P4T2	Ind P5T1	P5- Calpol	
	Ind P5T2	Ind P5T3	P5- Calpol	
	Ind P6T1	Ind P6T2	P6- Crocin Advance	
	Ind P7T1	Ind P7T2	P7- Crocin Advance	
	Ind P8T1	Ind P8T2	Tharfenac	325
	Ind P9T1		Paracip	650
Pakistan (n=2)	Pak P1T1	Pak P1T2	Panadol	
Nepal (n=8)	Nep P1T1	Nep P1T2	P1- Algina	500
	Nep P2T1	Nep P2T2	P2- Algina	
	Nep P3T1	Nep P3T2	P3- Niko	
	Nep P4T1	Nep P4T2	P4- Algina	
China (n=4)	Chn P1T1	Chn P1T2	P1- Tylenol	
	Chn P2T1	Chn P2T2	P2- Eurocetamol	
UAE (n=4)	UAE P1T1	UAE P1T2	P1- Pmol	
	UAE P2T1	UAE P2T2	P2- Adol	
Rwanda (n=6)	Rwa P1T1		P1- Ubithera	500
	Rwa P2T1		P2- Paradana	
	Rwa P3T1	Rwa P3T2	P3- Pharmaquick	
	Rwa P4T1	Rwa P4T2	P4- Eskay	
Ghana (n=4)	Gha P1T1	Gha P1T2	P1- Ayrton	
	Gha P2T1	Gha P2T2	P2- Cetapol	
Nigeria (n=4)	Nig P1T1	Nig P1T2	P1- Agary	
	Nig P2T1	Nig P2T2	P2- Seebest	
Jamaica (n=4)	Jam P1T1	Jam P1T2	P1- Panadol	
	Jam P2T1	Jam P2T2	P2- Panadol	

Note: n = number of samples, *P1T1= Pack 1 Tablet 1 and so on.

Table 3. 3 Multiple API tablets from the UK containing paracetamol analysed using ATR-FTIR

Tablet(s)	API(s) Expected in Tablets	Amount of API expected in tablet (mg)
PC A# 1	Paracetamol and Caffeine	Paracetamol-500,
PC A# 2		Caffeine- 65
PC B # 1	Paracetamol and Caffeine	Paracetamol- 500
PC B # 2		Caffeine- 65
PC C# 1	Paracetamol and Caffeine	Paracetamol- 500
PC C# 2		Caffeine- 65
PAC A#1	Paracetamol Aspirin, and Caffeine	Paracetamol- 200, Aspirin- 300, Caffeine- 45

Table 3. 4 Chloroquine tablets analysed, their countries of origin and expected amount

Country (Number of Tablets)	Tablets Analysed*	Expected Amount (mg)
UK (n=2)	UK C1T1 UK C1T2	250
Belgium (n=2)	Bel C1T1 Bel C1T2	
India (n=5)	Ind C1T1 Ind C1T2 Ind C2T1 Ind C2T2	
	Ind C3T1	500
Nepal (n=2)	Nep C1T1 Nep C1T2	250
Kenya (n=1)	Ken C1T1	
Nigeria (n=1)	Nig C1T1	400

Note: n = number of samples, *C1T1= Pack 1 Tablet 1 and so on.

3.2.3 Instrumentation

All spectra were recorded on the Alpha Bruker FTIR spectrometer (Bruker Corporation, Germany) equipped with the ATR platinum diamond sampling stage to provide robustness and durability. Spectral acquisition was done using OPUS software version 7.5 (Bruker Corporation, UK).

For qualitative analysis of tablet samples, the Spectrum Search facility within the OPUS 7.5 software was used for comparing sample spectra to those in local reference library. For paracetamol, quantitative measurements were carried out using two methods namely:

- Manual integration of characteristic peaks identified using the integration mode facility on the software.

- Automatic multivariate partial least squares (PLS) calibration by using the Quant 2 facility within the software to provide calibration spectra from different concentrations (%w/w) of paracetamol prepared in selected excipients.

Quantification of chloroquine was based on Quant 2 methods validated for paracetamol providing calibration spectra from different concentrations (% w/w) of chloroquine in maize starch.

3.2.4 Methods

3.2.4.1 Reference Spectra

All sample spectra were measured in absorbance mode. A fresh background spectrum was measured against air before starting measurements and subsequently after every 5 runs. Small amounts of finely ground samples of the individual reference materials, paracetamol, chloroquine, other APIs and excipients often found in pharmaceutical formulations, were placed on the diamond sampling crystal and pressed using a clamp to ensure proper contact. Each spectrum was measured by averaging 20 scans over the range 4000-400cm^{-1} and spectral resolution for measurement was 2cm^{-1}. Estimated scan time for spectral acquisition was 25 seconds. This process was repeated 5 times to ensure replicate data was produced. The platinum diamond sampling surface was cleaned after each sample using paper tissue with isopropanol and allowed to dry. Recorded fingerprint spectra for the reference materials were assessed for spectral reproducibility by comparing replicate spectra. Reproducible spectral data were used to create a local reference library to help in the identification of sample spectra. Identification was based on characteristic peaks chosen by

comparing test sample to reference. Members of the same excipient groups, starches, stearates and celluloses all had very similar spectra (Fig 3.5). Characteristic regions of paracetamol and chloroquine spectra were identified where there was little or no interference from other materials.

3.2.4.2 Preparation of API-excipient calibration mixtures
3.2.4.2.1 Paracetamol calibration

Excipients used were representative of different excipient groups commonly used in formulation for particular functions. Microcrystalline cellulose (binder, disintegrant), maize starch (diluent, binder) and magnesium stearate (lubricant) were the excipients selected as examples of excipients commonly used in paracetamol formulations (Haywood and Glass, 2011; Rowe, 2012).

For calibration by manual integration of peaks, different concentrations of paracetamol at 20, 30, 40, 60 70, 80 and 90% w/w in magnesium stearate were prepared mixing paracetamol and the excipient for 120 seconds. For the API/excipient mixtures, a uniform total weight of 200mg was measured each time. These concentrations covered the different dosages in common over-the-counter medicines. Calibration curves based on the recorded spectra of the paracetamol/excipient mixtures were prepared by determining the areas under the paracetamol peaks where there was no contribution from the excipients using the manual Integration facility on the OPUS 7.5 software. The final result was the mean of three separate runs per sample.

The same protocol was followed for preparation of paracetamol/ excipient mixture for calibration using the automatic Quant 2 method but concentrations of paracetamol at 10.0%, 20.0%, 30.0%, 50.0%, 70.0% and 90.0% w/w in maize starch, MCC and magnesium stearate respectively were prepared. Calibration data was generated for the different paracetamol concentrations in the three

excipients considered. Final results were the mean of five replicate runs per sample.

3.2.4.2.2 Chloroquine calibration

Quant 2 methods based on validated data from paracetamol analysis were adopted for analysis of chloroquine tablets. Different concentrations of chloroquine in maize starch (10.0%, 30.0%, 50.0%, 60.0%, 70.0% and 90.0% w/w) were prepared by mixing chloroquine in maize starch for 120 seconds. Maize starch was used as the only excipient for analysis of chloroquine tablets as it was identified to produce more accurate and reproducible results in the analysis of paracetamol tablets. Areas under characteristic peaks for chloroquine were determined for the calibration curve using the Quant 2 application on the software. The automatic Quant 2 application on the software was the only approach used for chloroquine analysis since it was quicker and gave more reproducible results for paracetamol tablet analysis making it more efficient. Furthermore, considering good reproducibility obtained for paracetamol studies three separate runs were performed per chloroquine sample. Hence, final results were the mean of three separate runs.

3.2.4.3 Preparation of test tablets- Qualitative analyses

Individual tablet samples were taken out of their blister packs and placed on to the diamond crystal on the sampling head of the ATR unit. Measurement was done using the same parameters as with the reference spectra in section 3.2.4.1. Several points on each side of the tablet were assessed with spectral data recorded each time in order to investigate reproducibility and to check if the presence of the API could be confirmed based on the data.

3.2.4.4 Preparation of test tablets- Quantitative analyses

Each tablet was weighed and then ground into fine powder using a mortar and pestle until a homogeneous mixture was obtained. Spectra of 5 individual powder samples per tablet were recorded. Characteristic peaks identified for paracetamol were then employed in assessing the potential of ATR-FTIR in the quantification of paracetamol in over-the-counter tablets using the OPUS 7.5 manual integration and Quant 2 applications respectively. For quantitative analysis of samples by manual integration, different integration modes provided by the software were applied as highlighted in Table 3.5.

Table 3. 5 Integration modes for ATR-FTIR quantitative analysis and functions

INTEGRATION MODE		FUNCTION
A		It performs the integration between the band, abscissa and the frequency limits defined.
B		It integrates the area above a straight line drawn between the peaks of the two frequency limits defined.
J		It measures the highest absolute peak intensity
K		It measures the peak intensity relative to the local baseline.

Effect of mixing time on the reproducibility of quantitative data for paracetamol tablets was also investigated by comparing quantitative data for selected tablet samples after mixing for 60 seconds and 120 seconds respectively.

For Quant 2 analysis the data was processed via the multivariate analysis capability of the OPUS 7.5 Quant 2 software

Quant 2 methods based on paracetamol analysis were adopted for chloroquine analysis. Spectra of three individual powder samples per tablet were recorded.

For both paracetamol and chloroquine analysis;

Measured levels of API were indicative of the percentage amount of API in tablet. The relationship between the results in % w/w and the actual dosage can be expressed as:

Actual Dosage of API in tablet (mg) = R x W
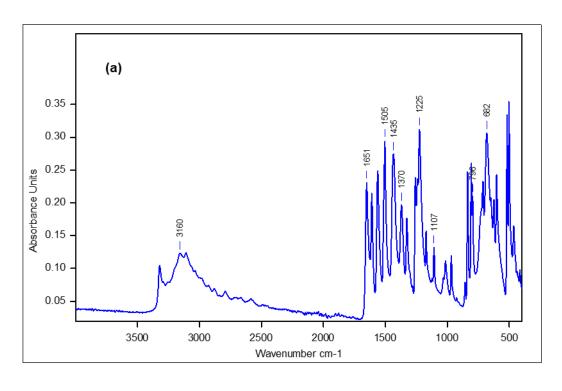
Where R= Concentration of API in % w/w and W= Total weight of the tablet (mg)

The levels of paracetamol and chloroquine in tablet medication obtained from the UK and several countries around the globe were then determined based on data obtained from the calibration mixtures.

3.3. Results and Discussion

3.3.1 Reference Spectra

Reference samples for several APIs were recorded as part of the local ATR-FTIR library created. Fig 3.2 (a) and (b) are the reference spectra for paracetamol and chloroquine respectively. These spectra saved in the reference library could then be used for identification purposes by the Spectrum search capability in the OPUS 7.5 software. If the spectral fingerprint of a test sample matched the reference wholly or in part the sample could be said to contain the same reference material. Reference spectra for other APIs are outlined in Figs 3.3 (a, b) and 3.4. (a, b). Fig 3.4(a) and 3.4(b) are artemether and artesunate which are artemisinin derivatives used as antimalarials. The obvious similarity in their spectra suggests that they belong to the same family of compounds or that they have similar structure. In addition, Fig 3.5 shows the spectra of different excipient groups (stearates, celluloses and starches) and the similarity between individual spectra within each group.
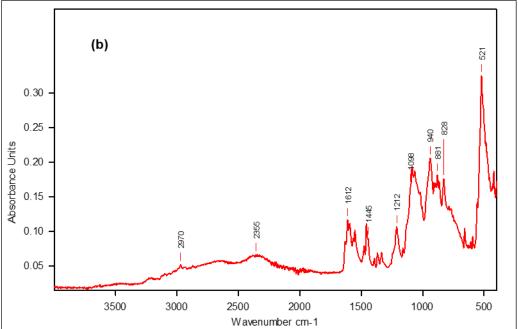
Figure 3. 2 ATR-FTIR reference spectra for (a) paracetamol (b) chloroquine
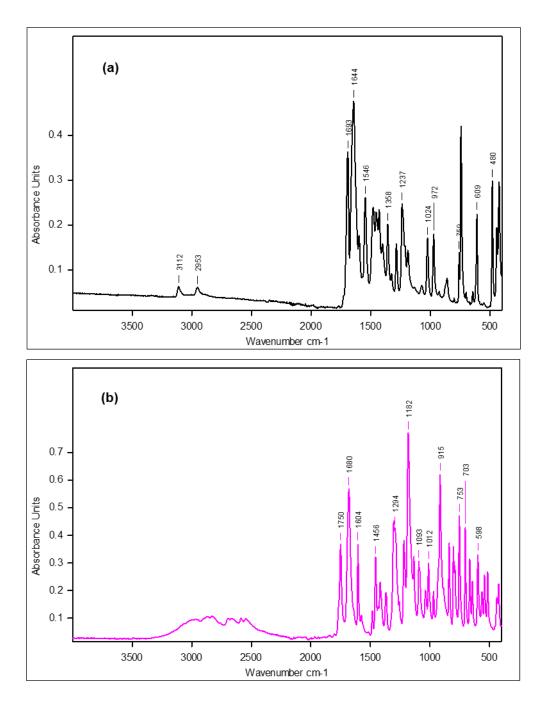
Figure 3. 3 ATR-FTIR reference spectra for (a) caffeine (b) aspirin
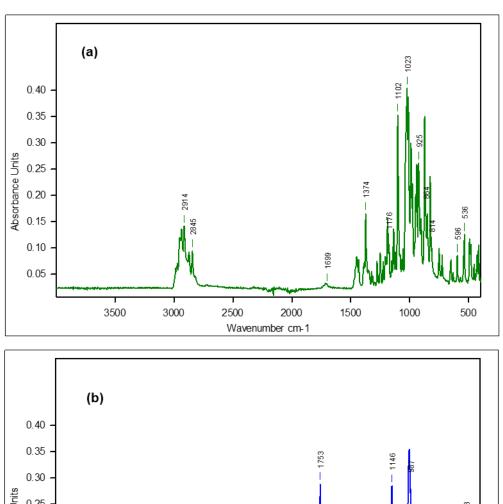
Figure 3. 4 ATR-FTIR reference spectra for (a) artemether (b) artesunate
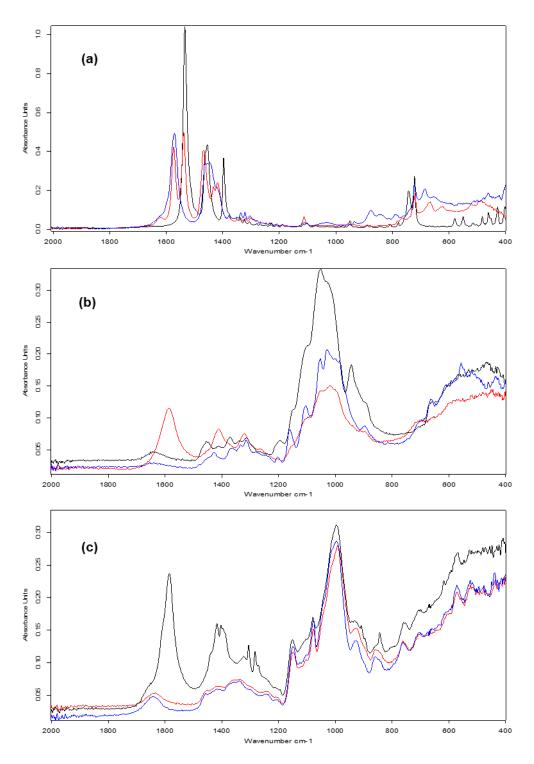
Figure 3. 5 Comparing members of the same excipient group (a) Stearates: magnesium stearate (Blue), calcium stearate (Red), zinc stearate (Black) **(**b) Celluloses: microcrystalline celluose (Blue), carboxymethyl cellulose (Red), methylcellulose (Black) (c) Starches: maize starch (Blue), potato starch (Red), sodium starch glycolate (Black)

3.3.2 Qualitative assessment of whole tablet samples

Data sufficiently reproducible for qualitative identification of paracetamol could be obtained from a whole tablet placed on the sampling port of the ATR-FTIR instrument. The data for both sides of the tablet are shown in the lower two (blue and red) traces in Fig. 3.6. The difference between these traces, whilst being qualitative, does not lead to reproducible quantitative data. This can be obtained from a crushed sample of the tablet as shown in the upper trace (black) in Fig. 3.6. Variation in the spectra obtained for the top, bottom and crushed tablet could be due to the degree of curvature of the tablet which affects contact with the sampling surface, poor homogeneity of the tablet or a combination of both. The irreproducibility of data based on whole tablet sample analysis meant that data based on the whole tablet was not appropriate for this work.
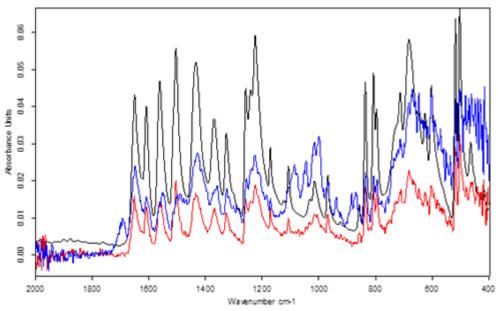
Figure 3. 6 Paracetamol crushed (Black), Tablet top (Blue), Tablet bottom (Red)

3.3.3 Spectral reproducibility

Overlays of replicate spectra of each reference material indicated that there were no detectable differences in peak position between replicate spectra of the same sample (Fig 3.7). Initial studies showed some variation in peak intensities

between replicate spectra (Fig 3.8) but this was resolved with improved homogenisation of samples and proper reproducible covering of the sampling surface. Optimised sample preparation methods therefore gave reproducible spectral data across the fingerprint range (2000 - 400 cm^{-1}).
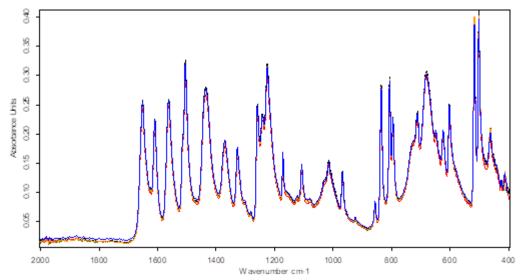
Figure 3. 7 Multiple spectra overlay of paracetamol reference showing reproducibility
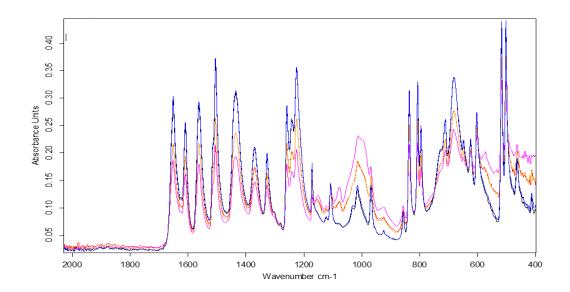
Figure 3. 8 Multiple spectra overlay of paracetamol reference showing variation in spectra due to improper homogenisation of samples

3.3.4 Identification of active pharmaceutical ingredient (API)

In order to identify the APIs (paracetamol and chloroquine) in the presence of excipients, a reference library containing spectra of reference material was created as discussed in 3.3.1. Replicate spectra of the reference samples (API and excipients) were recorded and there was no detectable difference in absorbance bands and peak data between individual replicates of the same material provided instrumental conditions remained constant. Spectral data based on both absorbance bands and peak intensities were reproducible.

3.3.4.1 Fingerprint and characteristic peaks for identification of APIs

The reproducible reference library formed the basis for identification of over-the-counter paracetamol tablet medications. Crushed tablet spectra were then recorded and compared with those in the reference library and if the peaks in the fingerprint region (2000 - 400cm^{-1}) matched, the presence of the API was confirmed. Aside from comparing the whole spectra, individual characteristic peaks were used to indicate the presence of a specified API in more complex tablet samples containing other APIs as well as excipients. This was achieved by selecting regions of the IR spectrum where there was little or no interference from the excipients. These characteristic peaks/ regions identified were then employed in the quantification of the API.

- For the manual integration method for the determination of paracetamol, characteristic peaks employed were identified by simply overlaying recorded spectra of the API (paracetamol) with the selected excipient in order to identify regions in the spectra with the least interference. Based on this, peaks at 1225 cm^{-1}, 1172cm^{-1}, 1108cm^{-1}, 603cm^{-1} were selected for calibration of paracetamol in magnesium stearate mixtures (Fig 3.9).
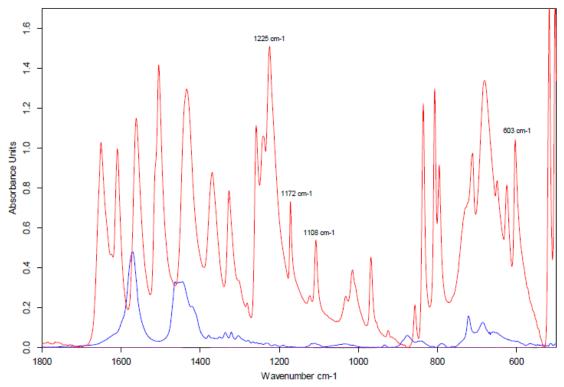
Figure 3. 9 Overlay of ATR-FTIR spectra for identification: Paracetamol reference (red) and magnesium stearate (blue).

- For Quant 2 analysis, paracetamol was compared with a more complex mixture of three excipients (maize starch, MCC and magnesium stearate). For example Figure 3.10(C), a mixture of excipients, shows little absorbance over the ranges 2000 - 1750cm^{-1}, 1600 - 1450cm^{-1} and 1300 -1100cm^{-1}. A comparison between the spectra for pure paracetamol (Fig 3.10(A)), a paracetamol tablet (Fig 3.10(B)) and a mixture of common excipients used in tablet formulations, (Fig 3.10) shows that peaks at 1505cm^{-1} and 1225cm^{-1} are indicative of the presence of paracetamol in the formulation.
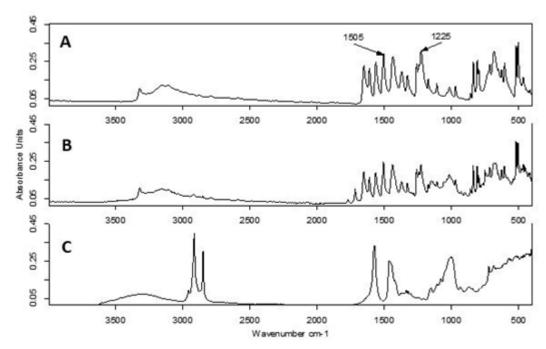
Figure 3. 10 Comparison of three spectra: (A) pure paracetamol, (B) a paracetamol tablet, (C) a mixture of three excipients (maize starch, magnesium stearate and microcrystalline cellulose).

Identification of the presence of paracetamol was possible down to about 5% w/w of API in excipient using the two characteristic peaks. The characteristic peaks for paracetamol at $1225cm^{-1}$ and $1505cm^{-1}$ correspond to the –OH in plane vibration and –CH_3 vibration respectively. The molecular structure of paracetamol is show in Fig 3.11.
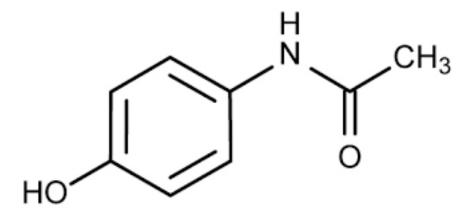
Figure 3. 11 Molecular Structure of Paracetamol (Acetaminophen)

Figure 3.12 shows identification of paracetamol based on a comparison of spectra from the paracetamol reference and a tablet formulation over the fingerprint region 2000 - 400cm^{-1}.
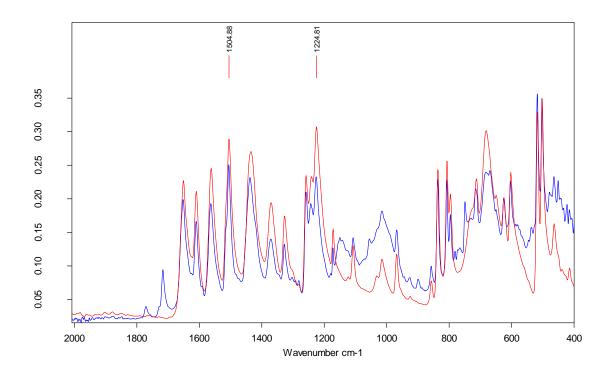
Figure 3. 12 Overlay of ATR-FTIR spectra for identification: Paracetamol reference (red) and paracetamol tablet (blue).

Figure 3.13 further confirms the peak at 1505cm^{-1} as a characteristic peak for paracetamol as it is identified in a complex multi API tablet containing aspirin and caffeine in addition to paracetamol
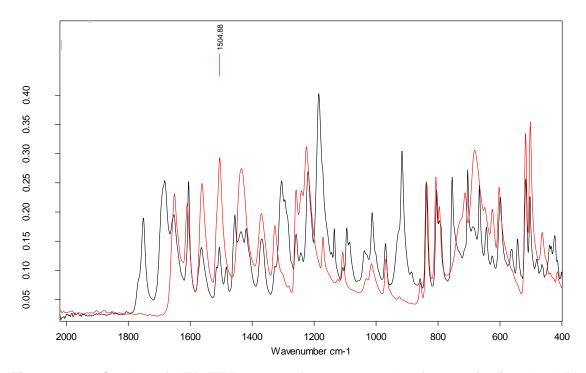
Figure 3. 13 Overlay of ATR-FTIR spectra for paracetamol reference (red) and 3-API tablet (black).

Similarly, characteristic peak for chloroquine was identified at 1212cm^{-1} as shown in Fig 3.14.
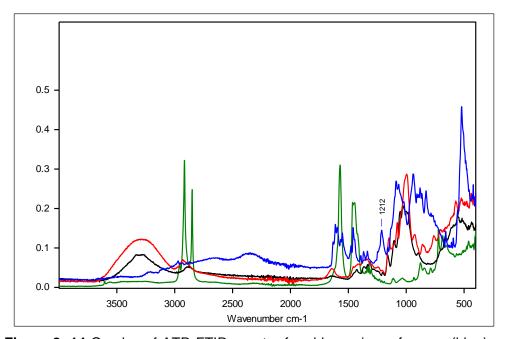
Figure 3. 14 Overlay of ATR-FTIR spectra for chloroquine reference (blue), magnesium stearate (green), MCC (black) and maize starch (red).

3.3.5 Quantification of Active Pharmaceutical Ingredients

Good grinding and homogenisation were essential in order to obtain well-defined, reproducible and quantifiable spectra. This was particularly important for the quantification of the API in tablet samples Effect of mixing time on reproducibility and quantification of data is explained in section 3.3.5.1.1 Grinding/mixing time was set at a minimum of 120 seconds per test tablet sample.

Different approaches to quantitative analysis of paracetamol were carried out by applying the OPUS 7.5 Integration and Quant 2 software applications respectively to the spectral data obtained from the calibration samples prepared. The OPUS 7.5 integration mode has four different operating functions highlighted in Table 3.5:

3.3.5.1 Quantification of paracetamol by manual integration of peak areas

During the preparation of the Paracetamol/magnesium stearate calibration mixtures, there was difficulty in obtaining an even mix of both samples. The difficulty in mixing magnesium stearate could be linked to its use as a lubricant in pharmaceutical formulations. Calibration graphs were plotted for each of the selected characteristic peaks using the different integration modes on the OPUS software 7.5. The OPUS 7.5 Integration application provides absorbance values for the area under the curve of selected spectral ranges using different modes as mentioned in section 3.2.4.4. Absorbance data for 3 replicate samples were recorded for each concentration. Table 3.6 indicates the calibration equation and the R^2 values for all calibration graphs for paracetamol in magnesium stearate prepared. Table 3.7 and Figure 3.15 show a representative example of the calibration data obtained.

Table 3. 6 Calibration curve equations and R^2 values using different integration modes for paracetamol in magnesium stearate calibration mixtures

PEAKS (cm^{-1})	MODE A	
	CALIBRATION EQUATION	R^2 VALUE
1225	y= 0.14x - 0.708	0.9922
1172	y= 0.0301x + 0.3367	0.9952
1108	y= 0.02x + 0.662	0.9879
	MODE B	
1225	y= 0.0387x – 0.5437	0.9743
1172	y= 0.0071x – 0.0486	0.9855
1108	y= 0.0067x + 0.0316	0.9877
603	y= 0.0277x – 0.3385	0.9927
	MODE J	
1225	y= 0.0043x – 0.0211	0.9837
1172	y= 0.002x + 0.0056	0.9905
1108	y= 0.0014x + 0.0307	0.9864
	MODE K	
1225	y= 0.0018x – 0.0323	0.9734
1172	y= 0.0011x – 0.0094	0.9807
1108	y= 0.0009x + 0.0005	0.9862
603	y= 0.0018x+ 0.0157	0.9969

Owing to the background effects observed with the peak at 603cm^{-1}, measurements could only be taken from the local baseline of the peak. Hence, the calibration data for the peak at 603cm^{-1} is only given in modes B and K.

Table 3. 7 Absorbance readings for paracetamol in magnesium stearate using mode A (Peak 1225cm^{-1})

MODE A (PEAK 1225cm^{-1})	AREA			
CONCENTRATION (% w/w)	Replicate 1	Replicate 2	Replicate 3	MEAN±SD
90	11.642	11.626	12.175	11.814±0.31
80	10.621	10.786	10.745	10.717±0.09
70	9.490	9.078	9.441	9.336±0.23
60	7.150	7.489	7.209	7.283±0.18
40	5.252	4.998	4.562	4.937±0.35
30	3.134	3.077	2.978	3.063±0.08
20	2.697	2.485	2.332	2.505±0.18
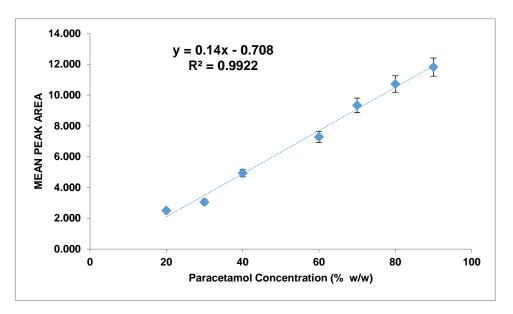
Figure 3. 15 Calibration curve for paracetamol in magnesium stearate at 1225cm⁻¹ using mode A (Data represents the mean±SD of 3 replicate samples).

Calibration data in Fig 3.15 indicates the reproducibility of the data obtained with a high correlation coefficient of 0.99. Similar results were obtained for calibration based on the characteristic peaks identified and the different integration modes as highlighted in Table 3.6. Correlation coefficients for all calibration curves were between 0.97 and 0.99 showing linearity of the data. Calibration equations were then used in working out the concentration of paracetamol in selected tablets (in % w/w).

3.3.5.1.1 Effect of grinding/ mixing on the quantification of paracetamol tablet samples

The effect of mixing time for crushed tablet samples on the quantification data for paracetamol tablets was assessed by mixing paracetamol tablets for 60 seconds and 120 seconds respectively   It was observed that good grinding/mixing to obtain fine powder was essential in the quantification of the API in the tablet samples. Grinding/mixing time of the tablet samples also had an impact on the final results obtained as shown graphically in Fig. 3.16a and 3.16b.
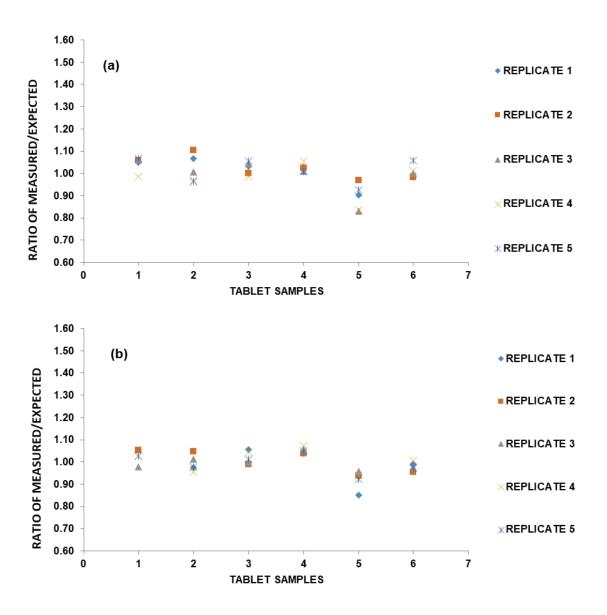
Figure 3. 16 Ratio of measured to expected amounts of paracetamol for 5 replicates each of 6 different paracetamol tablets after grinding/mixing (a) for 60 seconds (b) for 120 seconds

Results after grinding/mixing for 120 seconds were more precise as indicated by the spread of the replicate data for each tablet. 120 seconds was therefore the optimum mixing used for analysis.

3.3.3.1.2 Tablet Sample Analysis using OPUS 7.5 manual Integration

Individual tablets from selected samples were crushed and subjected to ATR-FTIR analysis with quantification by the OPUS Integration application. A representative

example of the quantitative paracetamol data for the characteristic peaks is displayed in Table 3.8 (% w/w) and Table 3.9 (in mg) for data based on Integration mode A. The same tablets were assessed based on the other integration modes (B, J and K) and a summary of the data is presented in Table 3.10. Final results for each tablet were the mean of results obtained for each integration mode as shown in Table 3.10.

Table 3. 8 Measured concentration of paracetamol (% w/w) using mode A calibration curve for Paracetamol in Magnesium stearate.

TABLET	Integration Mode A			MEAN±SD	Expected Concentration (% w/w)
	Peak 1225cm^{-1} (% w/w)	Peak 1172cm^{-1} (% w/w)	Peak 1108cm^{-1} (% w/w)		
UK P1T1	76.5	75.6	70.3	74.1±3.4	88.4
UK P1T2	78.5	75.5	70.4	74.8±4.1	89.7
Bel P1T1	84.5	82.6	83.5	83.5±1.0	85.8
Bel P1T2	85.5	83.7	83.8	84.3±1.0	86.0
Chn P1T1	71.2	69.8	68.6	69.9±1.3	84.7
Chn P1T2	74.0	73.6	73.6	73.7±0.2	85.4
Chn P2T1	78.9	78.9	72.1	76.6±3.9	80.7
Chn P2T2	80.6	81.2	75.4	79.1±3.2	77.2
Rwa P1T1	76.4	77.4	73.9	75.9±1.8	89.1
Rwa P2T1	78.2	77.0	73.7	76.3±2.3	87.5
Rwa P3T1	76.8	86.2	95.6	86.2±9.4	88.1
Rwa P3T2	75.1	83.0	91.7	83.3±8.3	77.6
Rwa P4T1	78.8	83.1	78.1	80.0±2.7	86.4
Rwa P4T2	72.1	80.6	75.6	76.1±4.3	87.3
Ind P1T1	72.6	72.4	69.1	71.4±2.0	75.6
Ind P1T2	69.8	68.0	63.3	67.0±3.4	75.0
Ind P2T1	73.4	82.3	95.9	83.9±11.3	84.0
Ind P2T2	72.6	84.0	96.2	84.3±11.8	82.1
Ind P3T1	72.2	78.5	83.0	77.9±5.4	84.9
Ind P3T2	72.8	77.4	81.1	77.1±4.2	83.7
Ind P3T3	71.9	75.4	78.5	75.3±3.3	84.1
Ind P4T1	73.7	79.0	85.6	79.4±6.0	85.9
Ind P4T2	74.1	81.5	87.4	81.0±6.7	84.4
Ind P5T1	71.7	70.3	67.0	69.7±2.4	79.5
Ind P5T2	70.6	69.5	65.2	68.4±2.9	77.0
Ind P5T3	69.1	68.6	63.8	67.2±2.9	79.9
Ind P8T1	56.6	29.5	102.5	62.9±36.9	42.7
Ind P8T2	57.9	29.9	103.3	63.7±37.0	42.5

Measured amounts of paracetamol (in mg) were deduced as outlined in section 3.2.4.4

Table 3. 9 Measured amount (in mg) of Paracetamol using Mode A calibration curve for paracetamol in magnesium stearate.

TABLET	Integration Mode A			MEAN±SD	Expected Amount (mg)
	Peak 1225cm^{-1} (mg)	Peak 1172cm^{-1} (mg)	Peak 1108cm^{-1} (mg)		
UK P1T1	432.4	427.3	397.5	419±19	500
UK P1T2	437.2	420.8	392.1	417±23	500
Bel P1T1	985.4	963.2	973.7	974±11	1000
Bel P1T2	994.1	973.2	974.3	981±12	1000
Chn P1T1	420.0	411.6	405.0	412±8	500
Chn P1T2	432.9	430.8	430.9	432±1.	500
Chn P2T1	488.9	489.0	446.9	475±24	500
Chn P2T2	521.7	526.0	488.1	512±21	500
Rwa P1T1	428.8	434.4	414.7	426±10	500
Rwa P2T1	446.8	439.9	421.0	436±13	500
Rwa P3T1	436.1	489.4	542.8	489±53	500
Rwa P3T2	484.1	535.0	590.5	537±53	500
Rwa P4T1	455.9	480.7	451.8	463±16	500
Rwa P4T2	441.5	461.5	432.9	445±15	500
Ind P1T1	479.6	478.8	456.8	472±13	500
Ind P1T2	465.3	453.7	422.0	447±22	500
Ind P2T1	436.6	489.8	570.5	499±67	500
Ind P2T2	442.1	511.1	585.6	513±72	500
Ind P3T1	425.5	462.4	489.0	459±32	500
Ind P3T2	434.8	462.4	484.4	461±25	500
Ind P3T3	427.2	448.1	466.6	447±20	500
Ind P4T1	429.1	459.5	498.2	462±35	500
Ind P4T2	438.9	482.7	517.8	480±40	500
Ind P5T1	450.9	442.4	421.4	438±15	500
Ind P5T2	458.4	451.6	423.6	445±18	500
Ind P5T3	432.1	429.2	399.2	420±18	500
Ind P8T1	430.9	225.1	780.8	479±281	325
Ind P8T2	443.2	228.8	790.2	487±283	325

ATR-FTIR quantitative results for paracetamol in modes A, B, J and K are mean results of the different fingerprint peaks considered. Ind P8 tablets notably had very high standard deviation (SD) values. This huge variation arises mainly from results at Peak 1108cm^{-1} with results obtained being more than double the expected amounts. Fig 3.17 clearly shows the difference in the IR spectra of the reference paracetamol and the Ind P8 tablet at peak 1108cm^{-1}. This variation could be due to the presence of an excipient or a different Active Pharmaceutical Ingredient (API) which absorbs in that region. This limits the use of peak 1108cm^{-1} in quantitative analysis of paracetamol.
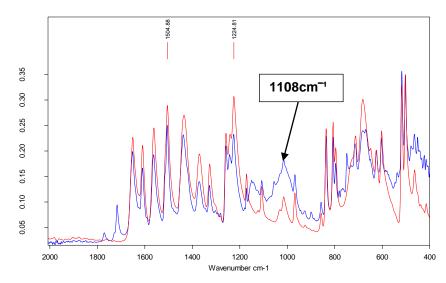
Figure 3. 17 Overlay of ATR-FTIR spectra of paracetamol reference (red) and Ind P8 tablet (blue).

Table 3. 10 Summary of the results of quantitative analysis of the Paracetamol tablets using manual integration method.

TABLET	MEASURED AMOUNTS OF PARACETAMOL				
	Mode A MEAN±SD (mg)	Mode B MEAN±SD (mg)	Mode J MEAN±SD (mg)	Mode K MEAN±SD (mg)	Expected Amount (mg)
UK P1T1	419±19	429±28	450±26	461±53	500
UK P1T2	417±23	442±32	456±31	476±58	500
Bel P1T1	974±11	977±78	1036±37	1050±127	1000
Bel P1T2	981±12	986±79	1049±44	1065±134	1000
Chn P1T1	412±8	410±36	441±23	450±62	500
Chn P1T2	432±1	419±39	455±21	456±66	500
Chn P2T1	475±24	431±24	492±32	468±54	500
Chn P2T2	512±21	457±24	529±27	494±56	500
Rwa P1T1	426±10	422±36	447±21	446±56	500
Rwa P2T1	436±13	440±36	466±25	475±60	500
Rwa P3T1	489±53	401±28	475±24	418±52	500
Rwa P3T2	537±53	448±38	524±21	472±63	500
Rwa P4T1	463±16	437±37	476±22	460±59	500
Rwa P4T2	445±15	422±37	456±23	440±57	500
Ind P1T1	472±13	448±42	497±29	489±71	500
Ind P1T2	447±22	445±41	480±38	487±73	500
Ind P2T1	499±67	392±40	472±30	400±56	500
Ind P2T2	513±72	394±44	479±33	409±59	500
Ind P3T1	459±32	394±38	454±8	421±61	500
Ind P3T2	460±25	407±40	464±5	439±63	500
Ind P3T3	447±20	405±40	455±7	435±62	500
Ind P4T1	462±35	412±37	462±7	435±60	500
Ind P4T2	480±40	410±43	473±11	436±62	500
Ind P5T1	438±15	434±39	465.±30	469±68	500
Ind P5T2	445±18	443±36	477±33	483±68	500
Ind P5T3	420±18	417±39	449±30	451±63	500
Ind P8T1	479±281	239±71	499±89	314±84	325
Ind P8T2	487±283	253±77	510±88	327±87	325

Note: Quantitative results in modes A, B, J and K are mean results of the different peaks considered (1225cm^{-1}, 1172cm^{-1}, 1108cm^{-1} and 603cm^{-1}).

A summary of the results based on different modes indicated that the paracetamol amounts in the tablets assessed were mostly within acceptable limits except the Ind P8 tablets which suggests interference with the peak at $1108cm^{-1}$ as mentioned earlier. The quantitative data is better appreciated as a plot of measured to expected amounts of paracetamol versus the individual tablets. These data for all integration modes is presented in Figs 3.18-3.21 with tablet samples split into regions.
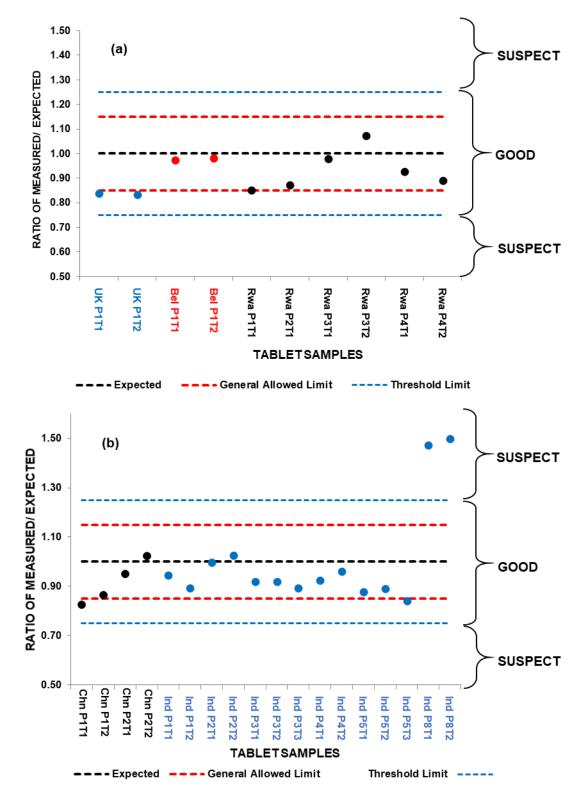
Figure 3. 18 Ratio of measured to expected paracetamol amounts in the tablets using integration **Mode A** for paracetamol in magnesium stearate calibration (a) 10 tablets from Europe and Africa (b) 18 tablet samples from Asia
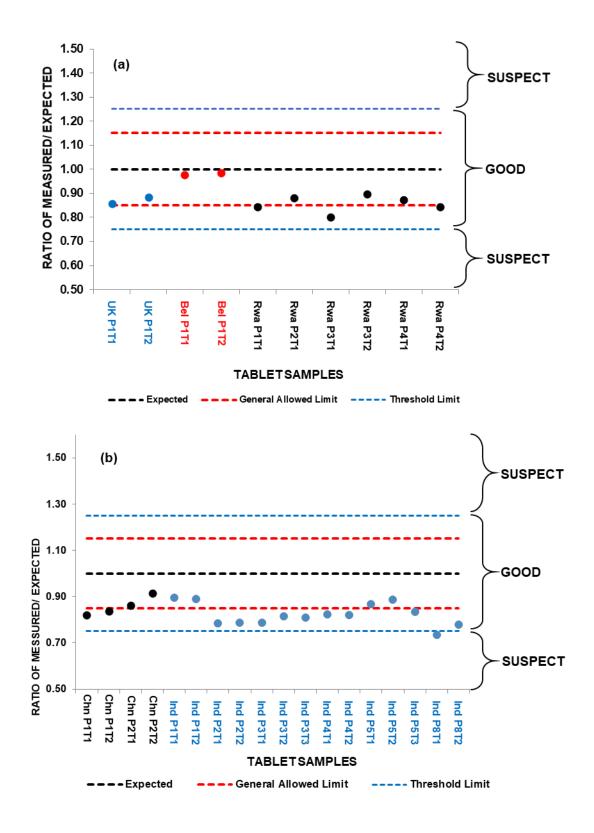
Figure 3. 19 Ratio of measured to expected paracetamol amounts in the tablets using integration **Mode B** for paracetamol in magnesium stearate calibration (a) 10 tablets from Europe and Africa (b) 18 tablet samples from Asia
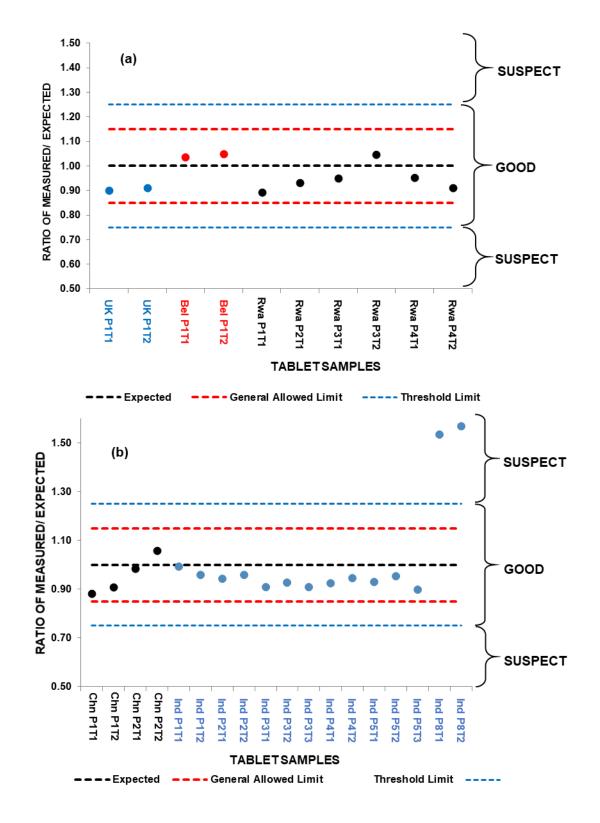
Figure 3. 20 Ratio of measured to expected paracetamol amounts in the tablets using integration **Mode J** for paracetamol in magnesium stearate calibration (a) 10 tablets from Europe and Africa (b) 18 tablet samples from Asia
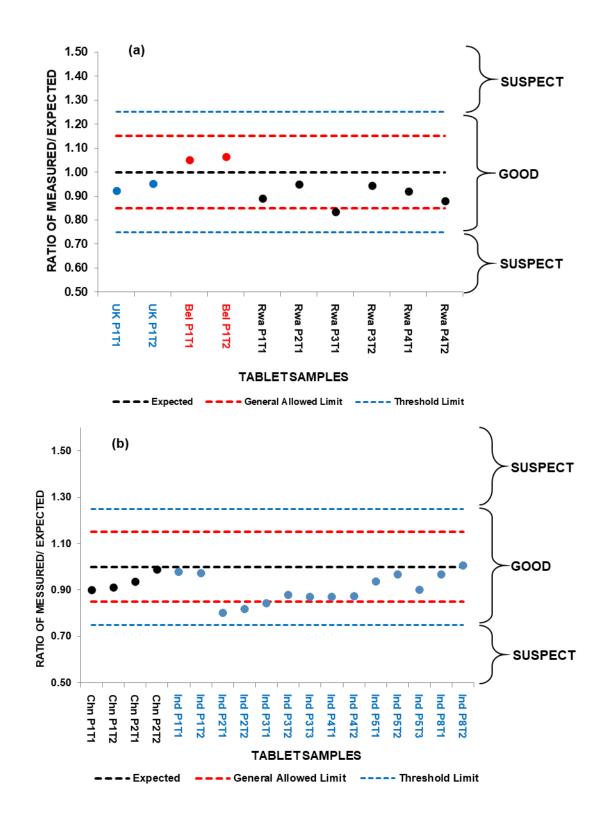
Figure 3. 21 Ratio of measured to expected paracetamol amounts in the tablets using integration **Mode K** for paracetamol in magnesium stearate calibration (a) 10 tablets from Europe and Africa (b) 18 tablet samples from Asia

According to British Pharmacopoeia's uniformity of dosage units test (BP, 2017), the test tablets comply and pass the test if not more than one tablet out of 30 tablets taken at random is outside the allowed limits of 85- 115% of the average content. The tablets fail the uniformity of dosage units test if more than one tablet is outside of the allowed limits of 85-115% of the average API content or if one tablet out of the 30 tablets taken at random, is outside the threshold limits of 75-125% of the expected API amounts. The FDA (FDA, 2014) also adopted the 75-125% threshold limit recommended by the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), which is an acceptance criteria harmonised between 3 different pharmacopoeias namely: the European Pharmacopoeia (Ph Eur), the Japanese Pharmacopoeia and the United States Pharamcopoeia (USP).

It is clear from Figs 3.18-3.21 that higher values for Ind P8 samples occurred when integration modes A and J were employed. Integration modes A and J carry out measurements relative to the axis and not the local baseline of the peaks as with modes B and K. The measurements relative to the axis might therefore be measuring extra background information in addition to the actual data from the peak analysed.

The data obtained from quantitative analysis of paracetamol via the integration method, showed that ATR-FTIR was not only able to identify the presence of the API but also quantify paracetamol amounts. This ATR-FTIR method was also able to identify suspect tablets (Ind P8) needing further analysis. The results therefore highlight the potential of the ATR-FTIR in the screening of medicines. Since the study is aimed at developing rapid analytical methods for the identification of falsified medicines, further analysis on paracetamol tablets was done using the automated Quant 2 facility on the OPUS 7.5 software. This would speed up the process since calibration is automated making the process even simpler. Quant 2 study is considered in the next section.

3.3.5.2 Quantification of Paracetamol by automated Quant 2 analysis (using a multivariate PLS calibration model)

The OPUS 7.5 Quant 2 application automatically employs a partial least square (PLS) regression approach to find the best correlation function between spectral and concentration data matrix. In addition to magnesium stearate used for calibration of paracetamol by integration, maize starch and MCC were also used as excipients for calibration via the Quant 2 method. Unlike with magnesium stearate, both maize starch and MCC were easy to mix with paracetamol but the lower density of MCC may be an issue when trying to ensure good contact on the sampling head.

The approaches assessed with Quant 2 included the use of the 3 individual excipients: MCC, maize starch and magnesium stearate with absorbance area measurements collected for:

- the ranges 1524 - 1493cm^{-1} and 1236 - 1210cm^{-1} corresponding to the 1505cm^{-1} and 1225cm^{-1} peaks,

- the range 1524 – 1210cm^{-1}

- the whole recorded spectral range 4000 – 400cm^{-1}.

Individual calibration curves for paracetamol in the three different excipients, were plotted using the selected spectral ranges.
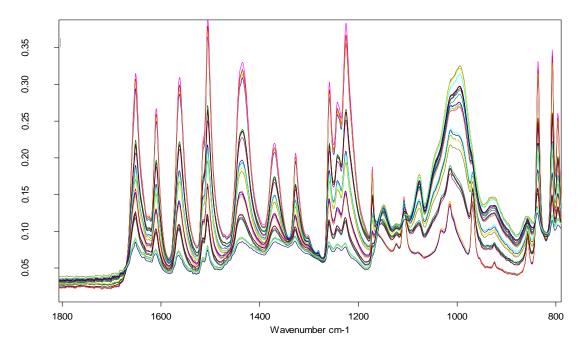
Figure 3. 22 ATR FTIR spectra of Paracetamol in maize starch standards between 1800-800cm^{-1}

Calibration graphs containing 10-90% paracetamol in the selected excipients were produced from recorded spectra (example in Fig. 3.22) with R^2 values between 0.98 and 0.99 for the different combinations as shown in Table 3.11.

Table 3. 11 PLS calibration data for paracetamol in selected excipients and across different ranges

Calibration	Range	Intercept	Slope	Correlation coefficient
Paracetamol in maize starch	1524 - 1493cm^{-1}	0.038	0.999	0.9996
Paracetamol in maize starch	1236 - 1210cm^{-1}	0.214	0.995	0.9976
Paracetamol in maize starch	1524 – 1210cm^{-1}	0.027	0.999	0.9997
Paracetamol in maize starch	4000 – 400cm^{-1}	0.029	0.999	0.9997
Paracetamol in magnesium stearate	1524 - 1493cm^{-1}	1.447	0.974	0.9869
Paracetamol in magnesium stearate	1236 - 1210cm^{-1}	0.248	0.996	0.9978
Paracetamol in magnesium stearate	1524 – 1210cm^{-1}	0.046	0.999	0.9996
Paracetamol in magnesium stearate	4000 – 400cm^{-1}	0.067	0.999	0.9994
Paracetamol in MCC	1524 - 1493cm^{-1}	0.191	0.996	0.9982
Paracetamol in MCC	1236 - 1210cm^{-1}	0.036	0.999	0.9997
Paracetamol in MCC	1524 – 1210cm^{-1}	0.012	1.000	0.9999
Paracetamol in MCC	4000 – 400cm^{-1}	0.062	0.999	0.9994
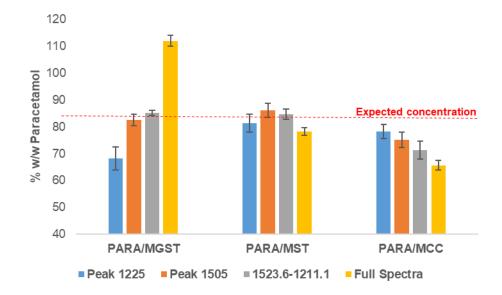
3.3.5.2.1 Methods validation for Quant 2 Analysis

The performance of the developed calibration data was assessed against a set of known typical OTC paracetamol tablets in which the paracetamol level had been measured by UV analysis. The tablets were assessed as containing 84% w/w paracetamol and the performance of the different ATR-FTIR approaches in assessing this value are shown in Table 3.12 and Fig 3.23. As expected the magnesium stearate samples show the widest variation with significant differences within the MCC samples. Furthermore the data reported for the 1505cm^{-1} peak and also when using maize starch as excipient was the most consistent through-out all the measurements. This is evident in the data shown

in Table 3.12 with lower standard deviation (SD) values for data obtained for the peak centred at 1505cm^{-1} and when using maize starch.

Table 3. 12 Assessing the performance of Quant 2 calibration methods for paracetamol in excipients across different spectral ranges

Calibration	Spectral Ranges				
Method	Peak 1225 (% w/w)	Peak 1505 (% w/w)	1523.6-1211.1 (% w/w)	Full Spectra (% w/w)	SD
PARA/MGST	68.1	82.5	85.2	111.9	18
PARA/MST	81.3	86	84.7	78.3	3
PARA/MCC	78.2	75.1	71.3	65.7	5
SD	7	6	8	24	

Where, Para/Mgst= Paracetamol in magnesium stearate; Para/MSt= Paracetamol in Maize Starch; Para/MCC= Paracetamol in Microcrystalline Cellulose. Expected concentration= 83.7 % w/w (results are the mean of 5 replicate readings).

Figure 3. 23 Chart showing measured amounts of paracetamol in tablets based on quantification methods using different absorbance bands and excipients.

As a result of these validation studies, the calibration method based on paracetamol in maize starch for the peak centred at 1505cm^{-1} was used to determine the level of paracetamol in the test tablets. A typical calibration curve for quantification of paracetamol using data for the peak centred on 1505cm^{-1} and covering the range 1524-1493cm^{-1} is shown in Fig 3.24. The R^2 value of

0.9996 demonstrates good correlation between the changes in spectral data with changes in paracetamol concentration. Figure 3.25 is a comparison of the expected (True) paracetamol concentrations with the measured concentrations (Fit) showing close correlation for the data obtained using the PLS calibration model.
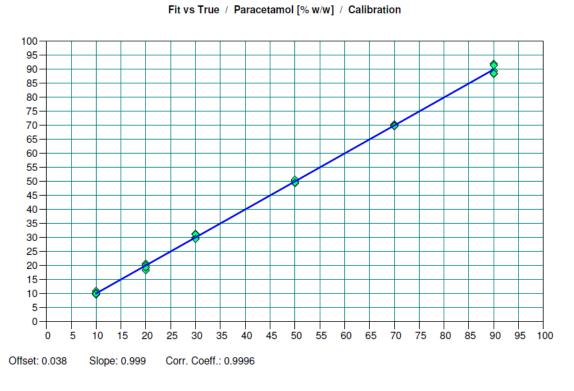
Figure 3. 24 PLS calibration plot over the range 1524-1493cm^{-1} (with peak centred at 1505cm^{-1})

	File Name	True	Fit	Residuum
1	10% PARAM	10	10.9	-0.898
2	10% PARAM	10	9.963	0.0368
3	10% PARAM	10	9.642	0.358
4	10% PARAM	10	10.26	-0.265
5	10% PARAM	10	9.904	0.0956
6	20% PARAM	20	20.63	-0.633
7	20% PARAM	20	20.07	-0.0725
8	20% PARAM	20	18.35	1.65
9	20% PARAM	20	20.07	-0.0745
10	20% PARAM	20	19.36	0.64
11	30% PARAM	30	29.87	0.134
12	30% PARAM	30	29.85	0.153
13	30% PARAM	30	31.29	-1.29
14	30% PARAM	30	31.12	-1.12
15	30% PARAM	30	29.48	0.517
16	50% PARAM	50	49.53	0.469
17	50% PARAM	50	50.16	-0.164
18	50% PARAM	50	49.41	0.585
19	50% PARAM	50	50.61	-0.612
20	50% PARAM	50	49.55	0.454
21	70% PARAM	70	70.36	-0.355
22	70% PARAM	70	70.23	-0.227
23	70% PARAM	70	70.06	-0.0599
24	70% PARAM	70	69.75	0.254
25	70% PARAM	70	69.85	0.154
26	90% PARAM	90	89.41	0.592
27	90% PARAM	90	88.39	1.61
28	90% PARAM	90	91.94	-1.94
29	90% PARAM	90	91.45	-1.45
30	90% PARAM	90	88.53	1.47

Figure 3. 25 Comparison of measured versus expected amounts of paracetamol in calibration mixtures based on the range 1524-1493cm^{-1}.

3.3.5.2.2 Analysis of paracetamol tablet samples using Quant 2 multivariate PLS calibration model

Individual tablets with paracetamol as single API from each of the separate samples were crushed and subjected to ATR-FTIR analyses. Quantification of paracetamol was based on the calibration for paracetamol in maize starch over the range 1524-1493cm^{-1} as mentioned earlier. The mean results for each sample set are recorded in Table 3.13. UV analysis was also conducted for validation of the ATR-FTIR paracetamol data (Chapter 6). Most of the ATR-FTIR data agreed within the general limits of ±15% of the expected dosage of the

paracetamol tablets (BP, 2017) but some significant differences were identified. Whilst there was close agreement for many of the samples the ATR-FTIR Quant 2 approach gave high values for some samples from Belgium (Bel P1T1 and Bel P1T2), India (Ind P8T1 and P8T2) and Cyprus (Cyp P1T1 and Cyp P1T2). Low levels of API for a tablet sample from Pakistan (Pak P1T1) were also obtained from Quant 2 ATR measurements.

The ATR-FTIR data is displayed as a plot of the ratio of expected to measured paracetamol levels versus sample origin as shown in Fig 3.26. This figure clearly shows that whilst the majority of tablet samples were within the general acceptable limits, seven samples merited further investigation. The first of these, the Belgian samples, gave high results using the Quant 2 ATR-FTIR tests but results based on quantification by the Integration mode were within allowed limits for these tablets. The Belgian samples would therefore be allowed to pass a screening test. Two of the Indian samples tested high on both Integration and Quant 2 ATR-FTIR methods for quantification but the ATR signal suggested the presence of other material in the fingerprint region and would therefore fail a screening test. Further investigation of the reason for failure would be required.

Table 3. 13 Summary of quantitative results using the Quant 2 method for paracetamol tablet analysis from around the world (results are the mean of 5 replicate samples)

Region	Country (Number of Tablets)	Tablets Analysed	ATR-FTIR Measured Content (mg)	Expected Amount (mg)
Europe (n=14)	UK (n=2)	UK P1T1	514±15	500
		UK P1T2	505±15	
	Cyprus (n=2)	Cyp P1T1	594±14	
		Cyp P1T2	591±5	
	Switzerland (n=2)	Swz P1T1	510±10	
		Swz P1T2	505±9	
	Spain (n=6)	Spn P1T1	514±15	
		Spn P1T2	497±18	
		Spn P2T1	663±19	650
		Spn P2T2	683±10	
		Spn P3T1	921±41	1000
		Spn P3T2	980±20	
	Belgium (n=2)	Bel P1T1	1196±55	
		Bel P1T2	1178±24	
Asia & Middle East (n=37)	India (n=19)	Ind P1T1	565±23	500
		Ind P1T2	558±21	
		Ind P2T1	462±11	
		Ind P2T2	469±16	
		Ind P3T1	493±16	
		Ind P3T2	500±7	
		Ind P3T3	493±9	
		Ind P4T1	489±8	
		Ind P4T2	481±25	
		Ind P5T1	533±20	
		Ind P5T2	542±16	
		Ind P5T3	509±25	
		Ind P6T1	545±6	
		Ind P6T2	540±8	
		Ind P7T1	530±23	
		Ind P7T2	529±15	
		Ind P8T1	464±12	325
		Ind P8T2	472±4	
		Ind P9T1	651±17	650
	Pakistan (n=2)	Pak P1T1	373±14	
		Pak P1T2	451±8	
	Nepal (n=8)	Nep P1T1	532±13	500
		Nep P1T2	526±18	
		Nep P2T1	501±17	
		Nep P2T2	461±17	
		Nep P3T1	535±13	
		Nep P3T2	555±7	
		Nep P4T1	499±24	
		Nep P4T2	536±11	

Table 3.13 (continued)

Region	Country (Number of Tablets)	Tablets Analysed	ATR-FTIR Measured Content (mg)	Expected Amount (mg)
	China (n=4)	Chn P1T1	494±21	
		Chn P1T2	500±9	
		Chn P2T1	516±11	
		Chn P2T2	546±6	
	UAE (n=4)	UAE P1T1	527±8	
		UAE P1T2	541±17	
		UAE P2T1	499±12	
		UAE P2T2	520±16	
Africa and Caribbean Islands (n=18)	Rwanda (n=6)	Rwa P1T1	508±9	500
		Rwa P2T1	525±8	
		Rwa P3T1	486±14	
		Rwa P3T2	536±11	
		Rwa P4T1	533±10	
		Rwa P4T2	519±14	
	Ghana (n=4)	Gha P1T1	496±20	
		Gha P1T2	510±11	
		Gha P2T1	530±5	
		Gha P2T2	490±13	
	Jamaica (n=4)	Jam P1T1	481±11	
		Jam P1T2	498±15	
		Jam P2T1	509±10	
		Jam P2T2	517±15	
	Nigeria (n=4)	Nig P1T1	488±13	
		Nig P1T2	503±10	
		Nig P2T1	522±11	
		Nig P2T2	519±33	

For the tablet sample from Pakistan (Pak P1T1), Quant 2 ATR-FTIR analyses showed clear evidence of insufficient levels of paracetamol. The samples from Cyprus would also fail a screening test with paracetamol levels higher than is generally allowed considering the Quant data in Fig 3.26. It is however important to note that the tablets from Cyprus were indicated as having glycerol as an excipient which was not the case for the other tablets assessed. Glycerol is not commonly used as a pharmaceutical excipient and its FTIR spectrum shows it has significant absorbance in the spectral range used for analysis of

paracetamol (1524-1493cm^{-1}) (Nanda et al, 2014). This could be the reason for the higher than expected ATR-FTIR signals for the Cyprus tablets. Consequently, this set of tablets identified as suspect, would require further analysis.

Based on the results obtained from the analysis of single API tablets containing paracetamol, ATR-FTIR spectroscopy showed its potential in rapidly identifying and quantifying paracetamol in tablet dosage forms. It was also able to identify suspect tablets based on the data obtained.
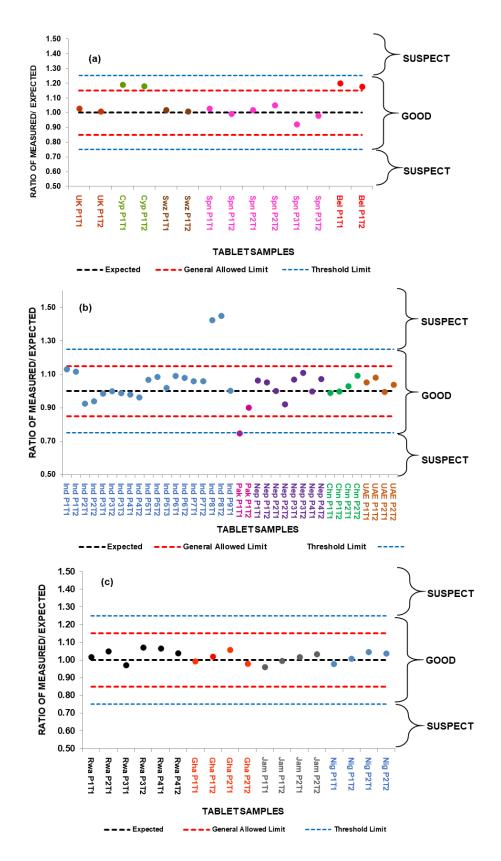
Figure 3. 26 Ratio of measured to expected amounts of Paracetamol in 69 samples of tablets from around the world based on calibration for peak at 1505cm^{-1} (a) 14 tablet samples from around Europe (b) 37 tablet samples from Asia and the Middle East (c) 18 tablet samples from around Africa and the Caribbean Islands.

Furthermore, the Quant 2 multivariate PLS calibration model was assessed against multiple API tablets containing paracetamol. The tablets were analysed using the same protocol for the single API tablets and with the validated calibration method for paracetamol in maize starch for the peak centred at 1505cm^{-1}. Quantitative data obtained for paracetamol are shown Table 3.14.

Table 3. 14 Measured amounts of paracetamol in multiple API tablets from the UK based on Quant 2 ATR-FTIR analysis

Tablet(s)	Measured amount of paracetamol (mg)	Amount of API expected in tablet (mg)
PC A# 1	502± 21	Paracetamol-500,
PC A# 2	504±18	Caffeine- 65
PC B # 1	493±9	Paracetamol- 500
PC B # 2	490±14	Caffeine- 65
PC C# 1	469±14	Paracetamol- 500
PC C# 2	480±10	Caffeine- 65
PAC A#1	132±7	Paracetamol- 200, Aspirin- 300, Caffeine- 45

Quantitative results for paracetamol in Table 3.14 indicate that paracetamol levels were generally within allowed limits for most of the multiple API tablets assessed. However, measured paracetamol amount for the 3 API tablet (PAC A#1) was lower than the threshold limits (75% of expected content). A comparison of the tablet spectra with reference spectra for paracetamol indicates notable absorbance in the spectral region being assessed as shown in Fig 3.27.
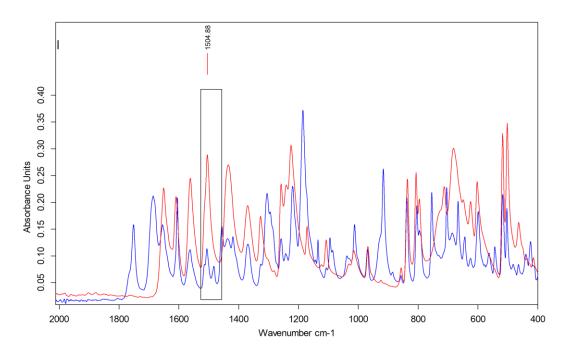
Figure 3. 27 Comparison of ATR-FTIR spectra for 3 API tablet (blue) with paracetamol reference (red) showing absorbance from other material in spectra range assessed.

Further comparison of the 3-API tablet spectra with the other APIs expected in the tablet suggests the material with absorbance in the selected region for measurement is aspirin shown in Fig 3.28.
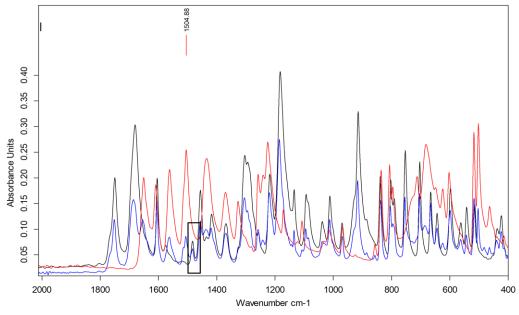
Figure 3. 28 Comparison of ATR-FTIR spectra for 3 API tablet (blue), paracetamol reference (red) and aspirin reference (black) identifying peak interfering with measurement range to be aspirin

Aspirin was also expected in higher concentrations (48% w/w) than paracetamol (32% w/w) in the tablet. Therefore quantification using ATR-FTIR might be a challenge in multiple API tablets containing more than two APIs especially when the target API is present in lower concentrations. However, for the purposes of this research considering simple and quick YES or NO assessments of tablet medicines, ATR-FTIR provides a valuable option in this regard.

3.3.5.2.3 Analysis of chloroquine tablet samples using Quant 2 multivariate PLS calibration model

The Quant 2 multivariate PLS calibration model was assessed against chloroquine tablets from around the world. Quantification of chloroquine in tablets was based on validated methods for paracetamol. Hence, calibration for chloroquine in maize starch for the spectral range 1256-1173cm^{-1} corresponding to the peak at 1212cm^{-1} (identified as characteristic peak) was employed in the assessment of chloroquine tablet samples. PLS calibration data for chloroquine is shown in Figs 3.29 and 3.30. The R^2 value of 0.9959 showed good correlation between spectral changes and changes in chloroquine concentration.
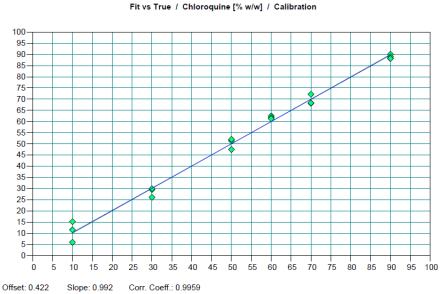
Figure 3. 29 PLS calibration plot for chloroquine over the range 1256-1173cm^{-1} (for peak at 1212cm^{-1}) using the Bruker OPUS 7.5 Quant 2 software. Data represents three replicate samples with maize starch as excipient.

	File Name	True	Fit	Residuum
1	10% chloro	10	15.16	-5.16
2	10% chloro	10	11.49	-1.49
3	10% chloro	10	5.927	4.07
4	30% chloro	30	26.05	3.95
5	30% chloro	30	29.51	0.486
6	30% chloro	30	29.92	0.0786
7	50% chloro	50	51.37	-1.37
8	50% chloro	50	47.46	2.54
9	50% chloro	50	51.97	-1.97
10	60% chloro	60	62.49	-2.49
11	60% chloro	60	61.71	-1.71
12	60% chloro	60	61.23	-1.23
13	70% chloro	70	68.1	1.9
14	70% chloro	70	68.25	1.75
15	70% chloro	70	72.17	-2.17
16	90% chloro	90	90.08	-0.0758
17	90% chloro	90	88.95	1.05
18	90% chloro	90	88.17	1.83

Figure 3. 30 Comparison of measured (Fit) versus expected (True) amounts of chloroquine in calibration mixtures over the range 1256-1173cm^{-1}.

Calibration data obtained was then used in the assessment of tablets containing chloroquine with a summary of the quantitative results shown in Table 3.15.

Table 3. 15 Summary of quantitative results for chloroquine tablet analysis form around the world (results are the mean of 3 replicate samples)

Country (Number of Tablets)	Tablets Analysed	ATR-FTIR Measured Content (mg)	Expected Amount (mg)	Weight of Tablet
UK (n=2)	UK C1T1	240±5	250	338.8
	UK C1T2	257±23	250	370.4
Belgium (n=2)	Bel C1T1	301±14	250	337.6
	Bel C1T2	288±5	250	332.7
India (n=5)	Ind C1T1	321±11	250	320.5
	Ind C1T2	311±3	250	317.4
	Ind C2T1	348±1	250	311.6
	Ind C2T2	339±7	250	306.5
	Ind C3T1	468±7	500	627.1
Nepal (n=2)	Nep C1T1	308±17	250	314.6
	Nep C1T2	314±2	250	315.0
Kenya (n=1)	Ken C1T1	1517±81	250	501.7
Nigeria (n=1)	Nig C1T1	287±8	400	480.4

Results in Table 3.15 show generally high levels of chloroquine in the tablets except with the UK tablets. The tablet from Nigeria also showed values lower than the threshold limit. This could be due to differences in the more complex formulation compared to the calibration made from binary mixtures. In addition, spectral peaks for

chloroquine are not well-defined (separated) as with the paracetamol peaks. This could be a challenge when comparing peaks for identification.

Although results obtained for most of the tablets were generally high, the measured values of chloroquine in the tablet sample from Kenya (Ken C1T1) were anomalously higher than all the other tablets. The values for Ken C1T1 were about five times the total weight of the tablet. The results are better appreciated with a plot of the ratio of measured to expected chloroquine amounts in the tablets as shown in Fig 3.31 (a and b).
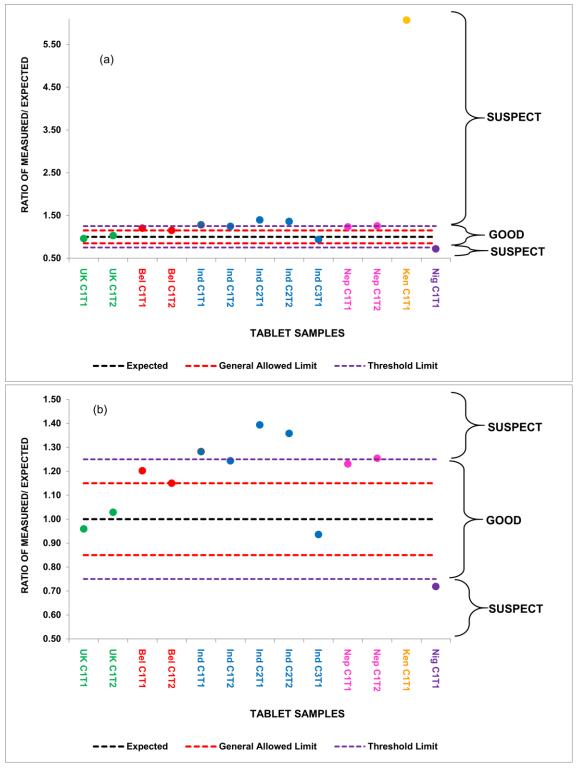
Figure 3. 31 Ratio of measured to expected amounts of chloroquine in tablet samples from around the world based on peak over the range 1256-1173cm^{-1}.(for the peak centred at 1212cm^{-1}). (a) with Ken C1T1 tablet sample (b) zoomed in excluding Ken C1T1 sample

Based on the results obtained for the tablet from Kenya (Ken C1T1), the tablet spectra was compared with the UK tablet and the chloroquine reference as shown in Fig 3.32.
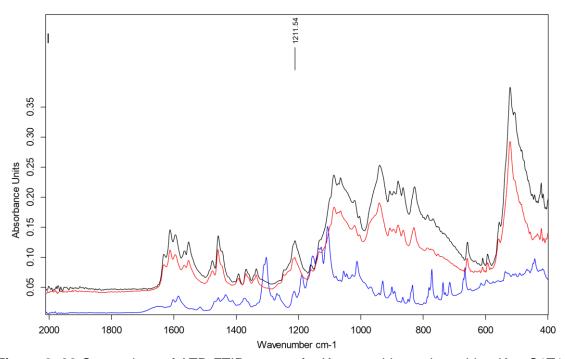
Figure 3. 32 Comparison of ATR-FTIR spectra for Kenyan chloroquine tablet- Ken C1T1 (blue), UK chloroquine tablet- UK C1T1 (red) and chloroquine reference (black) identifying peak showing differences in Ken C1T1 spectra.

Figure 3.32 shows marked differences between the FTIR spectra for the chloroquine tablet from Kenya and the spectra for the UK tablet and chloroquine reference. Consequently, the tablet from Kenya (Ken C1T1) would fail a screening test. It is also important to note that the expected concentration of chloroquine for all other chloroquine tablets was between 67-82% w/w. On the other hand, chloroquine levels in the Kenyan tablet were expected to be about 50% w/w. This difference in expected chloroquine levels implies that the Kenyan tablet would have higher excipient levels than with the other chloroquine tablets assessed. This could be the reason for the difference in spectra. Overall, the Kenyan chloroquine tablet would fail a screening test needing further analysis.

Although there were limitations in quantifying chloroquine tablets using the ATR-FTIR method (as with the 3-API tablet containing paracetamol), the ATR-FTIR method was able to identify suspect samples like the Ken C1T1. Identifying the suspect sample is priority in the quick screening of medicines and this is the purpose for which the ATR-FTIR is being assessed.

One advantage of the approach taken here, recording reference and sample spectra over the range 4000-400cm^{-1} means that the Spectrum Search capabilities of the OPUS 7.5 software can be used. This approach should allow identification of the compound/s contributing to anomalously high peak areas in characteristic spectral ranges for measurement of target APIs as demonstrated in this research.

The limit of quantification (LOQ) of the method is dependent on the type of excipient used and the weight of the tablet and so is not fixed for all investigations. Consequently, quantification of APIs in the presence of excipients was possible down to concentrations between 10-20% w/w. These results were based on the excipients considered in the study and the actual weight of the tablets.

Overall, this novel method developed based on the ATR-FTIR technique has a few advantages as it not only identifies the presence or absence of the API but also indicates how much of the API could be in the tablet in a short time. It also reduces exposure to toxic chemicals used in solvent extraction of the API(s) for analysis using conventional pharmacopoeia approved methods. Its portability makes it valuable for in-field analysis such as quality control by pharmaceutical companies and post marketing surveillance by regulatory bodies. Furthermore, its potential in the identification of FSMs with incorrect amounts of API will also reduce the public health risk posed by these medications such as antimicrobial resistance and ultimately therapeutic failure. The dangers of under dosing or

exceeding the allowed limits for API(s) in medication especially those with a narrow therapeutic range will also be reduced (Blackstone et al, 2014). Economically, funds spent on these medications which are toxic or have no therapeutic effect will be reduced.

3. 4 Conclusion

Based on the rapid ATR-FTIR method examined, this study was able to identify 10% (7 tablet samples) of the paracetamol tablet samples and 8% (1 tablet) of the antimalarial chloroquine tablets as suspect samples needing further analysis. These results are also in line with the WHO estimates of the level of FSMs globally, highlighting the wide range of samples assessed.

This study shows that the simple ATR-FTIR approach employed has the capacity to rapidly identify and also quantify paracetamol in the presence of excipients and other APIs. The whole process of crushing, identifying and quantifying a tablet would take about 5 minutes per tablet sample after the method has been optimised. However, this is not meant to replace the more established and highly sensitive conventional solvent extraction methods but to serve as a valuable alternative to the expensive Raman systems as an in-field technique for quick screening of medicines. It is also a green technique as the elimination of solvent extraction of APIs reduces the amounts of toxic chemicals used thus, reducing chemical waste. Furthermore, the technique will enable quick withdrawal of counterfeit medicines from the market thereby reducing the threat to public health. It is also relatively inexpensive and easy to use compared to the pharmacopoeia approved techniques so can also be used in developing countries where facilities are not readily available. This approach employed in the identification and quantification of paracetamol could potentially be applied in the analysis of other APIs in tablet dosage forms.

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CHAPTER FOUR

Raman Spectroscopy for rapid discrimination of tablet medicines

4.1 Introduction

As mentioned in Chapter 2, several techniques can, and have been used for the confirmation of FSMs, depending on the type of information needed. Among these techniques, Raman spectroscopy is one of the most commonly used for assessing FSMs (Dégardin et al, 2011; Kwok and Taylor, 2012; Neuberger and Neusüß, 2015; Peters et al, 2016) because it is largely non-destructive and sample measurement can be made directly through packaging. In addition, Raman spectroscopy as with other spectroscopic techniques like the ATR-FTIR, does not require sample preparation and is environmentally friendly. These features of the Raman technique along with availability of portable handheld models of the equipment makes it valuable for fast in-field screening of medicines (Dégardin et al, 2017).

Raman spectroscopy investigates vibrational transitions in molecules and is similar to both mid and near infrared (IR) spectroscopy. However, Raman spectroscopy depends on light scattering while IR spectroscopy is hinged on absorption. Monochromatic laser light (whether visible or near-IR) is used to irradiate samples in Raman spectroscopy. At the same wave number, most of the light with which the sample is irradiated is scattered back and this is called Rayleigh scattering (Gala and Chauhan, 2015). However, in a very small percentage of cases this light is scattered at wave numbers which are slightly higher (anti-Stokes scattering) or, less often, at lower wave numbers (Stokes scattering). Collectively, this change from the exact wave number of irradiation is known as Raman scattering, although Stokes scattering is more commonly reported since anti-Stokes is much weaker. The difference between the wavenumber of the incident radiation and the scattered radiation amounts to vibrational energy transitions. Consequently, it is this vibrational energy change, indicated at the corresponding wavenumber, Raman spectroscopy detects (Strachan et al, 2007; Gala and Chauhan, 2015). Not only are there vibrational transitions within the molecule but changes

between electronic states can also occur giving rise to fluorescence. Fluorescence is evident as a non-uniform increase in the base line level in the recorded spectrum. Sample fluorescence masks the Raman response and posed a huge challenge for the use of Raman spectrometers in the pharmaceutical industry until the advent of novel experimental methods such as charge coupled device (CCD)-based experiments and Fourier transform (FT) spectrometers.

Raman scattering is dependent on a change in the electronic polarisability of a molecule along with the vibrational mode. Polarisability refers to the electron charge being susceptible to having an induced dipole within it. As a result, large polarisability changes and in turn strong Raman scattering, are generally linked to molecules with delocalised electron systems (for example, aromatic conjugated systems). On the other hand, polar molecules exhibit the strongest IR absorption. Therefore, information provided by IR and Raman spectroscopy are complementary (Strachan et al, 2007; Gala and Chauhan, 2015).

Several practical features of Raman spectroscopy make it attractive for solid-state analysis in the pharmaceutical industry. Most active pharmaceutical ingredients (APIs) are aromatics making them strong Raman scatterers. In contrast, many excipients are aliphatic and as a result produce much weaker Raman spectra. Thus, Raman spectroscopy has its advantages compared to mid- and near-IR spectroscopy when studying APIs in formulations, especially in low dosage formulations. Furthermore, while it may be very challenging to conduct mid- and near-IR spectroscopic analyses in aqueous environments, this will not be the case with Raman spectroscopy since water is a very poor Raman scatterer. Consequently, Raman spectroscopy will be suitable for solid-state analysis even in the presence of water (Strachan et al, 2007; Gala and Chauhan, 2015).

Several applications of Raman spectroscopy in pharmaceutical research have been evaluated from drug formulation to quality control in the pharmaceutical industry (Izake, 2010; Gala and Chauhan, 2015). Kalyanaraman et al (2012) in their review discuss the general use of the Raman device in the detection of falsified medicines. Luczak and Kalyanaraman (2014), evaluated results for falsified medicines of three products using both portable and bench top Raman technologies. A case study assessing the use of the Raman handheld device for the authentication of medicines in Nigeria is presented by Spink et al (2016). Hajjou et al (2013) evaluated the specificity and precision of the handheld Raman device in the screening of six pharmaceutical products including analgesics, antimalarial and antidiarrheal medicines. Assi (2016) successfully used dual laser handheld Raman spectroscopy for the authentication and chemical characterisation of type 5 phosphodiesterase inhibitors with concentrations of API in the range 5-15% w/w.

Raman spectroscopy has its own draw backs such as fluorescence of some samples which hampers the accuracy of analytical data and the possibility of samples getting burnt due to overheating when illuminated with the laser beam. Assi (2016) suggests different laser excitation wavelengths should be explored to identify wavelength at which fluorescence is least observed. The difficulty however would be getting a balance between the right laser excitation wavelength and sensitivity. Overheating or burning of test samples while using Raman spectroscopy is more likely when the sample is illuminated with the laser beam on the same spot for a long period. Shin and Chung (2013) proposed rotating of test samples during analysis to reduce overheating or burning of the sample. Beyond qualitative analysis and general characterisation of medicines, Raman spectroscopy has proven useful in the quantitative determination of APIs in solid dosage forms. Just as with the optimised methods based on ATR-FTIR in Chapter 3, quantitative determination of tablet samples using the Raman device has

been done mostly in combination with chemometric analysis (Strachan et al, 2007; Fransson et al, 2010; Fraser et al, 2013; Shin and Chung, 2013; Griffen et al, 2015). Cailletaud et al (2017) in their review and Zheng et al (2014) considered different ways of improving sensitivity of the Raman device for low dosage quantification. Tondepu et al (2017) considered the use of portable Raman spectroscopy for the qualitative analysis and quantification of antiviral and antibiotic drugs but solvent dissolution of samples was involved. On the other hand, Rebiere et al (2018) applied Raman spectroscopy and chemometric methods in the screening and direct quantification of Viagra® and Plavix® test tablet samples and their results showed the deviation of quantitative data from the reference method was between −15% and +24% of the API content which was considered acceptable for screening purposes.

Besides the quantitative abilities of the Raman device, it can also be used in forensic analysis of medicines. Neuberger and Neusüß (2015) considered the use of Raman spectroscopy in the characterisation of medicines based on differences in the their excipients, API amounts and mixing quality but they achieved this by extensive pre-processing of data including normalisation, baseline correction of the spectra and a reduction in spectral range used for analysis. For valuable forensic information to be presented in court for instance, it is important that analysis is done using the raw data to obtain as much information as possible.

This chapter explores the potential of Raman spectroscopy in forensic analysis of FSMs, its ability to discriminate between different batches of medicines and characterise them from raw data obtained without any data pre-processing. This research also proposes its potential use in collecting quick valuable forensic information tenable in a law court during investigations.

4.2 Materials and Method

4.2.1 Materials

Pharmaceutical tablet formulations containing paracetamol and the antimalarial medicine, chloroquine were obtained for analysis from various locations across the globe. Table 4 .1 and 4.2 outlines details of paracetamol and chloroquine tablets accessed respectively.

Table 4. 1 Paracetamol tablets analysed and their origin

Region	Country (Number of Tablets)	Tablets Analysed*	Expected Amount (mg)
Europe (n=16)	UK (n=2)	UK P2T1, UK P2T2	500
	Cyprus (n=2)	Cyp P1T3, Cyp P1T4	
	Belgium (n=2)	Bel P2T1, Bel P2T2	
	Spain (n=6)	Spn P1T3, Spn P1T4	
		Spn P2T3, Spn P2T4	650
		Spn P3T3, Spn P3T4	1000
	Belgium (n=2)	Bel P1T3, Bel P1T4	
Asia (n=34)	India (n=14)	Ind P11T1, Ind P11T2, Ind P14T1, Ind P14T2, Ind P15T1, Ind P15T2, Ind P16T1, Ind P16T2	650
		Ind P10T1, Ind P10T2	500
		Ind P12T1, Ind P12T2	300
		Ind P13T1, Ind P13T2	250+15mg Caffeine- Yellow
	Indonesia (n=1)	Indn P1T1	500
	Nepal (n=8)	Nep P1T3, Nep P1T4, Nep P2T3, Nep P2T4, Nep P3T3, Nep P3T4, Nep P4T3, Nep P4T4	
	Thailand (2)	Thai P1T1, Thai P1T2	
	UAE (n=2)	UAE P1T3, UAE P1T4	
	China (n=7)	Chn P1T3, Chn P1T4	
		Chn P2T3, Chn P2T4	500+65mg Caffeine- Pink
		Chn P3T1, Chn P3T2,	650
		Chn P4T1	300
Africa (n=16)	Rwanda (n=4)	Rwa P1T2, Rwa P2T2, Rwa P3T3, Rwa P4T3	500
	Ghana (n=4)	Gha P1T3, Gha P1T4, Gha P2T3, Gha P2T4,	
	Nigeria (n=4)	Nig P1T3, Nig P1T4, Nig P2T3, Nig P2T4,	
	South Africa (n=2)	SthAfr P1T1, SthAfr P1T2	
	Kenya (n=2)	Ken P1T1, Ken P1T2	500+65mg Caffeine

Note: n = number of samples, *P1T1= Pack 1 Tablet 1 and so on. All tablets are white except where colour is indicated

Table 4. 2 Chloroquine Tablet Samples analysed and their origin

Country	Tablet	Expected Amount (mg)
UK (3)	UK C1T3, UK C1T4 UK C2T1	250
Nepal (2)	NEP C1T3, NEP C1T4	250
India (4)	IND C3T2, IND C3T3	500
	IND C2T3, IND C2T4	250
Nigeria (1)	NIG C1T2	400

Note: n = number of samples, *C1T1= Pack 1 Tablet 1 and so on. All tablets are white except where colour is indicated

4.2.2 Instrumentation

The Foram 785 HP bench top Raman spectrometer by Foster + Freeman, UK was used to collect spectra. The software (Foram 3 by Foster + Freeman) was used to control the spectrometer during acquisition of spectral data and analysis. In addition, the Foram 3 software package had local spectral libraries (including APIs, and excipients) and chemometric algorithms (Metrohm, Runcorn, UK) such as principal component analysis (PCA).

Spectrometer parameters used are outlined below:

Laser excitation wavelength: 785nm

Wavenumber range: 300-3000cm^{-1}

Scan time - 3 seconds

Detector: Front illuminated charge-coupled device (CCD)

Laser power output: 2.8mW at 100% laser output

4.3 Method

Calibration of the Raman system was done before any tablet sample measurements using a polystyrene reference sample. Test samples were measured in three formats:

- Whole tablets removed from the packaging

- Powder samples from crushed tablets

- Tablet samples retained in the original blister packaging.

Samples were measured by placing them on the XYZ translation stage which allows adjusting the tablet samples in different directions to enable good measurement or well defined Raman spectra using the integral video microscope. Each tablet was measured in triplicate from different spots on the tablet surface to improve sample representation. All measurements were taken from the plain, unmarked (without embossing or debossing) side of the each tablet to ensure the laser beams was focussed accurately on the tablet/sample surface. For powders the sample was placed on a slide to help position it under the lens. For the 'in-package' samples care was needed to ensure the laser beam was focussed on the tablet surface and not the packaging. Background spectral data from the packaging was also collected. The 5x objective lens was used for locating the sample while 20x lenses were used for collecting Raman spectra. Raw data was collected for each sample, recorded and subsequently background corrected using the inboard software. The raw data was also subjected to automatic PCA analysis using the Foram 3 software.

4.4 Results and Discussion

4.4.1 Raman Analysis of Paracetamol Tablet Samples
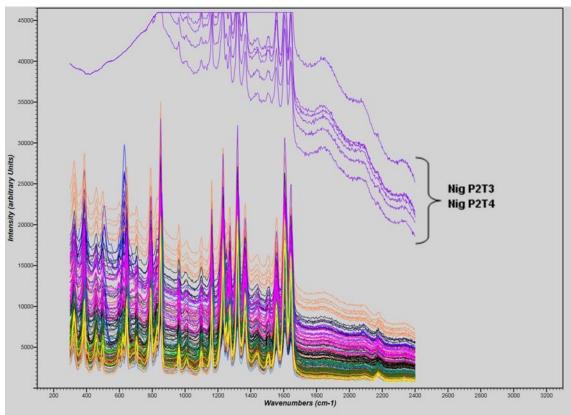
Figure 4. 1 Raman spectra for all paracetamol tablets assessed- Non Baseline Corrected

Figure 4.1 is an overlay of Raman spectra for all paracetamol tablets assessed. The similarity in most of the spectra suggests the presence of the same API in the tablets. The only spectra with marked differences from the other tablets are those at the top of the figure identified as the spectra for tablet samples Nig P2T3 and Nig P2T4 from Nigeria. This difference in spectra could be due to fluorescence (a problem with Raman device mentioned earlier) with any of the excipients in the tablets. Therefore, by visual observation of spectra as presented in Fig 4.1, the tablet samples could be said to contain the same API (paracetamol), with tablets, Nig P2T3 and Nig P2T4 identified as being suspect samples needing further analysis. Inspection of Table 4.1 reveals that several tablet samples contain both paracetamol and caffeine but these cannot be identified in Fig 4.1. Furthermore two of the samples were coloured and these were not

differentiated in Fig 4.1. Raman alone is insensitive to small differences which may be readily revealed by Principal Component Analysis (PCA) of the raw data.

4.4.1.1 Principal Component Analysis of Paracetamol Test Tablet Samples

Figure 4.2 shows a principal component plot of all paracetamol tablets assessed with the test tablets separated into different groups based on the spectra presented. For forensic data, it is important that there is minimal manipulation or pre-processing of the initial data obtained so raw (non-baseline corrected) spectra for test tablets were used for initial PCA study.
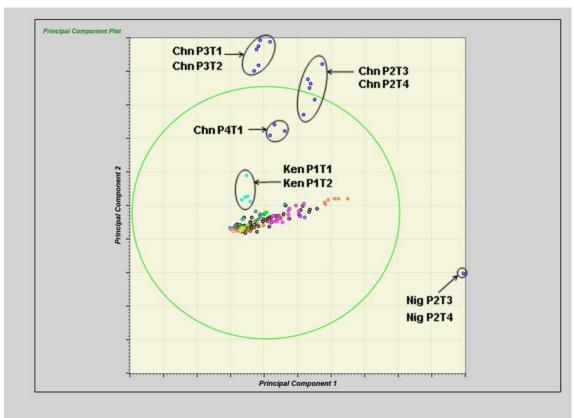
Figure 4. 2 PCA for all paracetamol tablets assessed- Non-Baseline Corrected

The data in Fig 4.2 indicates a similarity between most of the test samples analysed as highlighted by the cluster in the middle of the circle. This similarity can be traced to the presence of the API, paracetamol in the test tablets. However, more variation is observed with data from the PCA study since more tablets (in addition to the 2 identified by visual observation of spectral data in 4.4.1) are highlighted to be outside

the general group in the middle of the circle. It was therefore important to understand the basis for the exclusion of the tablets found outside the general group.

As indicated in Table 4.1, tablets from packs identified as Chn P2 and Ind P13 (in general group) are coloured. This could be a basis for discrimination by PCA since all other tablets are white. Interestingly, Chn P2 tablets are some of those outside of the general group. On the other hand, Ind P13 tablets are highlighted as part of the general group suggesting there might be other properties that make them closer related to the general group such as having similar excipients in addition to the paracetamol API present. Nevertheless, for forensic investigation purposes on the field, coloured samples can be spotted by visual observation and so will be removed. Figure 4.3 shows a principal component plot of all the remaining white paracetamol test tablets.
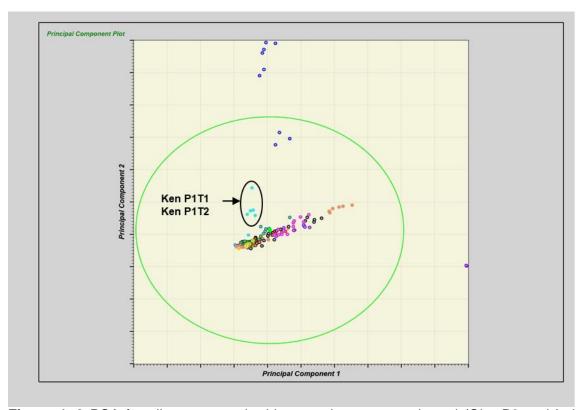
Figure 4. 3 PCA for all paracetamol tablet samples except coloured (Chn P2 and Ind P13) tablets- Non-Baseline Corrected.

Figure 4.3 shows once again that most test tablets fall within the same cluster in the middle of the circle confirming the similarity in the tablets. With the coloured samples

excluded from the white test tablet samples, fewer test tablets were found outside of the general group as expected.

Another basis for exclusion of samples was the presence of other APIs apart from paracetamol and as highlighted in Table 4.1, test tablets from the pack Ken P1 contained caffeine in addition to paracetamol. For this reason, Ken P1 samples were again excluded from PCA study with details shown in Fig 4.4.
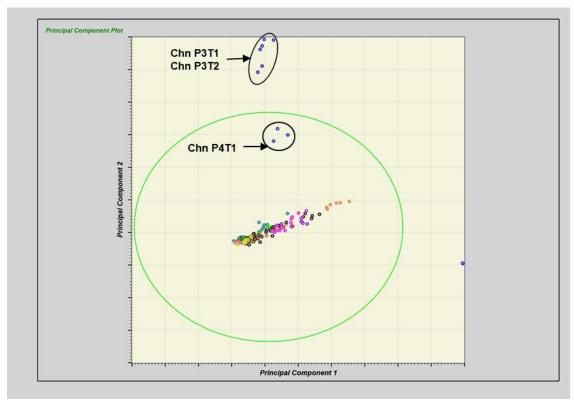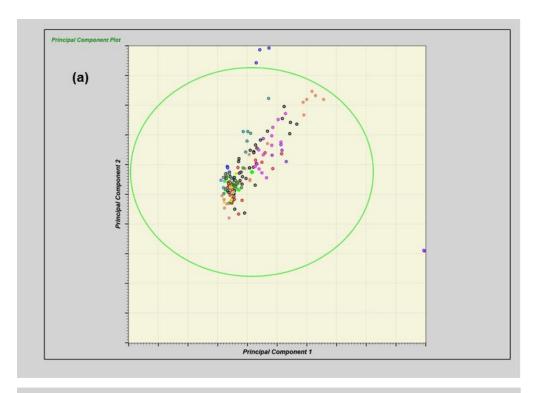
Figure 4. 4 PCA for all paracetamol tablets in Fig. 4.3 not containing other APIs like caffeine (Ken P1) – Non-Baseline Corrected

Figure 4.4 shows further reduction in the number of outliers based on the PCA study with exclusion of the Ken P1 test tablet samples containing caffeine in addition to paracetamol.

Test tablets were then assessed once again in the PCA study with outlier samples from Chn P3 and Chn P4 excluded as shown in Fig 4.5a and 4.5b respectively.
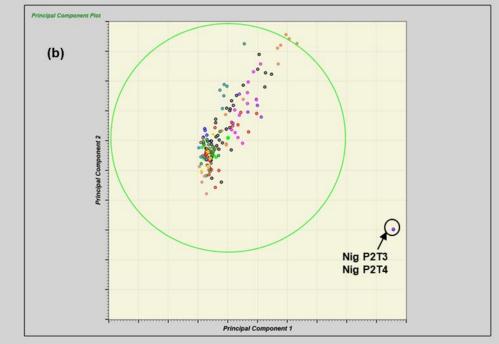
Figure 4. 5 (a) PCA for all paracetamol tablets in Fig 4.4 without outlier Chinese samples (Chn P3T1 and Chn P3T2) – Non-Baseline Corrected (b**)** PCA for all paracetamol tablets in Fig. 4.5a without outlier Chinese sample (Chn P4T1) – Non-Baseline Corrected

It is not clear why the Chn P3 and Chn P4 samples are highlighted as significantly

different from the rest of the tablets assessed. However, it is worthy of note that though

Chn P3 and Chn P4 tablet samples were obtained from different locations in China and

were expected to contain different dosages of paracetamol (650 and 300mg respectively), they are produced by the same manufacturer. Therefore, there might be similarities between Chn P3 and Chn P4 and as such, the basis for the exclusion of these tablets by the PCA study might be the same.

The next exclusion criteria was based on the initial visual inspection of test sample spectra which indicated Nig P2 test tablet samples as being different from the others and requiring further analysis. These test tablets (Nig P2) were therefore taken off the principal component plot as shown in Fig 4.5b resulting in the principal component plot in Fig. 4.6.
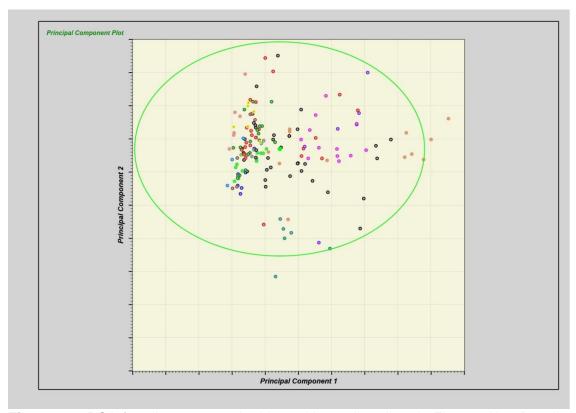
Figure 4. 6 PCA for all paracetamol tablets without all outliers in Fig 4.2- Non-Baseline Corrected

The principal component plot in Fig 4.6 indicates the remaining test tablet samples generally fall within the same group since samples are spread across the circle. The few cases spotted outside the circle could be due to slight variation in the spectra

recorded from different portions of a tablet since data from the same tablet spectra are also found within the circle.

Generally, PCA study was able to confirm similarity between test tablet samples based on the presence of the expected API (paracetamol) and also identify marked differences in several tablets; some of which were not identified by visual observation of spectra. Raman spectroscopy and chemometrics has been used extensively in the quantitative analysis of tablet medicines (as mentioned earlier) so quantitative determination of tablet medicines using Raman spectroscopy and chemometrics is not addressed in this study.

4.4.1.2 Comparing Non Baseline Corrected vs Baseline Corrected Data for Paracetamol Tablet Samples

Most studies using Raman spectroscopy involved pre-processing of the Raman spectra by either baseline correction or normalisation of the data in order to obtain valuable spectral data. To ascertain that valuable forensic data could be obtained without manipulating or pre-processing data, raw (non-baseline corrected) spectral data for selected test paracteamol tablet samples were compared with pre-processed (baseline corrected) spectral data.

Table 4. 3 Selected paracetamol tablet samples used for comparing non baseline corrected vs baseline corrected tablet samples

Country	Tablet
UK	UK P2T1, UK P2T2
Belgium	BEL P1T3, BEL P1T4, BEL P2T1, BEL P2T2
China	CHN P3T1, CHN P3T2 CHN P4T1
India	IND P11T1, IND P11T2
South Africa	STHAFR P1T1, STHAFR P1T2
Nigeria	NIG P2T3, NIG P2T4
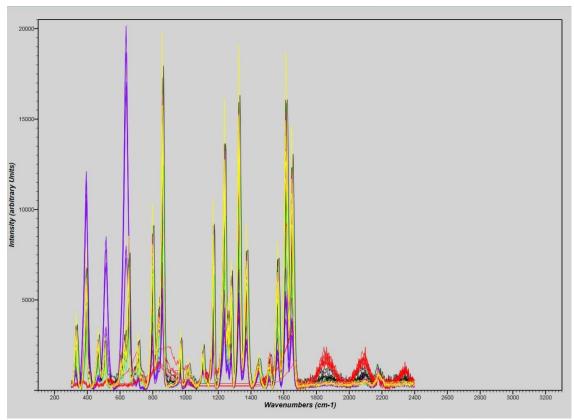
Figure 4. 7 Raman spectra for selected paracetamol tablets including Nig P2 (Red) - Baseline Corrected

Baseline corrected spectral data for all the tablets assessed were similar suggesting the presence of the same constituents. In addition, the effect of fluorescence observed with the non-baseline corrected spectral data, especially for the for Nig P2 tablets, was eliminated with baseline correction. However, even though there are similarities between the baseline corrected spectral peaks for Nig P2 tablets (red) and baseline corrected spectra for the other tablets, there are still noticeable differences as observed between 800 and 1200 wavenumbers (red spectra) in Fig 4.7.

PCA study for baseline corrected spectra of the selected tablet samples was also conducted to check if PCA results were significantly affected by data pre-processing. A principal component plot of the selected paracetamol test tablet samples is shown in Fig. 4.8.
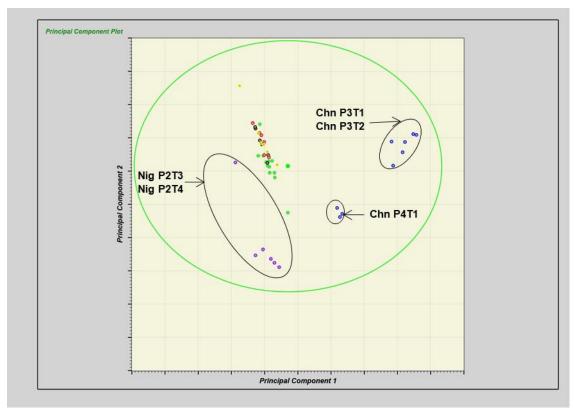
Figure 4. 8 PCA for selected paracetamol tablets listed in Table 4.3 - Baseline Corrected

PCA study of baseline corrected tablet spectra suggests there was no significant difference in PCA results since outlier samples based on baseline corrected spectra was generally consistent with results obtained using non baseline corrected samples as shown in Fig 4.8.
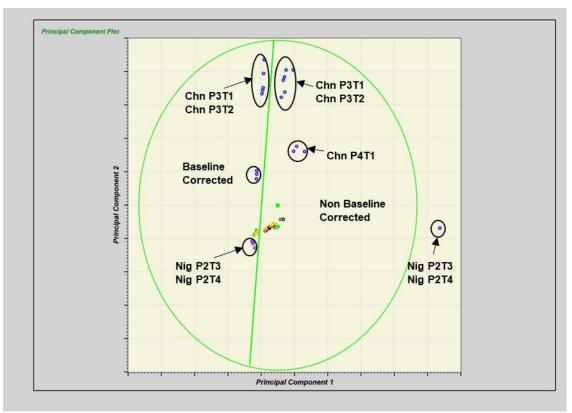
Figure 4. 9 PCA for selected paracetamol tablets - Baseline Corrected vs Non-Baseline Corrected

Both baseline corrected and non-baseline corrected spectra for the selected paracetamol tablets were subjected to PCA study at the same time to check if the system could identify differences between raw and pre-processed (manipulated) data. Figure 4.9 indicates the system is clearly able to discriminate between baseline corrected and non-baseline corrected spectra. It can also be observed that variation between baseline corrected spectra is not as pronounced as with non-baseline corrected spectra.

4.4.1.3 Comparing Raman data for whole tablet vs crushed (powder) samples of selected paracetamol tablets

Raman data for selected whole paracetamol tablets (Table 4.4) was compared with crushed powder samples of the same tablets to check if this made a huge difference on the final outcome. Non baseline corrected spectral data was used for this.

Table 4. 4 Selected Paracetamol tablet samples used for comparing tablet vs powder data

Country	Tablet
Belgium	BEL P2T1
Spain	SPN P1T3
China	CHN P2T3
Nepal	NEP P1T3
UAE	UAE P1T3
Rwanda	RWA P3T3, RWA P4T3
Nigeria	NIG P1T1, NIG P2T3
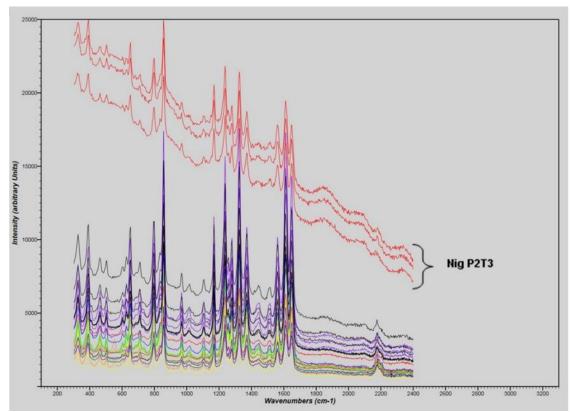
Figure 4. 10 Spectra for powder samples of selected paracetamol tablets- Non-Baseline Corrected

Figure 4.10 shows non-baseline corrected spectra for powder samples of the selected paracetamol tablets. The same peaks are present in the spectra of the powdered samples but at significantly reduced intensity. The effect of fluorescence on the spectra for the crushed Nig P2 tablet is not as strong as it was with the whole tablet since spectral peaks are better defined. PCA studies were also conducted for the powder samples as highlighted in Fig 4.11.
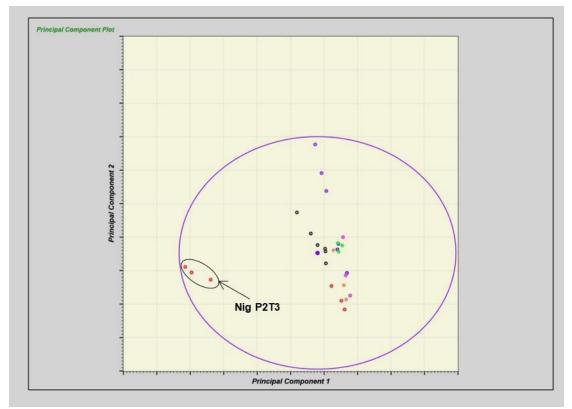
Figure 4. 11 PCA for powder samples of selected paracetamol tablets in Table 4.4- Non-Baseline Corrected

PCA for powder samples in Fig 4.11 shows similarity between most of the tablets except Nig P2T3 which was once again identified as an outlier. Therefore, results based on powder samples were in agreement with those obtained using the whole tablet. This implies that valuable forensic data can be obtained using the whole tablet for Raman analysis which will in turn save time of analysis. It is however important to note that coating on tablets surfaces could reduce the Raman effect or cause fluorescence.
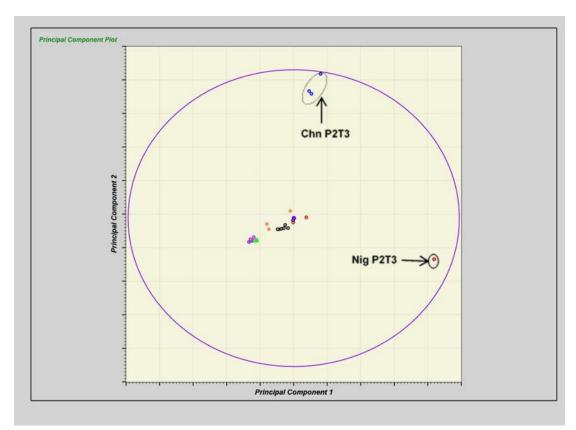
Figure 4. 12 PCA for whole paracetamol tablets selected in Table 4.4- Non-Baseline Corrected

Figure 4.12 is a principal component plot for selected whole tablets of paracetamol outlined in Table 4.4. Interestingly, there is a slight difference observed between the principal component plot for whole paracetamol tablets selected and the principal component plot for the crushed tablets. Chn P2T3 identified as an outlier using whole tablet data in Fig 4.12 is not an outlier when crushed but part of the general group. This suggests that the property or constituent of Chn P2T3 which makes the system identify it as an outlier in tablet is suppressed or eliminated when it is crushed. This will explain similarity with other tablets after it is crushed. It might therefore be important to check powder samples of tablets initially identified as outliers to rule out false positives as was observed with Chn P2T3.

The details for the powder samples in Fig 4.11 and whole tablets in Fig. 4.12 are compared in a combined principal component plot in Fig. 4.13.
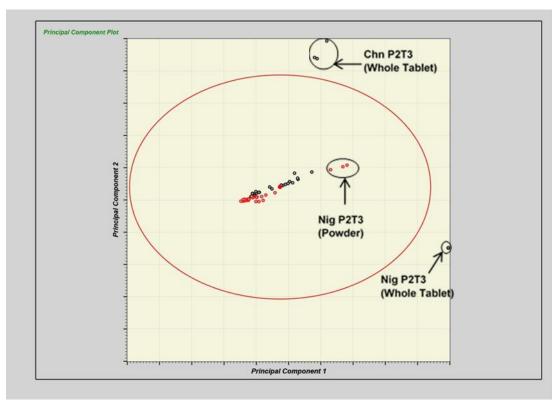
Figure 4. 13 PCA for Powder (Red) vs Whole tablet (Black) Raman data for selected paracetamol tablets in Table 4.4 – Non-Baseline Corrected

Figure 4.13 shows similarities between data based on the whole tablet and powdered form of most of the selected tablets. The exceptions are with Chn P2T3 (mentioned earlier) and Nig P2T3. Although Nig P2T3 is identified as an outlier when both whole tablet and crushed tablet are assessed, it is important to note that the variation compared to the other tablets is reduced considerably when the crushed tablet is used.

4.4.1.4 Comparing Raman data for whole tablets vs non-invasive analysis through blister pack for selected paracetamol tablet samples

Since one of the advantages of the Raman device is its ability to screen samples even through packaging, data obtained by direct analysis of whole tablets was compared to data obtained without taking the tablets out of their blister packs. Selected paracetamol tablet samples were used as outlined in Table 4.5.

Table 4. 5 Selected Paracetamol tablet samples used for comparing whole tablet data vs data through blister packs

Country	Tablet
Spain	SPN P1T3
Nepal	NEP P1T3
UAE	UAE P1T3
Nigeria	NIG P2T3

Figure 4. 14 Non-transparent side of a blister pack for paracetamol

Figure 4.14 is an example of a blister pack of paracetamol showing the non-transparent side. Raman spectra for the selected paracetamol tablets were collected through the non-transparent side of the blister pack and recorded as shown Fig. 4.15. This data from the non-transparent (aluminium foil) side of the blister pack was used as background reference data when analysing through the transparent side of the blister since there is little or no Raman response through the aluminium foil or through coating.
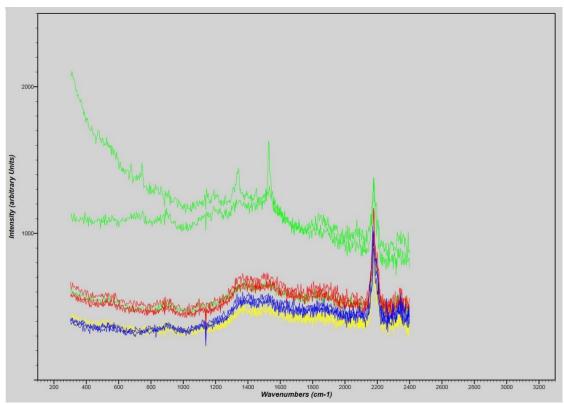
Figure 4. 15 Spectra for selected paracetamol tablets through the non-transparent side (Fig 4.14) of the blister pack – Non Baseline Corrected

Figure 4.15 shows data collected from the non-transparent side of the blister indicating a signal of very low intensity and poorly defined peaks. Signals at this level will pose little or no interference with the spectra obtained through the transparent side of the blister pack or direct analysis of the whole tablet. The transparent side of the blister pack (Fig 4.16) was then assessed for its potential in producing good Raman spectra for analysis.

Figure 4. 16 Transparent side of a blister pack for paracetamol
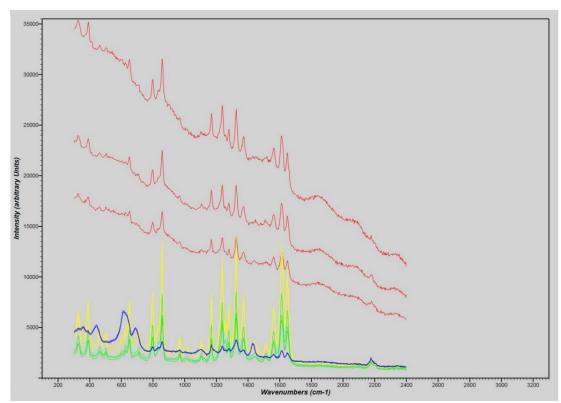
Figure 4. 17 Spectra for selected paracetamol tablets through the transparent side (Fig 4.16) of the blister pack - Non Baseline Corrected

Figure 4.17 indicates that the transparent side of the blister pack can be used to obtain spectra with defined peaks for analysis. However, the peak obtained via the transparent side of the blister pack might be weak. The Raman data obtained directly for the whole tablet, from powder samples and through the blister pack are compared in the next section.

4.4.1.5 Comparing Raman spectra collected directly from whole paracetamol tablets to spectra obtained through the blister pack and from powder samples
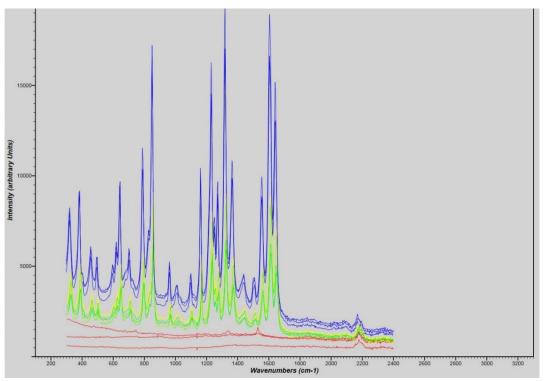
Figure 4. 18 Raman spectra for Paracetamol tablet sample from Nepal (Nep P1T3)(Blue-directly from tablet; Yellow- crushed /powder sample; Green- through transparent side of blister pack; Red- non transparent side of blister pack)
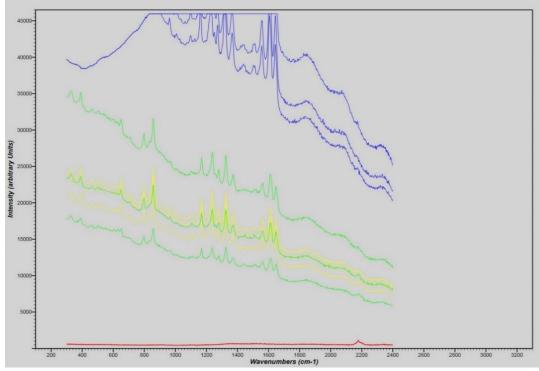
Figure 4. 19 Raman spectra for Paracetamol tablet sample from Nigeria (P2T3) (Blue-directly from tablet; Yellow- crushed /powder sample; Green- through transparent side of blister pack; Red- non transparent side of blister pack)
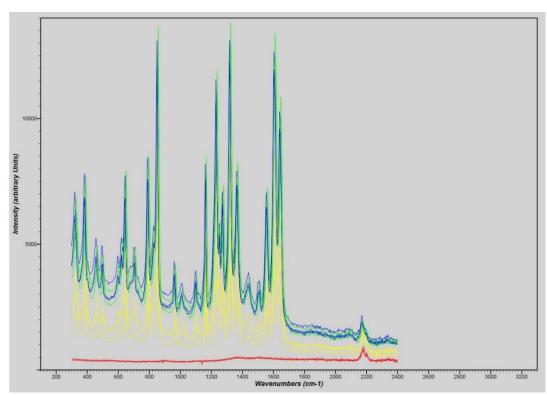
Figure 4. 20 Raman spectra for Paracetamol tablet sample from Spain (P1T3) (Blue-directly from tablet; Yellow- crushed /powder sample; Green- through transparent side of blister pack; Red- non transparent side of blister pack)
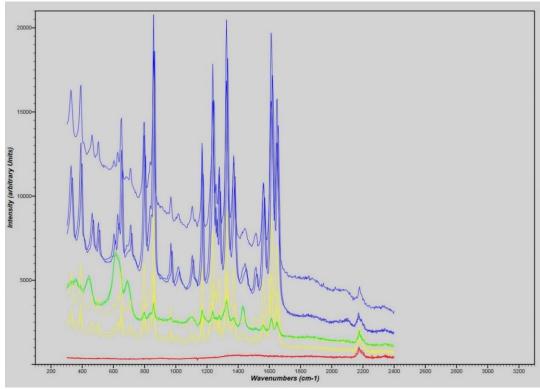
Figure 4. 21 Spectra for Paracetamol tablet sample from UAE (UAE P1T3) (Blue-directly from whole tablet; Yellow- crushed /powder sample; Green- through transparent side of blister pack; Red- non transparent side of blister pack)

Raman spectral data for the tablets assessed in Figures 4.18-4.21 show that the intensity of spectral peaks was generally highest for data obtained directly from the whole tablet (blue). This could be due to the compactness of the whole tablets. Intensities of Raman spectral peaks based on the powder sample (yellow) and through the transparent side of the blister pack (green) were not significantly different but less intense than those obtained directly from the whole tablet. The amount of information needed will therefore determine the approach taken in Raman analysis of test tablets. It is also important to note that the Raman response is dependent on the amount of analyte in the tablet. Therefore, analysis of low API tablet medicines might be a challenge especially with high levels of excipient that could cause fluorescence.

4.4.2 Raman Analysis of Antimalarial (Chloroquine) Tablets

The chloroquine tablets outlined in Table 4.6 were subjected to Raman analysis and spectral data was recorded as shown in Fig 4.22

Table 4. 6 Chloroquine Tablet Samples analysed and their origin

Country	Tablet	Expected Amount (mg)
UK	UK C1T3, UK C1T4 UK C2T1	250
Nepal	NEP C1T3, NEP C1T4	250
India	IND C3T2, IND C3T3	500
	IND C2T3, IND C2T4	250
Nigeria	NIG C1T2	400
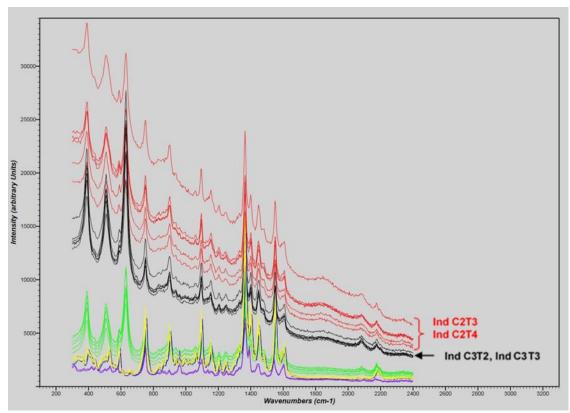
Figure 4. 22 Raman spectra for chloroquine tablet samples- Non Baseline Corrected

Figure 4.22 shows the Raman spectra of all the chloroquine tablets which contain the anticipated characteristic Raman peaks for chloroquine (740, 900, 1110, 1360, 1380 and 1550 wavenumbers) thus confirming the presence of the same constituent/API (chloroquine). The different levels of fluorescence (IndC2 vs Ind C3 suggests different excipient concentrations and the variations in the TiO_2 peaks (650, 500 and 370 cm^{-1}) confirm this. Baseline corrected data for the chloroquine tablets are displayed in Fig 4.23.
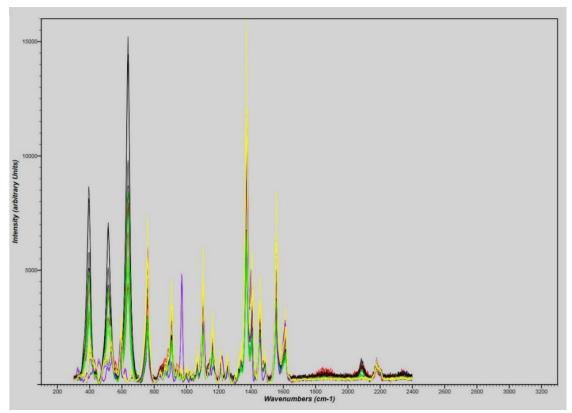
Figure 4. 23 Raman spectra for chloroquine tablet samples- Baseline Corrected

The baseline corrected spectra for chloroquine tablets in Fig 4.23 eliminates the background due to fluorescence in the samples from India with all sample spectra closely matched in the overlay further indicating that tablets contain principally the same chemical compound (chloroquine). PCA studies were carried out for all chloroquine tablets based on non-baseline corrected data and are highlighted in Fig 4.24.

4.4.2.1 Principal Component Analysis of Chloroquine Test Tablet Samples
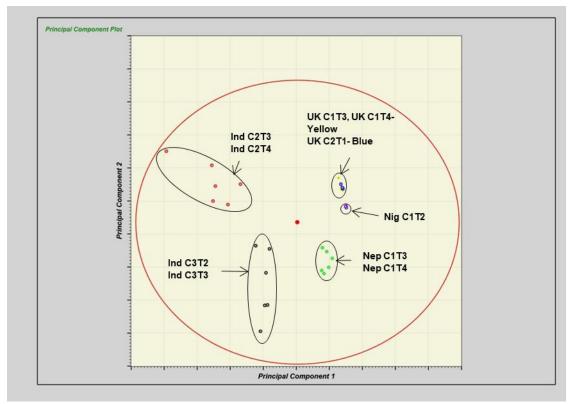
Figure 4. 24 PCA for all chloroquine tablet samples - Non Baseline Corrected

Based on the PCA data presented in Fig 4.24, four distinct groups are highlighted, corresponding to the spectral differences noted for Fig 4.22. Applying the same approach used in the PCA study for the paracetamol tablet Nig P2 (which exhibited fluorescence but was eliminated by baseline correction), PCA study for baseline corrected chloroquine samples was conducted and results recorded (Fig 4.25).
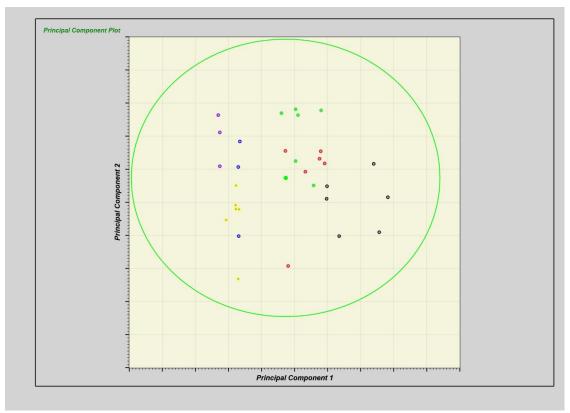
Figure 4. 25 PCA for all chloroquine tablet samples - Baseline Corrected

PCA for baseline corrected spectra for all chloroquine tablets in Fig 4.25 shows all the samples (including Ind C2 and C3) as part of the same group. This suggests that fluorescence was the reason for exclusion of the Indian samples in Fig 4.24 but baseline correction of the spectra was able to eliminate the effect. Therefore, pre-processing of Raman spectra by baseline correction (as is commonly used in most studies) might be discarding valuable information needed to discriminate between tablet samples by the Raman equipment. Figure 4.26 is a combined PCA study comparing non baseline corrected data (Fig 4.24) for chloroquine to baseline corrected data (Fig 4.25).
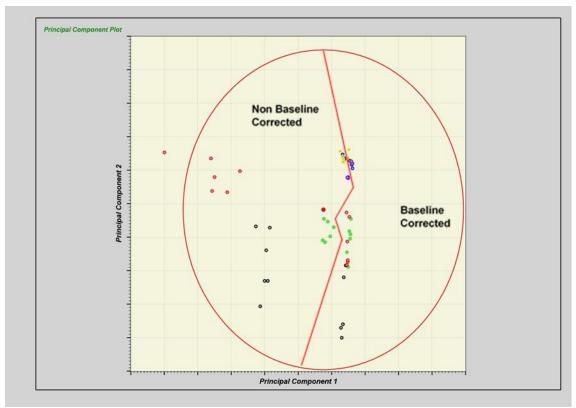
Figure 4. 26 PCA for all chloroquine tablet samples – (Baseline Corrected vs Non Baseline Corrected)

Figure 4.26 shows clearly the difference between non baseline corrected data and baseline corrected data for the chloroquine tablets. The figure also shows how baseline correction masks the obvious differences in the tablets highlighted by the non-baseline corrected data.

Table 4. 7 Paracetamol tablets analysed and their weights and expected amounts (%w/w)

Country (Number of Tablets)	Tablets Analysed*	Tablet Weight (mg)	Expected percentage of paracetamol in Tablet (% w/w)	Expected Amount (mg)
UK (n=2)	UK P2T1	575.1	86.9	500
	UK P2T2	593.1	84.3	
Cyprus (n=2)	Cyp P1T3	627.0	79.7	
	Cyp P1T4	628.7	79.5	
Belgium (n=2)	Bel P2T1	584.4	85.6	
	Bel P2T2	583.0	85.8	
Spain (n=6)	Spn P1T3	598.8	83.5	
	Spn P1T4	585.8	85.4	
	Spn P2T3	863.8	75.2	650
	Spn P2T4	860.3	75.6	
	Spn P3T3	1308.3	76.4	1000
	Spn P3T4	1310.1	76.3	
Belgium (n=2)	Bel P1T3	1150.3	86.9	
	Bel P1T4	1159.1	86.3	
India (n=14)	Ind P11T1	806.6	80.6	650
	Ind P11T2	802.3	81.0	
	Ind P14T1	741.5	87.7	
	Ind P14T2	748.6	86.8	
	Ind P15T1	855.5	76.0	
	Ind P15T2	844.0	77.0	
	Ind P16T1	778.2	83.5	
	Ind P16T2	785.7	82.7	
	Ind P10T1	587.7	85.1	500
	Ind P10T2	586.3	85.3	
	Ind P12T1,	440.0	68.2	300
	Ind P12T2	446.2	67.2	
	Ind P13T1	580.7	43.1	250+15mg Caffeine-Yellow
	Ind P13T2	586.2	42.6	
Indonesia (n=1)	Indn P1T1	594.1	84.2	500
Nepal (n=8)	Nep P1T3	599.9	83.3	
	Nep P1T4	620.0	80.6	
	Nep P2T3	594.6	84.1	
	Nep P2T4	612.3	81.7	
	Nep P3T3	610.6	81.9	
	Nep P3T4	618.5	80.8	
	Nep P4T3	593.0	84.3	
	Nep P4T4	600.2	83.3	
Thailand (2)	Thai P1T1	575.9	86.8	
	Thai P1T2	556.6	89.8	
UAE (n=2)	UAE P1T3	605.3	82.6	
	UAE P1T4	609.0	82.1	

Table 4.7 (continued)

Country (Number of Tablets)	Tablets Analysed*	Tablet Weight (mg)	Expected percentage of paracetamol in Tablet (% w/w)	Expected Amount (mg)
China (n=7)	Chn P1T3	592.5	84.4	
	Chn P1T4	592.9	84.3	
	Chn P2T3	629.4	79.4	500+65mg Caffeine- Pink
	Chn P2T4	638.3	78.3	
	Chn P3T1	785.9	82.7	650
	Chn P3T2	784.6	82.8	
	Chn P4T1	456.3	65.7	300
Rwanda (n=4)	Rwa P1T2	588.3	85.0	500
	Rwa P2T2	576.8	86.7	
	Rwa P3T3	564.7	88.5	
	Rwa P4T3	559.4	89.4	
Ghana (n=4)	Gha P1T3	625.8	79.9	
	Gha P1T4	537.7	93.1	
	Gha P2T3	543.4	92.0	
	Gha P2T4	527.4	94.8	
Nigeria (n=4)	Nig P1T3	593.2	84.3	
	Nig P1T4	584.0	85.6	
	Nig P2T3	583.1	85.7	
	Nig P2T4	544.0	91.9	
South Africa (n=2)	SthAfr P1T1	602.3	83.0	
	SthAfr P1T2	584.9	85.5	
Kenya (n=2)	Ken P1T1	671.5	74.5	500+65mg Caffeine
	Ken P1T2	673.4	74.3	

Note: n = number of samples, *P1T1= Pack 1 Tablet 1 and so on. All tablets are white except where colour is indicated

4.5 Conclusion

Current work indicates the potential of Raman spectroscopy in providing valuable forensic data in the screening of FSMs. Results suggest the use of chemometrics (PCA) facilitates the recognition of minor spectral differences which might be difficult to detect by simple visual comparison of unique Raman spectral regions or peaks.

The factors that may affect the Raman spectra include:

- The wrong level (concentration) of API

- The wrong API or more than one API

- Different excipient mixtures

- Surface coatings or colours

The data recorded in Table 4.7, for European samples, shows that tablets covering 500, 650 and 1000mg all contain about the same concentration (68-85%) of paracetamol on a w/w basis. The spectra are very similar and the PCA signals all cluster in the centre of Figure 4.2 Similarly all the other samples with a paracetamol concentration within the range 68-85% lie within the central group on Fig 4.2 except for the Chn P2 and Ken P1 tablets which both contain a second API and are therefore outliers. Of the remaining outliers samples Nig P2 clearly has different excipients present and ChnP4 has a reduced concentration of paracetamol (66%).

One sample, Ind P13 challenges the Raman/PCA methodology reported here. These tablets contain 2 APIs, are coloured only contain 250mg of the API and yet appear to be bona fide 500mg tablets.

In addition to the spectroscopic techniques considered in Chapters 3 and 4, the next chapter will focus on direct insertion probe mass spectrometry (DIP-MS) and its potential use in the fast detection of falsified and substandard medicines.

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CHAPTER FIVE

Falsified and Substandard Medicines: Probe Mass Spectrometry for rapid characterisation of tablet content

5.1 Introduction

As established earlier, falsified and substandard medicines pose a huge threat to public health not only in many small and middle-income countries but also in developed countries as well. In a recent report, key malaria treatment was unable to cure four patients in the UK who had all visited Africa suggesting early signs of the parasite being resistant to the medication (Gallagher, 2017). The United Nations Office on Drugs and Crime has also identified medicine counterfeiting as a global threat (Kaur, 2015).

One way to prevent the spread of falsified and substandard medicines as spelt out in Chapter 2 is to carry out onsite screening of medicines. This type of screening is generally done either by batch number/ bar code searching using special apps on mobiles phones or by simple colorimetric or thin layer chromatography (TLC) kits. There is also the issue of sensitivity (false positives) and the largely qualitative nature of these TLC tests which neglects or doesn't take into account falsified medicines with insufficient amounts of the active ingredient (Rebiere et al, 2017; Zou et al, 2017). In addition, it is even more difficult to prevent the spread of clones of particular tablet medications especially where chiral compounds are involved with cheaper analogues substituted for genuine and more expensive APIs. With the analogues being very similar in that they have the same chemical species or functional groups and are basically mirror images of each other, they are more difficult to detect (Kovacs et al, 2014; Rebiere et al, 2017).

Mass spectrometry has been identified as one of the most popular techniques for chemical analysis due to its high accuracy, selectivity and quantitative capabilities provided that the analytes in question can be converted efficiently to gas phase ions.

While results that are more accurate are obtained using mass spectrometers than with conventional immunoassay kits such as the GPHF Minilab and simpler techniques like the TLC, mass spectrometers until recently, were considered to be too complex and expensive to be used for *in situ* screening of pharmaceutical formulations. It is in a bid to address the challenge posed by using large laboratory based equipment for in-field analysis that efforts towards the miniaturisation of mass spectrometers have been on the rise in recent times (Kumano et al, 2015).

5.1.1 Introduction to Mass Spectrometry

Mass spectrometers need a vacuum system to function and measure the mass to charge ratio of ions in order to determine the identity of a sample. For many years mass spectrometers have been used, generally in combination with a gas chromatograph (GC), to identify the components of mixtures. The identification of the components of mixtures which could be in different physical states (solid, liquid or gas) have been based on the characteristic fragmentation patterns produced by high energy electron impact ionisation processes employed in the mass spectrometer. The GC acts both as a separation technique and a means of introducing the samples into the MS. Separation of mixtures by GC requires the mixture components to be both readily volatile and thermally stable but this is unlikely to be the case for the components of a tablet and so an alternative means of introducing a solid sample into the mass spectrometer is needed. It was common practice in the 60s and 70s to use a heated direct insertion probe (DIP), via a vacuum lock, to introduce samples directly into the ion source of a mass spectrometer where the sample was volatilised, ionised and analysed. This technique is referred to as DIP-MS and can be applied to crushed tablet samples with no other sample preparation required prior to analysis. Conventionally, identifications are based on the characteristic positive ion fragmentation patterns

produced by the high energy electron impact (EI) ionisation processes used in the mass spectrometer. This process can be summarised:

$$M + e^-_{(H)} = [M^+] + 2e^-_{(L)} = m_1^+ + m_2^+$$

Note $e^-_{(H)}$ = high energy electron and $e^-_{(L)}$ = low energy electron

$[M^+]$ = excited short-lived molecular ion

It is also possible for the sample to be ionised by a low energy electron to produce a negatively charged molecular ion which is unlikely to fragment. Both approaches have been used here and the results compared with data from an example of the new atmospheric pressure or ambient ionisation technique (Culzoni et al, 2014, Fernandez et al, 2014). In these techniques, the ionisation processes can be represented as:

$M + H^+ \longrightarrow (M+H)^+$ where $(M+H)^+$ is known as the pseudomolecular ion. Atmospheric pressure or ambient ionisation techniques offer two technical differences to the EI-MS process. In these systems ions are created outside of the MS vacuum system, usually by the addition of a proton rather than the removal of an electron. Under these conditions, the energy transferred to the target molecule is low and therefore little fragmentation is observed and the target signal is the anticipated molecular weight (Mwt) plus 1 mass unit.

Exactly what is meant by a molecular ion needs to be clarified. There are three different ways to calculate the molecular mass from the empirical formula:

Average mass – based on the average atomic weights over all isotopes

Monoisotopic mass – based on the mass of the most abundant isotopes

Nominal mass – based on the integer mass of the most abundant isotopes

In mass spectrometry the monoisotopic mass should be used and thus any compound containing chlorine, for example, will have two molecular ions separated by 2 mass units corresponding to the 35 and 37 isotopes of chlorine.

Ambient sampling/ionisation MS has shown great potential (Culzoni et al, 2014, Fernandez et al, 2014) with direct analysis in real time (DART) and desorption electrospray ionisation (DESI) identified as the most frequently used techniques (Bernier et al, 2016). Culzoni et al (2014) asserts that high throughput and very sensitive ambient mass spectrometry techniques will enhance the investigation of pharmaceutical formulations with little or no sample preparation. Bernier et al (2016) employed DART ionisation coupled with a compact single quadrupole mass spectrometer in fingerprinting antimalarial tablets and compared them to high-resolution equipment with both systems producing similar results thereby suggesting its possible use on the field. There have also been instances where thermal desorption of the sample was followed by ambient ionization. Atmospheric pressure chemical ionisation (APCI), dielectric barrier discharge ionisation (DBDI), electrospray ionisation and atmospheric pressure photoionization are further examples of ambient ionisation methods coupled with thermal desorption (Culzoni et al, 2014). All these methods have proven to be useful but Kumano et al (2015) points out the challenge using bulky equipment for a portable spectrometer.

Efforts to miniaturize mass spectrometers making them more portable have targeted improving sensitivity (Snyder et al, 2015; Bernier et al, 2016; Brown et al, 2016; Lawton et al, 2017; Li et al, 2017). The weight of the mass spectrometer is predominantly attributed to the vacuum pump. As such, reducing the weight of the whole system will involve using a smaller pump but this in turn lowers pumping speed which results in lower sensitivity for analysis due to the necessary reduction in sample size (Kumano et al, 2015).

Several techniques have been developed to address this lower sensitivity challenge. Contreras et al (2008) employed a portable mass spectrometer with a gas chromatograph, a toroidal iron trap and an electron ionisation source where a solid

phase micro extraction method was used in concentrating the sample. This in turn improved sensitivity. Ouyang et al (2009) designed a portable mass spectrometer with an electrospray ionisation source and a rectilinear ion trap. They enhanced the efficiency of ion transfer (from an ionisation source at atmospheric pressure to a mass analyser in a vacuum) by designing a discontinuous atmospheric interface.

Kumano et al (2015) in their quest to find an alternative to the use of immunoassay kits (involving solvent extraction) for the screening of illicit drugs, developed a prototype of a portable mass spectrometer. It employs a low-pressure dielectric barrier discharge ionisation (LP-DBDI) source which is highly sensitive. To allow for efficient transport of the sample vapour to the LP-DBDI source, a vacuum headspace method and a discontinuous sample gas introduction technique was used. The spectrometer had the ability to detect 0.1 ppm of methamphetamine in a liquid, making it adequately sensitive for the screening illicit of drugs. The limitation with this portable mass spectrometer however, is that it can only be used for analysis of liquid samples. Kumano et al (2015) in their work also suggested that a portable mass spectrometer with solid sampling capabilities will be required to and possibly enhance the early detection of illicit drugs before they fall into the hands of criminals. To achieve this, they developed a probe heating method which they incorporated in the portable mass spectrometer to facilitate the screening of solid samples (illicit drugs). This technique (probe heating method), they identified as being important for public safety. Li et al (2017) also used portable ion mobility spectrometry in the rapid screening of non-steroidal anti-inflammatory drugs which were illegally added in anti-rheumatic herbal supplements. Furthermore, Ma and Ouyang (2016) are also of the opinion that integrating ambient ionization and miniature mass spectrometry systems would facilitate chemical and biological analysis.

Besides analgesics/ antipyretics, antimalarial medicines have been identified as one of the most falsified medicines globally and more prevalent in tropical low and middle

income countries (Chaccour et al, 2015). This is because individuals are poor and so will settle for cheaper options they can get even if they are not genuine. Karunamoorthi (2014), puts the global annual death rate due to falsified antimalarial medicines at nearly 660, 000. Chaccour et al, (2015) further indicates that over 584,000 deaths occurred in 2013 due to falsified antimalarial medicines with about 453,000 being children under 5yrs highlighting the severity of the situation. Different antimalarial combination therapies have been in use in malaria endemic countries for the treatment of drug-resistant malaria. Artesunate, artemether, lumefantrine, amodiaquine, sulfadoxine and pyrimethamine are key drugs used mostly in combined doses in the treatment of multi-drug resistant *Plasmodium falciparum* malaria (Kaur et al, 2016). Reports suggest that counterfeit antimalarials contain either insufficient amounts of API or, in some cases, no APIs (Chaccour et al, 2015). The ignorant consumer of such medicines is at substantial risk of not getting any therapeutic relief, becoming resistant to the illness and may even die due to ineffective treatment (Karunamoorthi, 2014).

In a bid to address the challenges encountered with ATR-FTIR outlined in chapter 3 (such as limit of detection and difficulty in characterising/quantifying mixtures of APIs due to complex non well-defined spectra), it was important to consider a more sensitive technique for example DIP-MS which is rapid and provides simpler and more compound specific information about both API(s) and other components of a tablet including impurities/contaminants. DIP-MS also has the potential to be used for in-field analysis of pharmaceutical tablet formulations. Until now, DIP-MS has not been identified for its use in the identification of falsified and substandard pharmaceutical tablet formulations.

This chapter describes the fast analysis of analgesic/antipyretic and antimalarial tablets by direct insertion probe mass spectrometry (DIP-MS). Furthermore, the potential of DIP-MS in the analysis of more complex tablets having multiple APIs/excipients and

those with API concentrations lower than the detection limits of the ATR-FTIR (e.g. antimalarials) were assessed. Tablets were assessed using conventional electron ionisation (EI) methods and the results were compared with data from ambient/atmospheric ionisation methods (atmospheric solids analysis probe - ASAP® mass spectrometry).

5.2 Principle and Potential Applications of Probe-MS
5.2.1 DIP-MS

The apparatus schematic (Fig 5.1) shows the demountable sample probe (DIP) which has a heated sample cup in the tip which is inserted into the MS ion source. To analyse a sample a small amount (a few milligrams) of the powdered sample is contained in a pyrex tube placed in the tip of the direct insertion probe (DIP). The probe is introduced into the vacuum system via a vacuum lock and positioned such that the top of the sample tube is close to the ionization chamber of the MS as shown in Figure 5.1.

IONISATION

FILAMENT

HEATED
PROBE TIP   ELECTRONS

SAMPLE
PROBE   IONS

                                          MASS
                                      SPECTROMETER
FLARED
SAMPLE
VIAL

ATMOSPHERIC PRESSURE   VACUUM
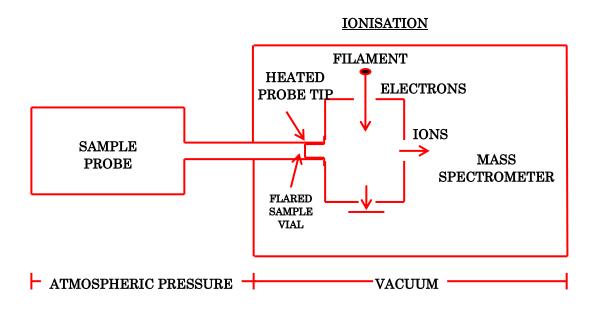
Figure 5. 1 Direct Insertion Probe Mass Spectrometry (with high energy electron ionisation- EI) Schematic.

The tip of the probe is then heated in a temperature controlled mode in order to volatilise the different components of the sample; a technique similar to the fractional distillation process but with the separation enabled by the dual effect of heat and the vacuum.

The temperature ramp rate chosen is very crucial in order to avoid a situation where the sample vaporizes too rapidly and the signal is saturated. As the volatile species are ionised by electronic impact the resultant mass spectra are monitored. Electron ionisation (EI) method in mass spectrometry has several advantages and disadvantages as outlined in Table 5.1.

Table 5. 1 Advantages and Disadvantages of Electron ionisation (EI) method in mass spectrometry

Advantages	Disadvantages
Sensitive	The molecule must be volatile
Simple	The molecule has to be thermally stable
Fragmentation enables characterisation of molecules	Extensive fragmentation leads to more complex data which is difficult to interpret

(Adapted from Gross, 2011)

It is important to ensure the molecules have a short residence time in the source so as to enhance rapid ionisation and prevent thermolytic degradation. In the DIP-MS, this can be achieved by;

- Very short distance between the tip of the probe and the ionisation source.

- Ensuring the number of molecules involved in collision with the electron flux is small.

- The turbomolecular pump system, coupled to the mass spectrometer, gives rise to a dynamic vacuum in the ionisation chamber in order to sustain high vacuum conditions.

- Molecule collision and recombination are minimised at low pressures (Flego and Zannoni, 2012).

The vacuum helps to lower the boiling temperature of compounds thereby allowing the vaporisation of these high-boiling point compounds at temperatures lower than expected (their true boiling points). The temperature controlled/programmed probe feature provided by DIP-MS enhances its versatility in data analysis because a controlled temperature increase allows sequential volatilisation of components in the sample. This effect can be seen in the total ion chromatogram - TIC profile (Fig 5.2) which plots the combined intensity of all the masses in each MS scan against the time and hence probe temperature of the analysis. Once the probe is in position, the mass spectrometer is started to carry out repetitive scans, over the selected mass range, as the probe tip is heated to volatilise the sample into the MS ion source. The mass spectrum from each scan is recorded and the Total Ion Intensity in each scan is plotted against time to produce the idealised data in Figure 5.2. In this Figure the horizontal axis contains data on time into the analysis, the mass spectral scan number and the probe tip temperature.
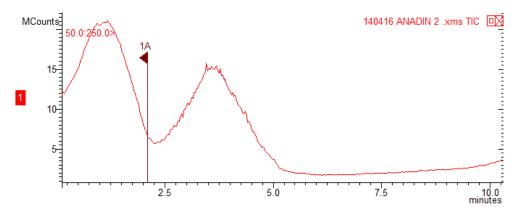
Figure 5. 2 TIC for UK tablet with multiple APIs/components.

The peaks in Fig 5.2 are indicative of the vaporisation of individual components corresponding to the evolution of different compounds from the tablet formulation as the temperature is increased with time. In this example, the maxima of the two humps/peaks correspond to 1.0 and 3.5 minutes into the run i.e. probe tip temperatures of ca 70°C and 150°C respectively. The flag on the figure indicates the point at which a

complete mass spectrum can be retrieved from the data system. This flag can be moved to any position on the TIC trace.

The mass spectrum like the TIC is representative of the total fragmentation patterns of all the species vaporised in that instance and may not be directly indicative of the fragmentation pattern of a single compound.

5.2.2 ASAP-MS

The Atmospheric Solids Analysis Probe – ASAP is an ambient desorption ionisation technique typically employed in the analysis of solid samples by MS but is capable of analysing a wide range of volatile and semi-volatile analytes (solid and liquid). Little or no work up is required before analysis of test samples and so unequivocal results can be obtained within 2-3 minutes.

The ASAP-MS technique is a soft ionisation process producing minimal fragmentation. As a result, majority of the ionised species are either the molecular or pseudo molecular ion. The mechanism of sample presentation is similar to DIP-MS but in this case a flowing stream of heated nitrogen gas is used to selectively vaporise the test sample into the ionisation region and the corona discharge triggers ionisation under ambient atmospheric pressure conditions as shown in Figure 5.3. The ions produced are focussed into the MS to be analysed.

Just as with the EI method, it is also important that the test sample loaded onto a glass sample vial is in close proximity to the ionisation region and the MS inlet to enhance sensitivity as well as ensure safety provided by the enclosed source housing. The design of the probe (having both fixed and removable sections) allows for rapid sample introduction without removing the complete assembly thereby ensuring stable source conditions. A corona discharge, atmospheric pressure chemical ionisation (APCI) source is used which leads to either charge transfer ionisation from nitrogen radical

cations or proton transfer reactions generated from moisture in the sample or ambient air.
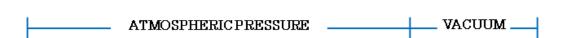
Figure 5. 3 Direct Insertion Probe Mass Spectrometry (ASAP-MS with low energy ionisation under atmospheric pressure) Schematic

The proposed methods do not need highly sophisticated and expensive high resolution mass spectrometers since they are mainly targeted towards screening for the presence of the compound(s) stated to be present (Gross, 2011; Flego and Zannoni, 2012). The accumulated data can be retrospectively searched for ions (m/z values) characteristic of the compounds expected to be present. The process is known as extracted ion analysis and is described in Section 5.4.

5.3 Materials and Methods
5.3.1 Materials

Reference samples of paracetamol (acetaminophen), aspirin, caffeine, chloroquine diphosphate, sulfadoxine were obtained from Sigma Aldrich, UK and the excipient, maize starch was obtained from Fisher Scientific Ltd, Loughborough, UK.

Test samples of over the counter paracetamol tablets (Table 5.2) were obtained opportunistically from various pharmacy outlets in the UK, across other countries in Europe, Africa, Southeast Asia and the Caribbean Islands as detailed in Table 5.2. In addition eight (8) analgesic tablets from the UK containing multiple APIs including paracetamol, caffeine and aspirin were also obtained for analysis. Six of the multiple API tablets were expected to individually contain 2 APIs (paracetamol and caffeine) while the other two were indicated as containing 3 APIs (paracetamol, aspirin and caffeine) (Table 5.3).

Table 5. 2 List of analgesic/antipyretic tablets containing paracetamol analysed using EI DIP-MS and their countries of origin

Tablet(s)	Number of Tablet Samples	Expected Amount (mg)
UK	2	500
Belgium	2	1000
Spain	2	500
Spain	2	650
Spain	2	1000
Cyprus	2	500
Switzerland	2	500
Pakistan	2	500
China	4	500
UAE	4	500
Rwanda	6	500
Ghana	4	500
India	16	500
India	2	325
India	1	650
Jamaica	4	500
Nepal	8	500

Table 5. 3 List of multiple API analgesic/antipyretic tablets from the UK containing paracetamol analysed using EI DIP-MS

Tablet(s)	Number of Tablet Samples	API(s) Expected in Tablets
PC A#1 & 2	2	Paracetamol and Caffeine
PC B #1 & 2	2	Paracetamol and Caffeine
PC C# 1& 2	2	Paracetamol and Caffeine
PAC A#1 & 2	2	Paracetamol Aspirin, and Caffeine

A range of over the counter antimalarial tablets (Table 5.4) from the UK, India, Zimbabwe and Ghana were subjected to EI DIP-MS analyses in order to determine if the appropriate active ingredient could be detected at the therapeutic levels present.

Table 5. 4 List of antimalarial tablets analysed using EI DIP-MS and their countries

Tablet(s)	Number of Tablet Samples	API(s) Expected in Tablets
AM. UK A#1 & 2	2	Chloroquine phosphate
AM. Zimbabwe A#1 & 2	2	Artemether and Lumefantrine
AM. Zimbabwe B# 1& 2	2	Artesunate and Amodiaquine
AM. India A#1 & 2	2	Artemether and Lumefantrine
AM. Ghana A# 1& 2	2	Sulfadoxine and Pyrimethamine

5.3.2 Instrumentation

5.3.2.1 Direct Insertion Probe Mass Spectrometry

DIP-MS analysis was performed using the Bruker 450-GC system coupled with a quadrupole mass spectrometer detector with electron impact ionization and also having a direct insertion probe module.

The MS operating parameters were:

Mass range	50-550m/z
MS Scan rate	33/min
Source temperature	150ºC
Electron Ionisation at	70eV or 20eV

Probe temperature was 40ºC to 350ºC at 30ºC/min. Once 350ºC is reached, cooling begins.

5.3.2.2 Atmospheric solids analysis Probe Mass Spectrometry (ASAP-MS)

ASAP-MS analysis was performed using the Advion Expression compact mass spectrometer (CMS) with atmospheric pressure chemical ionisation (APCI) and also having an Atmospheric Solids Analysis Probe (a direct insertion probe module). Operating parameters for CMS were as follows:

Ionisation mode:	APCI positive or negative
Nitrogen gas temperature:	300°C
Mass range	100-550m/z

5.3.3 Methods- Sample analysis

5.3.3.1 DIP-MS

Crushed tablet powder sample was placed in the sample vial using TLC spotters to ensure minimal amount of sample. Sample vial containing analyte affixed to the tip of the sampling probe was introduced into the mass spectrometer via a gate valve. This valve was only opened when the sample was introduced to maintain the vacuum state of the mass spectrometer. Once the sample was in position, the mass spectrometer was set to scan and controlled temperature increase on the system caused vaporisation of the sample which was then ionised and mass spectral data recorded. A blank (empty sample vial) was run after every measurement to avoid any carry over from previous run. After each run, cooling time was needed to allow both probe temperature and the ion source pressure to drop prior to further scanning.

For quantitative study using, different concentrations of paracetamol at 1, 2, 5, 10, 20, 30, 50, 70, and 90% w/w in maize starch were prepared. The data obtained was used in preparing a calibration curve (Section 5.4.5) to assess the potential of DIP-MS in direct quantification of tablet samples

First of all, the crushed test tablet sample (powder) was loaded onto the closed end of a glass melting point capillary tube by dipping the tip into the sample of interest and then placing it into the compact mass spectrometer (CMS) for analysis. For each run, after placing the melting point tube in contact with the test powder sample at the rim of the sample bottle, excess test powder was removed before inserting the probe into the CMS. With the ASAP probe already in the sealed MS source enclosure, and in order for MS acquisition to proceed, the flowing stream of heated nitrogen gas was rapidly heated to 300°C. Only relatively low temperatures, up to 300°C were applied in order to avoid pyrolysis and in-source fragmentation. To minimize any background ions, a clean, new and empty glass capillary tube was inserted into the MS and heated up for approximately 15seconds. The acquisition of MS data was initiated while the newly heated capillary tube was still inserted into the source in order to obtain reference background scans. When collection of MS data for the blank glass capillary tube was complete, the probe was taken out from the source and the test sample was applied. The probe was re-introduced into the MS source and data acquisition re-started.

5.4 Results and Discussion

5.4.1 Application of DIP-MS to Analgesics/Antipyretics

5.4.1.1 Analysis of Reference Samples for Common Analgesics/Antipyretics

High purity/reference samples of paracetamol, aspirin and caffeine were subjected to the DIP process and the mass spectra recorded at the maximum TIC signal using 70eV electron ionisation (EI) are shown in Fig 5.4. Molecular ions for paracetamol and caffeine were detected at *m/z* 151 and 194 respectively whilst both compounds produced fragment ions at *m/z* 109 (Fig 5.4a and 5.4b). In samples stated to contain paracetamol and/or caffeine the detection of these ions would be sufficient confirmation of the presence of the correct API.

The molecular ion for aspirin at *m/z* 180 was also detected though in low relative abundance with more prominent fragment ions at *m/z* 138, 120 and 92 (Fig 5.4c). This is consistent with results obtained by Modick et al (2013), Sneha et al (2017) and Chonnker et al (2016) for paracetamol, caffeine and aspirin respectively. The m/z values obtained for paracetamol, caffeine and aspirin also agree with NIST Mass Spec data (NIST online). The presence of the three APIs can therefore be inferred by the detection of the characteristic peaks m/z 151 (paracetamol), m/z 194 (caffeine) and both m/z 138 and m/z 120 (aspirin). It should be noted how few peaks are present in the mass spectra compared to the complex traces obtained from the ATR-FTIR analyses.
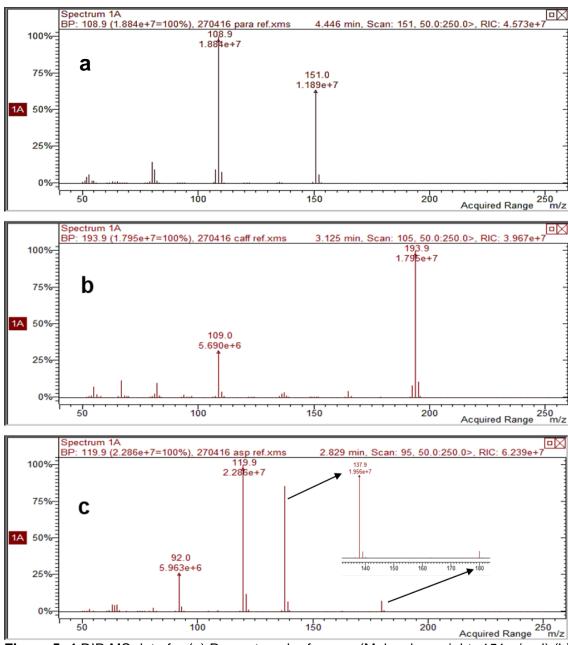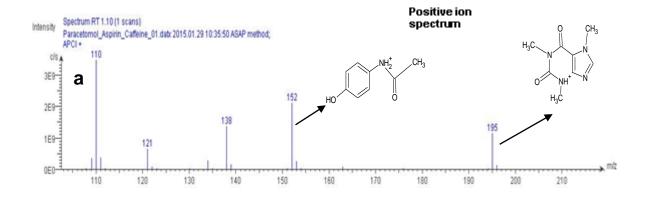
Figure 5. 4 DIP-MS data for (a) Paracetamol reference (Molecular weight- 151 g/mol) (b) Caffeine reference (Molecular weight- 194 g/mol) (c) Aspirin reference (Molecular weight- 180 g/mol)

Reference samples of paracetamol, aspirin and caffeine were also assessed using ambient ionisation ASAP-MS and mass spectra recorded. Figure 5.5 shows both positive (Fig 5.5a) and negative (Fig 5.5b) ion mass spectra of a mixture of reference samples of paracetamol, caffeine and aspirin obtained via ASAP-MS analysis.
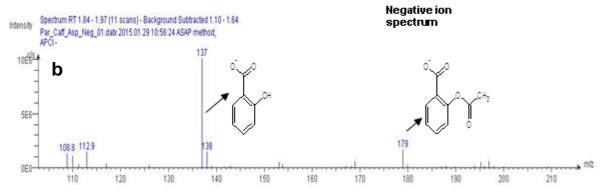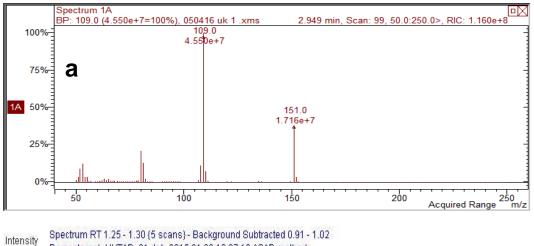
Figure 5. 5 ASAP data for a mixture of reference samples of Paracetamol, Caffeine and Aspirin (a) Positive ion spectrum (b) Negative ion spectrum.

Since ASAP-MS identifies the $[M + H]^+$ ions of analytes, *m/z* 152 and 195 in Fig. 5.5a indicate the presence of paracetamol and caffeine respectively. The fragment at m/z 110 is also the $[M + H]^+$ ion of the fragment at m/z 109 common to both paracetamol and caffeine as indicated in Fig 5.4 and so further confirmed the presence of both APIs. Interestingly the $(M + H)^+$ fragment ions for aspirin at m/z 121 and m/z 139 were detected in positive ion mode but in the negative ion mode (as highlighted in Fig 5.5b) the m/z values of 179 and 137 confirmed the presence of aspirin being the M-1 values for the molecular ion (m/z 180) and the fragment ion (m/z 138) respectively. The slight variation in mass spectral data (fragmentation patterns) between DIP-MS and ASAP-MS data for aspirin are due to the different energy transfer processes in the ionisation methods employed.

5.4.1.2 Paracetamol Tablet Results

Paracetamol tablets cited in Table 5.2 were then assessed to confirm the presence of the API. The mass spectrum in Fig 5.6 confirms the presence of the molecular ion for paracetamol, from a UK tablet as seen with the reference in Fig 5.4a. Similar results were produced by ambient ASAP-MS analysis with $[M + H]^+$ ions for paracetamol detected at m/z 110 and 152 for the fragment and molecular ion respectively (Fig 5.6b) further confirming the presence of the API, paracetamol.
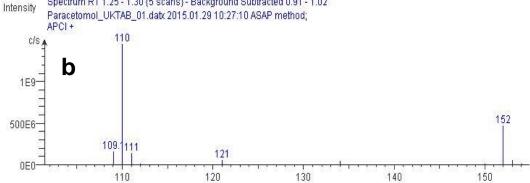
Figure 5. 6 Mass spectrum for Paracetamol tablet from UK (Molecular weight- 151 g/mol) (a) using high energy (EI) ionisation (b) using low energy ambient ionisation ASAP-MS.

Apart from the identification of APIs in test tablets, fragmentation patterns of analytes obtained using both ionisation methods for DIP-MS analysis might provide useful information for the characterisation of other compounds such as excipients and/or contaminants present in test tablets as well as valuable forensic data on the origin of the tablet samples.

Table 5.5 indicates the presence of paracetamol in the different test tablets obtained from different countries assessed via EI DIP-MS.

Table 5. 5 EI DIP-MS authentication of tablets containing paracetamol based on the presence of the API

Tablet(s)	Number of Tablets Assessed	Expected Amount (mg)	Paracetamol Present
UK	2	500	Y
Cyprus	2	500	Y
Switzerland	2	500	Y
Spain	2	500	Y
Spain	2	650	Y
Spain	2	1000	Y
Belgium	2	1000	Y
India	16	500	Y
India*	**2**	**325**	**Y**
India	1	650	Y
Pakistan	2	500	Y
Nepal	8	500	Y
China	4	500	Y
UAE	4	500	Y
Rwanda	6	500	Y
Ghana	4	500	Y
Jamaica	4	500	Y

Where n = number of tablets; Y = Yes/Paracetamol present; * = Suspect tablets based on ATR-FTIR study (Paracetamol detected along with other suspect components)

The previously reported (Chapter 3) ATR analyses indicated unexpected components present in the 325mg paracetamol tablet samples obtained from India. The data from the DIP investigation of these tablets (Figure 5.7) confirmed the presence of paracetamol and also other components with m/z values greater than 151. Inspection of these data (Fig 5.7) shows a recurring gap of 2m/z values between pairs of ions, notably m/z 214/216, 242/244, 277/279 and 353/355. In most cases a gap of 2 mass units indicates the presence of a halogen atom in the molecule usually chlorine, with isotopic masses of 35 and 37.
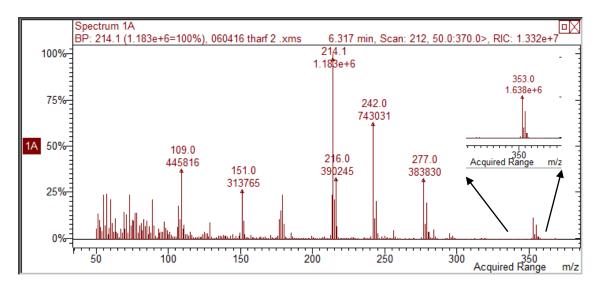
Figure 5. 7 Mass spectrum for suspect paracetamol tablet from India

Two compounds are often used in combination with paracetamol, diclofenac and aceclofenac. The mass spectral data (NIST), in decreasing intensity, for these compounds is:

Diclofenac   m/z 214, 242, 295, 216, 297 molecular ions m/z 295, 297
Aceclofenac   m/z 214, 242, 277, 216, 279 molecular ions m/z 353, 355

The mass spectral data is consistent with presence of aceclofenac in the tablet. White (2014), suggests this compound to be aceclofenac (nonsteroidal anti-inflammatory drug- NSAID) a common API used in combination with paracetamol generally used for rheumatoid arthritis and osteoarthritis. White (2014), also highlights that aceclofenac is not available over the counter in the US and is a prescription medication in countries like the UK, Italy and Spain though easily accessible through international internet purchases. White 2014, further points out that aceclofenac is widely utilised in India and some south-Asian countries and can be obtained over the counter or from street hawkers further confirming these results. White (2014), outlined the analytical profile of aceclofenac using several techniques including GC-MS. From the GC-MS study, the molecular ion was not detected but a fragment ion was detected at *m/z* 277. Other fragment ions were detected at *m/z* 242, 214 and 179 respectively.

The mass spectrum in Fig 5.7 confirms the presence of the fragment ions highlighted by White (2014), and the molecular ions at m/z 353/355 are also detected. The DIP-MS therefore provides a simpler and quicker method for detecting and identifying the compound which further highlights its potential in the authentication of medicines.

Table 5.6 outlines the tablets assessed via ambient ionisation ASAP-MS indicating the presence of paracetamol in all tablets analysed. However, the tablet sample from Hong Kong (as highlighted in bold in table 5.6) was found to contain another component not present in other tablet samples. The mass spectrum in Fig 5.8 again accentuates the efficiency of DIP-MS in the characterisation of test tablets samples. Ambient ionisation ASAP-MS enabled the characterisation of the paracetamol tablet from Hong Kong which identified the presence of paracetamol at m/z 110 and 152 $[M + H]^+$ but even more interestingly detected the presence of a chlorinated compound at m/z 230. This m/z value eliminates the presence of either diclofenac or aceclofenac and this could be a contaminant or undeclared constituent of the medication.

Table 5. 6 List of test tablets analysed using DIP-MS with low energy ambient ionisation (ASAP-MS) and their countries of origin

Tablet(s)	$[M+H]^+$ Detected (m/z)	API(s) Expected in Tablets
PCM UK A	110, 152	Paracetamol
PCM Nigeria A	110, 152	Paracetamol
PCM India A	110, 152	Paracetamol
PCM China A	110, 152	Paracetamol
PCM Hong Kong A*	**110, 152, 230***	**Paracetamol**

* = Suspect tablets another compound detected at m/z 230 in addition to Paracetamol

Contrastingly, though ATR-FTIR spectra confirmed the presence of paracetamol in the suspect tablet (PCM Hong Kong A), there was no noticeable difference in the tablet spectra indicative of the presence of another API, excipient or contaminant.
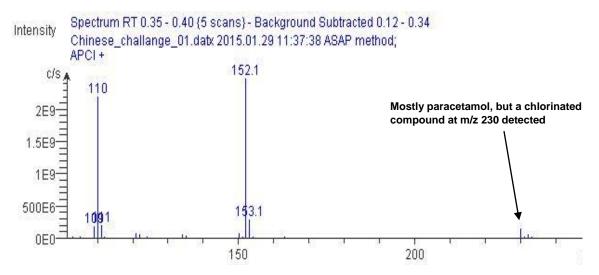
Figure 5. 8 ASAP-MS mass spectrum for suspect paracetamol tablet from Hong Kong.

Therefore, DIP-MS potentially provides a simple method for the characterisation of contaminants/adulterants to medicines along the supply chain and could enhance the capacity to trace the origin (post-marketing surveillance) when/where the medicines were interfered with. It could also be employed in the quality control process for monitoring production lines in pharmaceutical companies by carrying out checks at different points within the process to ensure there are no defects/errors or to identify the points these errors occur if they do. In addition, DIP-MS could provide an important technique not just for regulatory purposes but also for forensic investigations in the pharmaceutical manufacturing industry.

5.4.1.3 Evaluation of analgesic/antipyretic tablets containing multiple APIs

In the results discussed above in 5.4.1.2, the DIP system by EI demonstrated the presence of two APIs (paracetamol and aceclofenac) in the suspect tablet samples from Indian identified by the ATR-FTIR analysis in Chapter 3. The DIP assessment of tablets containing multiple APIs was therefore investigated. This is important because there have been reports of some falsified medicines containing other APIs like paracetamol which was not indicated on the tablet packaging (Höllein et al, 2016; Li et

al, 2017). It is also important to ascertain the ability of DIP-MS to screen for all APIs in tablet formulations simultaneously. OTC tablet samples from the UK, containing either 3 different APIs (Aspirin, Paracetamol and Caffeine) or 2 APIs (Paracetamol and Caffeine), as outlined in Table 5.3, were used for this study.

Referring back to Fig 5.2 in section 5.2.1, the TIC is the summation of all ions detected in an MS scan and it is therefore possible to search the recorded data (TIC) to see where particular ions (extracted ions) occur. This process is shown in Fig 5.9 where the TIC data from the 3 APIs tablet (red) is searched, (green) for m/z 151 –paracetamol, (orange) m/z 180- aspirin and (blue) m/z 194- caffeine. Temperature of the sample increases from left to right on the diagram and these compounds are volatilised at different temperatures as indicated by the traces.
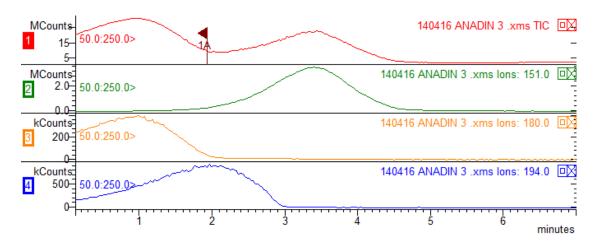
Figure 5. 9 Total ion chromatogram (TIC) and extracted ion chromatograms (EICs) for UK tablet containing 3 APIs (*m/z*- Paracetamol- 151, Aspirin- 180, Caffeine- 194).

Individual mass spectra will therefore vary depending on the time point selected within the total run time. Mass spectra recorded around the 2.0 minutes should contain ions from all the APIs as shown in Figure 5.9. This MS data is shown in Figure 5.10 where the ions from paracetamol (m/z 151) caffeine (m/z 194) and fragment ions from aspirin (m/z 138 and 120) are all visible.
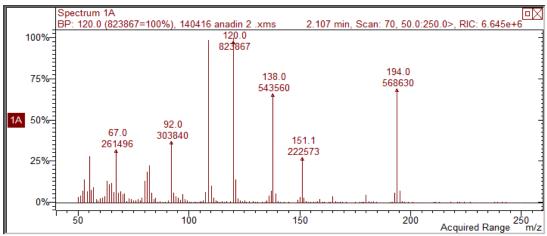
Figure 5. 10 Mass spectrum for UK tablet containing 3 APIs (*m/z*- Paracetamol- 151, Aspirin- 138, Caffeine- 194).

Both DIP-MS and ASAP MS were able to detect individual API ions from other analgesic tablets containing only two APIs (paracetamol and caffeine) (Fig 5.11).
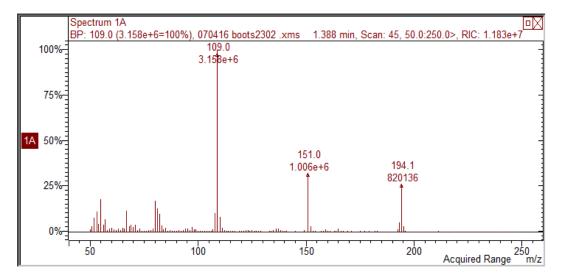
Figure 5. 11 Mass spectrum for UK tablet containing Paracetamol and Caffeine (*m/z*: Paracetamol- 151, Caffeine- 194).

Data in Table 5.7 represents details for individual test tablet samples with multiple APIs analysed via DIP-MS. In all cases, characteristic ions for individual APIs were detected enabling characterisation of the test tablet samples.

Table 5. 7 List of multiple API analgesic/antipyretic tablets containing paracetamol analysed using EI DIP-MS
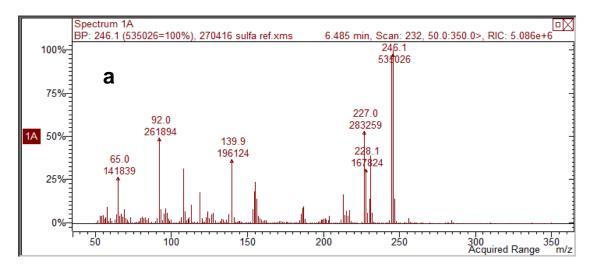
Tablet(s)	High energy DIP-MS data	ASAP-MS Data	API(s) Expected in Tablets
PC A1	M+ (m/z 151 and 194 observed for paracetamol and caffeine respectively); also fragment ion at m/z 109	-	Paracetamol and Caffeine
PC A2			
PC B1	M+ at m/z 151 and 194 observed low abundance; fragment ion at m/z 109	-	Paracetamol and Caffeine
PC B2			
PC C1	M+ at m/z 151 and 194 observed low abundance; fragment ion at m/z 109	-	Paracetamol and Caffeine
PC C2			
PCM UK B	-	M+1 (m/z 152 and 195 observed for paracetamol and caffeine respectively); also fragment ion at m/z 110	Paracetamol and Caffeine
PAC A1	Ions were at m/z 151, 194 and 180 with other fragment ions at m/z 92, 109, 120 and 138	-	Paracetamol Aspirin, and Caffeine
PAC A2			

This is very important in the screening for counterfeit medicines since most of these medicines are complex mixtures containing several APIs, excipients and other materials used by counterfeiters (contaminants/impurities). Furthermore, this individual m/z detection capability of DIP-MS or ASAP-MS in a complex mixture also lends itself to addressing one of the limitations of the spectroscopic techniques outlined in chapter 3 (ATR-FTIR spectroscopy) where there is difficulty in identifying individual components when the peaks are masked or overlap with those of other components.

5.4.2 Antimalarial medicines study

DIP-MS and ASAP-MS were used in the analysis of antimalarial tablet samples in order to avoid the limitations/difficultly encountered using the ATR-FTIR technique (Chapter 3) where the IR peaks were not well defined due to the complex nature of the APIs in these tablets. This complexity makes it difficult to fully characterise and quantify more than one API in the tablet.

Reference samples of two common APIs, sulfadoxine and chloroquine, found in antimalarial medicines were assessed using DIP-MS. Figure 5.12 (a and b) shows mass spectra of reference samples for the two APIs using ionisation at 70eV.
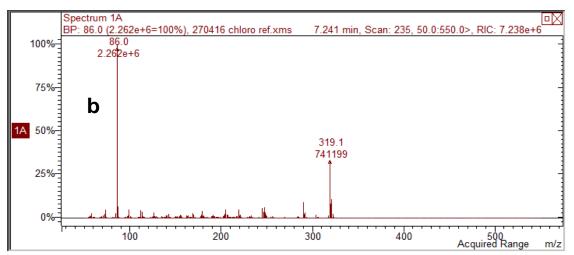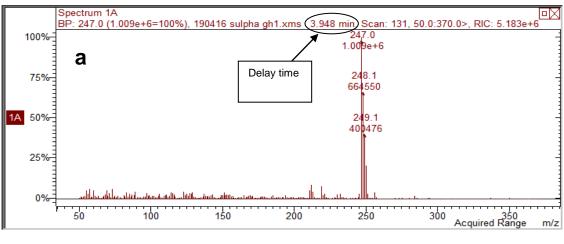
Figure 5. 12 Mass spectra for (a) sulfadoxine reference (Molecular ion: m/z 310) (b) chloroquine reference (Molecular ions: m/z 319 and 321).

For sulfadoxine (Mwt 310), the molecular ion was not detected but an intense peak was observed at *m/z* 246 and 245 with other fragment ions at *m/z* 227 and 228(Fig 5.12a). This was consistent with results obtained in previous studies (Florey, 1988) though there was no mass spectral data available for sulfadoxine in the NIST database. The molecular ion (M$^+$) for chloroquine was detected in low relative abundance at *m/z* 319 with the most abundant fragment ion at *m/z* 86 (Fig 5.12b) as observed in other studies

(Florey, 1984; Imran et al, 2016). The NIST data base also confirms mass spectral peaks at m/z 319 and 86 for chloroquine.

Antimalarial tablets containing these APIs (sulfadoxine and chloroquine) were then analysed. In addition, other tablets (Table 5.4 and 5.5) containing other antimalarial APIs, for example, arthemether, lumefantrine, pyrimethamine, amodiaquine and artesunate were also assessed and DIP-MS data compared with data from literature and the NIST online database to check the potential of DIP-MS for the identification of the API in antimalarial tablet medication.
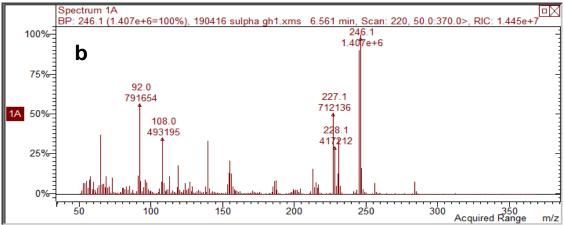
Figure 5. 13 Mass spectrum for Sulfadoxine and Pyrimethamine tablet 70eV- AM Ghana A (a) Delay time – 3.948min (b) Delay time – 6.561min.

A pack of tablets (AM. Ghana A) expected to contain sulfadoxine and pyrimethamine (Table 5.4) was assessed using 70eV DIP-MS and the presence of both APIs was

confirmed as indicated in the mass spectra in Fig 5.13a and Fig 5.13b. At a delay time of 6.561min the mass spectrum in Fig 5.13b was identical to the reference mass spectrum for sulfadoxine (Fig 5.12a) with similar delay time (6.485min). The base peak at *m/z* 246 and the fragment ion at *m/z* 227 were detected in the test tablet mass spectrum confirming the presence of sulfadoxine. The mass spectrum at delay time 3.948min (Fig 5.13a), showed the presence of pyrimethamine with m/z values at 247, 248, 249 and 250. The empirical formula of pyrimethamine is C12H13ClN4 and the two molecular ions are m/z 248 and 250 corresponding to the Cl 35 and 37 isotopes. The ions at m/z 247 and 249 correspond to the loss of a hydrogen atom from the molecular ions (Florey, 1983; Sandhya and Shijikumar, 2015). Again, these mass spectral data is consistent with the NIST database for pyrimethamine.
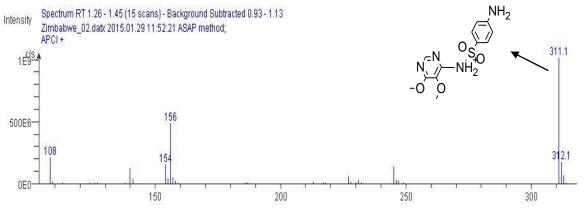
Figure 5. 14 ASAP-MS mass spectrum for Sulfadoxine and Pyrimethamine tablet

Interestingly, the 70eV DIP-MS mass spectral data in Fig 5.13 were noticeably different from those based on low energy ASAP-MS (Fig 5.14) for antimalarial tablet (AM. Zimbabwe C) expected to contain the same APIs (sulfadoxine and pyrimethamine). Low energy ASAP-MS confirmed the presence of the M+1 ion for sulfadoxine at m/z 311 with other fragment peaks at m/z 156 and 108. There was only a very low signal at the expected m/z values 247, 248 and 249 for pyrimethamine. Poor detection of pyrimethamine by ASAP-MS could be due to the soft ionisation method which might

not necessarily favour the production of the characteristic ions for pyrimethamine. Sensitivity of the equipment to the lower concentrations of API might be another point to note since the expected amount of pyrimethanime in the tablet is <5% w/w compared to about 80% w/w for sulfadoxine. Although the ASAP-MS data for sulfadoxine varies from the DIP-MS data, the ASAP-MS data for sulfadoxine is also consistent with GC-MS mass spectral data for sulfadoxine on the Drugbank (Drugbank Online) and the PubChem open chemistry (PubChem Online) databases. Since there could be variation in the ionisation/ fragmentation sequence depending on the ionisation method used during the Probe-MS process, it is important that mass spectrometric data obtained for test tablet samples be compared to reference samples assessed by the same analytical method.
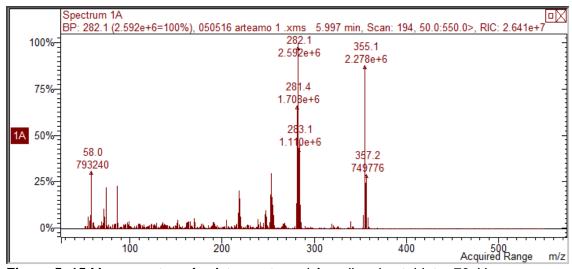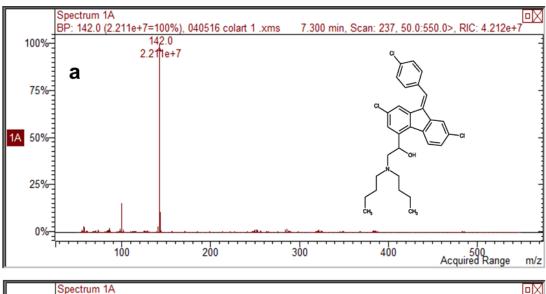
Figure 5. 15 Mass spectrum for Artesunate and Amodiaquine tablet – 70eV
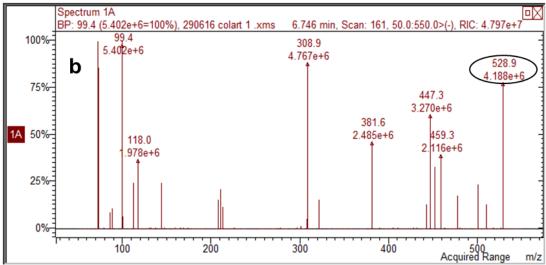
For high energy EI DIP-MS analysis of the antimalarial tablet containing amodiaquine and artesunate (AM. Zimbabwe B- Table 5.4), the molecular ions (M⁺) for amodiaquine at *m/z* 355 and *m/z* 357 were observed (Fig 5.15) indicative of the presence of a chlorine atom. A fragment ion at m/z 282 was present. These results are similar to those obtained by Florey (1992), Rathod et al (2016) and data available in the NIST database further confirming the presence of amodiaquine. On the other hand,

molecular ion for artesunate was not detected. Mass spectral data for artesunate is also not available in the NIST and DrugBank databases. This could be due to thermal stability issues attributed to artemisinin and its derivatives where degradation occurred when stored above room temperature (Ansari et al, 2013). Artesunate is a derivative of artemisinin so extreme temperatures might have caused degradation of the API during analysis. Furthermore, it is also possible that artesunate is not present in the sample as no other fragments were detected.

Two different packs of antimalarial tablets (AM. India A and AM. Zimbabwe A) with artemether and lumefantrine as the specified APIs were assessed using 70eV DIP MS. Under the current analytical conditions the APIs were volatilised between 6 and 8 minutes into the run. Examples of mass spectra recorded over this period are shown in Figure 5.16. In positive ion mode, the only ions observed were at m/z 142 and 100 (Fig 5.16a). Absence of any NIST mass spectral data for artemether and lumefantrine makes it difficult to characterise the individual APIs. However mass spectral data based on the Drugbank database identifies ions at m/z 142 and 100 as indicative of the presence of lumefantrine. Again, as observed with the tablet containing sulfadoxine and pyrimethamine, the difficulty in characterising API ions was found with the API of lower concentration (artemether). Expected concentration of artemether in each of these antimalarial tablets was about 8% w/w with much higher concentrations of 49% w/w expected for lumefantrine.

Molecular ion for lumefantrine at m/z 528.9 (Fig 5.16b) was also detected in EI negative ion mode. Results for lumefantrine were similar to those obtained by Bernier et al (2016). In the negative mode, an ion at m/z 298 was observed at an increased delay time of 7.6 minutes suggesting the presence of artemether in the tablet.
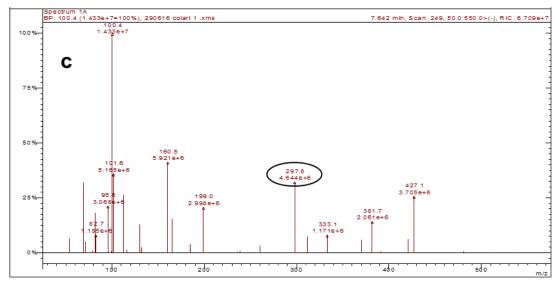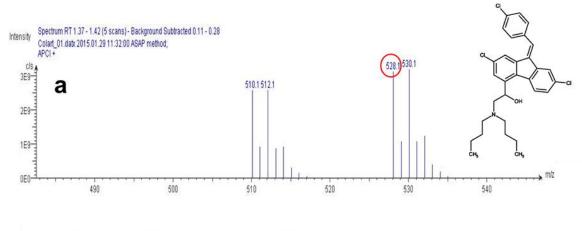
Figure 5. 16 Mass spectrum for antimalarial tablet containing artemether and lumefantrine -70eV (a) in positive ion mode (b) in negative ion mode showing molecular ion for lumefantrine at *m/z* 528.9 (c)) in negative ion mode showing molecular ion for artemether at *m/z* 298

Contrastingly, Carrà et al (2014) and Bernier et al (2016) did not detect the molecular ion at m/z 298 but other fragments like the [M+ NH$_4$] ion at m/z 316.21 and [M- OHCH$_3$ + H]$^+$ ion at m/z 267 which were not observed in this DIP MS study.
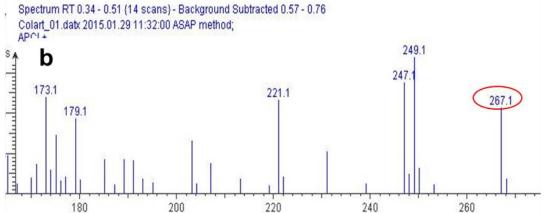
Figure 5. 17 ASAP mass spectrum for antimalarial tablet containing artemether and lumefantrine (a) showing molecular ion for lumefantrine at m/z 528 and 530 (b) showing fragment ion for artemether at m/z 267.

However, using ASAP-MS analysis, lumefantrine was detected as highlighted by ions at m/z 528, 530 and 532 indicative of the presence of chlorine atoms in the compound (see Fig 5.17a). Also, Fig 5.17b shows the ion at m/z 267 was detected suggesting the presence of the [M- OHCH$_3$ + H]$^+$ ion for artemether as highlighted by Bernier et al (2016). The difference in artemether data between both Probe-MS methods further suggests the ionisation methods employed could affect the fragmentation and hence, the ions produced because the mass spectral data for artemether based on ASAP-MS,

an ambient ionisation technique, are consistent with those obtained by Bernier et al (2016) who also used another ambient ionisation technique -DART-MS for analysis.

5.4.3 Effects of Probe Tip Temperature Ramp Rate on Signal Intensity

Since the rate of vaporisation of the sample is dependent on the temperature-programmed mode of the probe, this temperature also affected the detection of the API (Paracetamol) and the signal intensity. The relationship between the probe temperature and signal intensity was investigated for temperature ranges between 30 - 325°C using the reference paracetamol standard. Fig 5.18 and 5.19 show total ion chromatograms (TICs) and mass spectra for paracetamol analysed with initial probe temperature at 30ºC and 40ºC respectively while all other conditions remained the same.
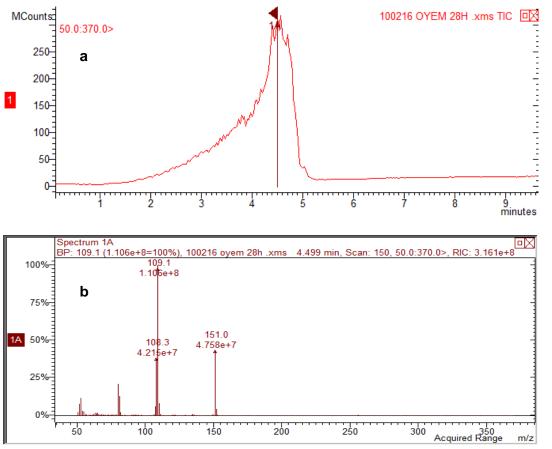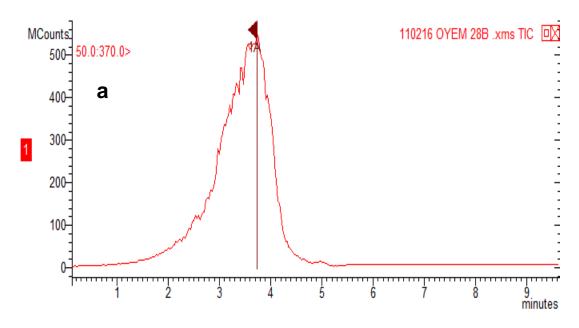
Figure 5. 18 Paracetamol reference- where temperature at TIC max is **165ºC** (a) TIC with initial probe temperature of **30ºC** (b) Mass spectrum with initial probe temperature of **30ºC**
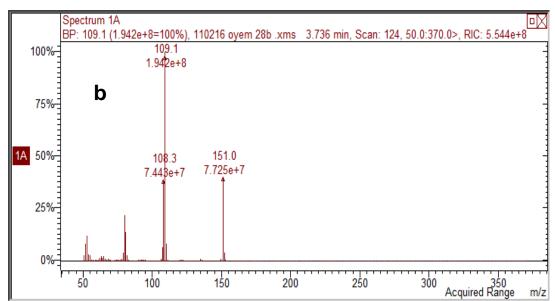
Figure 5. 19 Paracetamol reference - where temperature at TIC max is **152⁰C** (a) TIC with initial probe temperature of **40⁰C** (b) Mass spectrum with initial probe temperature of **40⁰C**.

Comparing the TICs in Fig 5.18a and 5.19a, it is observed that at lower initial probe temperature (30⁰C), paracetamol peak rises with a gentle slope to a maximum of about 300 MCounts while at 40⁰C, a peak which is more well defined and requires less time to arrive at a much higher count of about 500MCounts is observed. Analysis of paracetamol with initial probe temperature of 40⁰C was found to be a more efficient method for the samples investigated and so was the preferred initial temperature of the probe for analysis. Delay time was also found to be quicker (3.736min) using 40⁰C

initial probe temperature than with 30ºC (4.499min). In addition, considering the mass spectra in Fig 5.18b and 5.19b, the signal intensity showed progressive increase as temperature increased. For example the molecular ion at *m/z* 151 increased from 4.758e+7 at 30ºC initial probe temperature to 7.725e+7 at 40ºC. This was the same situation for the fragment ion at *m/z* 109 from 1.106e+8 at 30ºC to 1.942e+8 at 40ºC. This suggests that higher temperatures may lead to more highly sensitive signal depending on the boiling point of the sample.

On the other hand, temperature at TIC max for Fig 5.18 and 5.19 were 165ºC and 152ºC respectively. Since the ramp rate was the same for both runs (30ºC/ min), it is expected that the TIC max for both runs would be the same regardless of the initial probe temperature. The slight difference in TIC max temperature for both runs could be due to the sensitivity of the equipment to the amount of sample introduced into the probe tip as approximate amounts of sample were used for each run and not the exact same mass of sample per run. The minute amount of sample needed in the vial for each run therefore made it difficult to obtain the same sample mass before analysis. Thus, this variability in the amount of powder sample affixed to the probe tip may have obscured the effects of a change in probe temperature on the signal intensity.
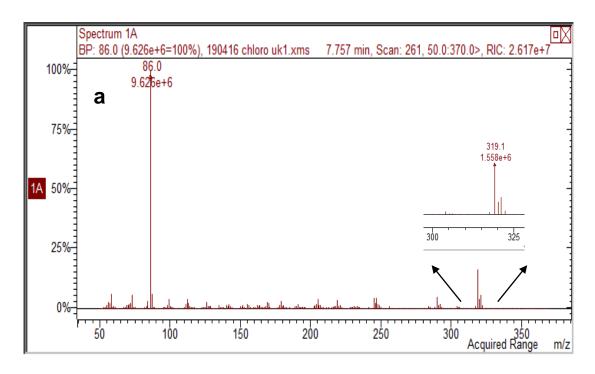
Ramping however must be controlled to avoid the sample vaporising too quickly or saturation of the signal as mentioned earlier. It is also important that start temperature (when heating the probe) in the probe programme is lower than the actual boiling point of the solid sample to enable proper ionisation and enhance signal intensity. This is important in order to prevent a situation where most of the sample on the probe tip is vaporised before it is introduced into the ionisation chamber.

5.4.4 Effects of electron voltage on the mass spectrum produced

Electron energy at 70eV has been identified to provide optimum sensitivity in mass spectrometry analysis since at this electron energy, all atoms/molecules can be ionised

(Gross, 2011). Electron ionisation (EI) spectra recorded at 70eV have also been identified as being more informative owing to the large number of fragments presented and the fact that these spectra are reproducible providing library searchable fingerprint spectra (Drugbank; NIST Online).

On the other hand, large number of fragments observed at 70eV could mean low abundance of the molecular ion making it more difficult to interpret spectra (Gross, 2011). Using chloroquine as an example, decreasing electron energy to ≤ 20eV had a crucial effect on the final spectrum obtained as demonstrated by a comparison of the data in Figure 5.20. Most of the fragment ions observed using 70eV electrons (Fig 5.20a) were no longer detected at 20eV leaving only the most characteristic fragment ions and the molecular ion for chloroquine at m/z 319 (Fig 5.20b). This is because electron energies higher than the ionisation energy of the test sample are required to facilitate its ionisation but subsequent fragmentation only reduces the level of the molecular ion signal but can provide some structural information concerning the molecule. In this research detection of the molecular ion is important.
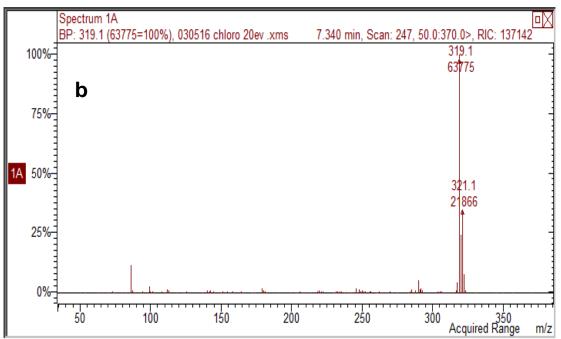
Figure 5. 20 Mass spectrum for Chloroquine tablet UK (a) Electron energy- 70eV (b) Electron energy– 20eV.

Table 5.8 is a list of tablets analysed and comments on the mass spectral data observed at different at 70ev and 20eV respectively.

Table 5. 8 DIP-MS data for antimalarial tablets with 70eV and 20eV

Tablet(s)	70eV	20eV	API(s) Expected in Tablets
AM. UK A1 & A2	M+ at *m/z* 319- low abundance; fragment at *m/z* 86 – high abundance	M+ at *m/z* 319 enhanced- high abundance; fragment at *m/z* 86 low abundance	Chloroquine phosphate
AM. Zimbabwe A1 & A2	**For artemether**: Ion(s) not detected. **For lumefantrine**: Ion at *m/z* 142 – high abundance; at m/z 100 – low abundance	**For artemether**: Ion(s) not detected. **For lumefantrine**: Ion at *m/z* 142 – high abundance; at *m/z* 100 – not detected	Artemether and Lumefantrine
AM. Zimbabwe B1 & B2	**For artesunate**: Ion(s) not detected. **For amodiaquine:** The molecular ion at *m/z* 355 and 357 were detected with the fragment ion at *m/z* 282.	**For artesunate**: Ion(s) not detected. **For amodiaquine:** The molecular ion at *m/z* 355 was detected but fragment ion at *m/z* 282 was not	Artesunate and Amodiaquine
AM. India A1 & A2	**For artemether**: Ion(s) not detected. **For lumefantrine:** Ion at m/z 142 – high abundance; at m/z 100 – low abundance	**For artemether**: Ion(s) not detected. **For lumefantrine:** Ion at *m/z* 142 – high abundance; at *m/z* 100 – not detected	Artemether and Lumefantrine
AM. Ghana A1 & A2	**For sulfadoxine**: Intense peaks at *m/z* 246 and 245 were detected with the fragment ions at *m/z* 227 and 228. **For pyrimethamine:** Molecular Ion at *m/z* 248 indicative of the Cl 35 isotope was detected with ions at *m/z* 247 and 249 indicating loss of hydrogen ion in molecular ions at *m/z* 248 and 250 (Cl 37 isotope) respectively. Ion at *m/z* 247 was the most prominent	**For sulfadoxine**: Ion at m/z 246 detected with high abundance like 70eV but fragment ion was diminished/in low abundance. **For pyrimethamine:** Molecular Ion at *m/z* 248 was detected with ions at *m/z* 247 and. Ion at *m/z* 248 was the most prominent	Sulfadoxine and Pyrimethamine

5.4.5 Limit of Detection (LOD) and Quantification Study

The potential of DIP-MS in the quantification of APIs in tablet medication was considered. Standard calibration mixtures of paracetamol (API) in maize starch

(between 10-90% w/w) were employed for this study. These calibration mixtures covered the range of concentrations within which paracetamol was present in OTC medicines (See Chapter 3).

The ion signal intensities at 70eV for each paracetamol standard mixture at the start, the apex and end of the peak (Fig 5.21) on the TIC were recorded as shown in Table 5.9 for paracetamol m/z values at 151. The mean of the ion signal intensities based on the three points (start, apex and end) of the paracetamol peak was also deduced for each paracetamol in maize starch calibration mixture. Since the TIC is indicative of the vaporisation of the individual components of the sample as highlighted in section 5.2.1, area under the curve was not feasible as reproducibility was a challenge. These data in Table 5.9 was recorded in a bid to ascertain the point on the TIC for the API (paracetamol) where quantitative analysis based on the mass spectrum ion signal intensities might be most feasible. The start and end points were determined by the point where paracetamol ions where first observed on the mass spectra and the point just before it was completely vaporised respectively.
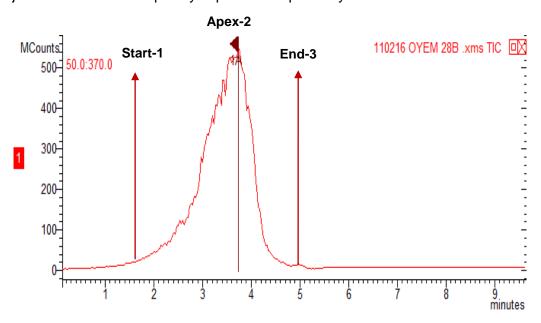
Figure 5. 21 TIC of a paracetamol test sample showing 3 approximate points (start, apex and end) on the paracetamol peak where mass spectral data was collected for each calibration standard sample considered for potential quantitative analysis of test tablet samples.

Table 5. 9 Ion signal intensities for calibration standard mixtures of paracetamol in maize starch (*m/z* 151) at different points on the TIC

Paracetamol Concentration (%w/w)	Peak Start (Ion Signal Intensity)	Peak Apex (Ion Signal Intensity)	Peak End (Ion Signal Intensity)
90	4.17E+06	6.67E+07	2.67E+07
70	1.08E+07	7.38E+07	3.73E+06
50	5.19E+06	9.81E+07	5.16E+06
30	3.43E+06	3.68E+07	1.29E+06
20	1.71E+06	1.16E+07	1.06E+06
10	1.10E+06	8.52E+06	6.81E+05
5	5.57E+05	4.73E+06	3.72E+05
2	5.26E+05	1.99E+06	3.63E+05
1	3.24E+05	1.15E+06	1.75E+05

Figure 5.22 indicates that there was poor linear correlation between paracetamol concentration and ion signal intensity across all the points considered on the TIC for paracetamol. As stated earlier, minimal amounts of sample were required for analysis; hence, the amount of paracetamol test tablet powder in the sample vial was not exactly the same between measurements.
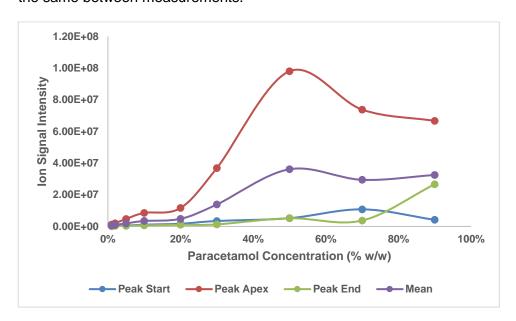
Figure 5. 22 Calibration curve for paracetamol in maize starch standard mixtures (1-90% w/w) using ion signal intensity for molecular ion at *m/z* 151.

Due to the high sensitivity and minimal amount of sample used for analysis with DIP-MS, slight variations in the distribution or homogeneity of the components of the mixture will have a huge impact on signal intensity. As such, direct quantification of the

API using DIP-MS could be a challenge. Therefore, to improve direct quantification of tablet samples, it might be important to ensure equal amounts of the crushed tablet samples are introduced into the vials each time and this could be achieved by suspending/dispersing known amounts of the powdered tablet sample in a medium that is heat stable.

Despite the fact that there was poor linear correlation between paracetamol concentration and ion signal intensity, further analysis of the quantitative data for paracetamol calibration samples based on the ion signal intensities showed improved linear correlation between ion signal intensity and paracetamol concentrations ≤ 10% w/w (Fig 5.23). It is not clear why this is the case but standardising the amount of test sample introduced in the probe per run could provide more information regarding this phenomenon (improved linearity for lower paracetamol concentrations).
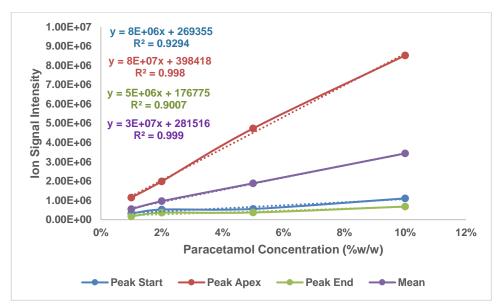
Figure 5. 23 Calibration curve for paracetamol in maize starch standard mixtures (1-10% w/w) using ion signal intensity for molecular ion at *m/z* 151.

The ion signal intensity at the apex of the peak represents data when paracetamol ions are most prevalent/fully ionised in the system during the run. Therefore, calibration based on the ion signal intensity at the apex of the peak might be most useful/ efficient in the direct quantitative analysis of tablet medication but further investigations will be

required to clarify this as data available so far are not enough to make any conclusive statements.

Figure 5.24 shows that the presence of the API, paracetamol could be identified down to 0.1% w/w of the paracetamol/maize starch powder mixture. In addition, the amount of test sample powder used is much less than the amount needed for analysis in the spectroscopic techniques thereby further reducing contact and exposure to the samples/chemicals.
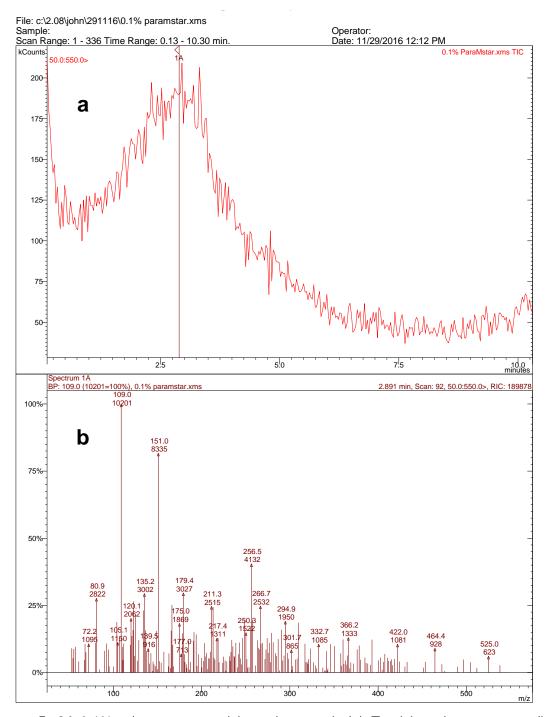
Figure 5. 24 0.1% w/w paracetamol in maize starch (a) Total ion chromatogram (b) Mass spectrum.

The ability of DIP-MS to identify much lower levels of particular compounds in a mixture could even be more valuable in the detection of toxic impurities in pharmaceutical tablet samples. DIP-MS was therefore found to be much more sensitive than the

spectroscopic techniques even when analysing the powder mixture directly without using any solvent extractive techniques and in full scan mode.

Consequently DIP-MS shows its propensity in the simple and rapid analysis of tablet samples with concentrations much lower than the limit of detection of ATR-FTIR spectroscopy. It is also non-destructive since no treatment is required for samples before analysis safe crushing of the tablet.

5.5 Conclusion

DIP-MS therefore provides another simple and more sensitive option in the event that samples to be analysed exceed the limit of detection of the cheaper but less sensitive spectroscopic techniques. This is observed by its ability to identify individual components in analgesic and antimalarial tablets. The probe MS method was able characterise and identify unknown material in suspect tablets. Furthermore, samples were analysed directly without solvent extraction thereby saving time spent in sample preparation and the associated costs of solvent consumption. Probe MS data were obtained in less than 10 minutes. Its non-destructive feature and the speed of analysis compared to conventional solvent extraction techniques makes DIP-MS well-suited for assessing possible falsified and substandard medicines. The potential of DIP-MS in the direct quantification of APIs in pharmaceutical solid dosage forms can be further explored.

Though the pharmacopoeia approved solvent extraction techniques are generally employed in the analysis of known chemicals (including APIs, excipients and possible impurites/contaminants) in pharmaceutical tablet formulations, these techniques might not be the most efficient for the forensic analysis of unknowns. DIP-MS on the other hand enables identification of unknowns as individual masses of the components molecular or fragment ions are recorded which further helps in elucidation of the structure of the compound.

Moreover, with the advent of mobile fibre optics spectrometers and their increasing availability, portable spectrometry based instruments can be deployed for in-field analysis of medicines.

Operation of the DIP-MS instrument including spectral interpretation can be automated by comparing recorded mass spectra with those available in a local reference library which in turn helps to provide logical results/data to non-specialist personnel in the field.

In addition to the simpler and quicker analytical techniques already proposed in Chapters 3 -5 for the screening of medicines in LMICs, conventional solvent extraction techniques were examined in order to validate results based on the rapid methods developed. Ultraviolet- visible (UV-vis) spectroscopy and Liquid Chromatography-Mass Spectrometry (LC-MS) will be the solvent extraction techniques considered in the next two chapters.

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CHAPTER SIX

Ultraviolet-Visible (UV-Vis) Spectrophotometry for validation of quantitative ATR-FTIR data

6.1 Introduction

Advancement in technology has made authentication of medicines and pharmacovigilance in general very complex as established in Chapter 2. Consequently, it is essential that methods employed in the screening of medicines facilitate the prompt removal of these medicines from the market (Dégardin et al, 2015).

Quantification of the API is very crucial in the quality control of medicines since inconsistency in API content or absence of the API could pose a serious threat to public health as highlighted in Chapter 2 (Dégardin et al, 2014). This is because medicines are beneficial not just when the APIs are present but when the APIs are present in the right amount. Quantitative analysis of medicines will help guard against instances of drug toxicity due to higher than expected API or resistance to medication as a result of lower than expected API (under dosage) as seen with antimalarials and antibiotics (Kelesidis and Falagas, 2015; Koczwara and Dressman, 2017). It is therefore very important that measures be put in place (as part of the pharmacovigilance process) to determine drug content in medicines at every point in the pharmaceutical distribution chain. In Chapter 3, this study demonstrates the novel use of ATR-FTIR in the rapid quantification of APIs in tablet dosage forms and its potential in the screening of medicines in-field and in LMICs. It is therefore important that the data obtained via the proposed ATR-FTIR method be validated using conventional approaches or pharmacopoeia approved methods. An assessment of the correlation between the proposed ATR-FTIR method and conventional methods will help in providing more comprehensive data about tablet medicines sent for test in order to confirm if they are genuine.

Ultraviolet-Visible (UV-Vis) spectrophotometry is one of the most commonly used techniques in pharmaceutical analysis (Behera et al, 2012; Ojeda and Rojas, 2013). Misiuk (2010), identifies UV-Vis spectrophotometry and the liquid chromatography as

the most frequently applied in pharmacopoeial monographs. This technique (UV-Vis), can be used for the quantification of the Active Pharmaceutical Ingredient (API) in medicines while screening for FSMs, since the absorbance patterns are indicative of the materials present and the intensity of the absorbance bands is directly proportional to the concentration of the API being assessed (Rote et al, 2012; Figueroa et al, 2015).

Several studies have investigated the use of UV-Vis in the quantitative assessment of medicines (Kumar Talluri et al, 2012; Khanage et al, 2013; Islam et al, 2016). Kumar Talluri et al (2012) and Khanage et al (2013) considered the simultaneous determination of paracetamol in the presence of other APIs (Iornoxicam and Eperisone respectively) in pharmaceutical solid dosage forms. Islam et al (2016), on the other hand developed UV-Vis methods for the determination of calcium orotate in tablet dosage forms. UV-Vis has also been used together with chemometric methods in the quantitative determination of paracetamol in pharmaceutical tablet formulations (Khajehsharifi et al, 2010; Glavanović et al, 2016).

Although UV-Vis has proven useful in the quantitative assessment of medicines, it is not as efficient as a qualitative analytical methodology since species are identified based on absorbance ranges (with maximum absorption- λ_{max}) in the ultraviolet or visible region rather than a definite wavelength. This is because there could be shifts in the absorption maxima (characteristic wavelength) of the species being assessed and this has been linked to the use of different solvents or the ratios of the solvents employed in the investigation (Figueroa et al, 2015).

Figueroa et al (2015) in their study using UV-Vis to detect counterfeit pharmaceuticals, also attribute the decrease in the absorption of paracetamol at 245nm and slight increase in other parts of the spectrum to contaminants which interact with the benzene ring of paracetamol. Although the use of UV-Vis spectrophotometry for simply identifying test species (analytes) is a challenge, Figueroa et al (2015) propose the

potential use of UV-Vis spectrophotometry in the identification of suspect samples based on the change in absorbance at particular wavelengths as well as the unique wavelength shifts observed when different solvents are employed on a particular analyte.

Although UV-Vis has been used extensively for analysis of medicines, the approach requires access to solvents and laboratory facilities which for the purpose of this study are considered beyond the reach of the people in the LMICs. In addition since UV-Vis is a solvent extraction technique, suggesting it requires a proper understanding of the analyte in question. This entails training which might be a challenge in areas where funds are not readily available.

Hence, this chapter describes UV-Vis as a technique used in the validation of quantitative paracetamol data for tablet samples obtained via ATR-FTIR in Chapter 3. Since there were challenges quantifying chloroquine based on the proposed ATR-FTIR, only the paracetamol tablet data were considered for UV-Vis validation.

6.1.1 Ultraviolet-Visible Spectrophotometry Principle

As a quantitative technique, UV-Vis estimates the amount of molecular species based on the radiation absorbed. UV-Vis use is however limited to the analysis of substances that can absorb light in the UV-Vis region (200-700nm) (Behera et al, 2012; Rote et al, 2012). As such, UV-Vis spectrophotometry is not useful in the direct determination of substances with poor light-absorbing capacity (e.g. aminoglycosidic antibiotics) (Ojeda and Rojas, 2013). The groups within a molecule that are able to absorb light are referred to as chromophores (Almeling et al, 2012). This technique is cheaper than other pharmacopoeia approved techniques like the LC-MS but is also a solvent extraction technique as mentioned earlier. Sample preparataion will therefore involve

lengthy processes. Quantitative UV-Vis spectrophotometric analysis of substances is fundamentally governed by the Beer-Lambert's law which states that:

When a beam of light is passed through a transparent cell containing a solution of an absorbing substance, attenuation of the intensity of light may occur (Behera et al, 2012). Beer-Lambert's law is expressed mathematically as;

$A = abc$

Where, A= absorbance or optical density, a= absorptivity/ extinction coefficient, b= radiation path length through sample (cm), c= concentration of solute in solution
a and b are constant hence, A (absorbance) is directly proportional to c (concentration).

It is also important to note that solutions to be analysed have to be dilute enough as absorbance values are measured between 0 and 1.

Therefore, to quantify pharmaceutical solid dosage forms using the UV-Vis spectrophotometer, a solution of the test tablet sample in transparent solvent is prepared and its absorbance is measured at a suitable wavelength. Normally, the wavelength of choice is the wavelength of maximum absorption (λ_{max}) where there is negligible effect on the measured absorbance even in the event of small errors in setting the wavelength scale (Behera et al, 2012; Figueroa et al, 2015). To assay a single component sample containing other absorbing substances, the absorbance values of a range of standard solutions of the reference sample are recorded and used to produce a calibration curve. The concentration of the analyte in the test solution is then deduced from the calibration curve as the concentration corresponding to the measured absorbance of the solution. Figure 5.1 is UV-Vis spectra for paracetamol showing the $\lambda_{max.}$
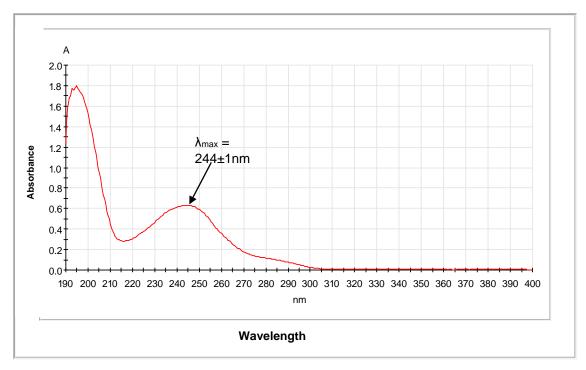
Figure 6. 1 UV-Vis spectra of paracetamol standard showing λ_{max} at 244nm

6.2 Materials and Methods

6.2.1 Materials and Reagents

Reference sample of paracetamol was obtained from Sigma-Aldrich Company, Dorset UK. UV grade methanol was obtained from Fisher Scientific Ltd. Loughborough, UK.

6.2.2 Test Tablet Samples

OTC paracetamol tablets obtained from different countries around the world which had been quantitatively screened using ATR-FTIR (Chapter 3) were subjected to UV-Vis analysis for the purpose of validation. Table 6.1 provides a summary of the countries of origin and the expected dose of the tablets analysed.

Table 6. 1 List of analgesic/antipyretic tablets containing paracetamol analysed using UV-Vis and their countries of origin and expected amounts

Tablet(s)	Number of Tablet Samples	Expected Amount (mg)
UK	2	500
Belgium	2	1000
Spain	2	500
Spain	2	650
Spain	2	1000
Cyprus	2	500
Switzerland	2	500
Pakistan	2	500
China	4	500
UAE	4	500
Rwanda	6	500
Ghana	4	500
India	16	500
India	2	325
India	1	650
Jamaica	4	500
Nigeria	4	500
Nepal	8	500

6.2.3 Instrumentation

6.2.3.1 UV-Vis Analysis

UV spectra were collected using UV-Visible spectrophotometer, Helios Gamma (Thermo Electron Corporation England). The studied spectral range was 190-400nm with a scan interval of 0.5nm. Quantitative readings for paracetamol were taken at 244nm. The UV-Visible spectrophotometer was piloted using the VisionLite software 2.2 (Ueberlingen, Germany).

6.2.4 Methods

Pulverized and properly homogenised paracetamol tablet samples already used for ATR-FTIR analysis were subjected to UV-Vis analysis to verify results obtained by the

ATR-FTIR. The protocol used by Behera et al (2012) was adopted for this part of the study.

6.2.4.1 Preparation of paracetamol standard solutions for calibration graph

10mg Paracetamol standard was dissolved in 15ml methanol. 85ml double-distilled water was then added to make up the volume to 100ml (100ppm). From the 100ppm solution, 2ml was taken and made up to 20ml (10ppm) with diluent. UV grade methanol and double-distilled water (15:85 v/v) was used as the diluent. Further dilutions of the 10ppm paracetamol standard were made to obtain concentrations between 2ppm and 10pmm (2, 4, 6, 8, and 10ppm) for paracetamol.

6.2.4.2 Test Tablet Sample Preparation for UV analysis

Each tablet was weighed and pulverised. 10mg of the pulverised paracetamol tablet was weighed and dissolved in 15ml methanol in a 100ml volumetric flask. 85ml double-distilled water was added to make up the volume to 100ml. The solution was also filtered using Whatman® filter paper and 2ml was taken out of the filtered solution and made up to 20ml with diluent in a 20ml volumetric flask (10ppm). Working solutions of each tablet sample along with the paracetamol standard solutions were then subjected to UV-Vis analysis.

6.2.4.3 Recording UV-Vis Spectra

All UV spectra were measured in absorbance mode. The solution to be analysed was placed in a quartz cuvette and scanned across the range 190-400nm in the UV-Vis spectrophotometer. Each test sample solution was analysed in triplicate to ensure reproducibility and blanks were run after every sample. The cuvette was cleaned after each measurement with diluent and dried with paper tissue before placing the next sample in the cuvette to avoid contamination or sample carry over.

6.2.4.4 Quantitative analysis of test tablet samples and calculations

All quantitative data was processed using Microsoft Excel 2016. Measured levels of paracetamol in working solution were obtained by substituting absorbance values for the samples in the calibration equation. The measured levels were indicative of the amount of the API in the 10ppm working solution of the test sample.

Sample calculation for the amount of paracetamol in mg for each tablet is outlined in section 6.2.3.4.1.

6.2.4.4.1 Sample Calculation for amount of Paracetamol based on portion of tablet

Considering a 10ppm solution of a solvent extracted portion (10mg) of a paracetamol tablet with mean absorbance found to be 0.558 for instance,

Mean absorbance reading= 0.558, calibration equation: y= 0.0635x + 0.0122

Substituting the absorbance value for y in the calibration equation, 0.558= 0.0635x + 0.0122

x = (0.558 – 0.0122)/0.0635 = 8.59ppm

10ppm tablet solution = 8.59ppm paracetamol and 10ppm=10mg/l

Therefore,

10mg of tablet powder contains 8.59mg paracetamol

Actual amount of paracetamol in tablet = (Tablet weight/10)* Amount in 10mg of the tablet

For tablet investigated, Tablet weight before crushing = 557.4mg

Actual amount of paracetamol in tablet = (557.4/10) * 8.59= 55.74* 8.59 = 479mg

6.2.4.5 Validation of method

The adopted method for paracetamol was validated in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy and precision.

6.2.4.5.1 Linearity

Linearity was assessed based on the correlation coefficient (R^2 - value) of the calibration curve for the mean absorbance versus the paracetamol standard concentrations. LOD and LOQ were determined based on the 3.3 σ/s and 10 σ/s criteria, respectively; where σ refers to the standard deviation of peak area and 's' is the slope of the calibration curve. Paracetamol showed good linearity for the concentration range assessed

6.2.4.5.2 Accuracy

To check accuracy of the method, an incurred sample analysis was employed in assessing recovery for paracetamol standards based on the calibration curve. Absorbance values, for test samples, were substituted into the calibration curve equation to obtain measured concentrations of paracetamol standards and these were compared to expected concentrations of the paracetamol standards.

6.2.4.5.3 Precision

Precision of the method was based on repeatability and variation in terms of the level of the expected API shown by the relative standard deviation (%RSD).

6.3 Results and Discussion

6.3.1 UV Spectra

Maximum absorbance was at 244nm±1 which was in agreement with Behera et al (2012) and Nnadi et al (2013) suggesting the presence of paracetamol in the samples analysed.

6.3.2 Quantification data: Linearity, Limit of Detection (LOD) and Limit of Quantification (LOQ)

Calibration curves were plotted for paracetamol based on data shown in Table 6.2. Linearity of paracetamol data obtained using the method is highlighted by correlation coefficients ≥ 0.998. Relative standard deviation (RSD) values are also highlighted in addition to the mean and standard deviation (SD). Figure 6.2 shows the calibration curve with data points representing the mean±SD of 3 replicates. Correlation coefficient of the mean reading is also indicated.

Table 6. 2 Paracetamol standard concentrations and absorbance values for UV-Vis calibration curve

Concentration (ppm)	Replicate 1	Replicate 2	Replicate 3	Mean±SD	RSD
2	0.113	0.110	0.112	0.112±0.002	1.79
4	0.229	0.228	0.229	0.229±0.001	0.45
6	0.364	0.363	0.364	0.364±0.001	0.27
8	0.496	0.490	0.493	0.493±0.004	0.81
10	0.631	0.635	0.633	0.633±0.003	0.47
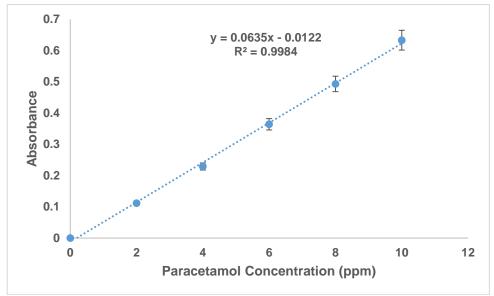
Figure 6. 2 UV-Vis calibration curve for paracetamol standards over the range 2-10ppm (Data points represent the mean±SD of 3 replicates.

The data in Table 6.2 highlights the reproducibility of the data obtained. Using the equations mentioned earlier in section 6.2.3.5.1, LOD and LOQ for the method were 1.0ppm and 3.0ppm respectively.

6.3.3 Accuracy/ Incurred sample analysis

Reference standards were analysed using the developed protocol. Mean absorbance values obtained were resubstituted in the calibration equation to check closeness of the measured concentration values based on the method to the expected concentrations as outlined in section 7.2.4.5.2. Results obtained were satisfactory as shown by the data in Table 6.3. Mean recovery of measured concentration values compared to expected concentrations was 99±3%.

Table 6. 3 Comparison of measured versus expected concentrations of paracetamol in standard calibration solutions

Standard	Expected concentration (ppm)	Measured concentration (ppm)	Recovery (%)
1.	2.0	2.0	100.0
2.	4.0	3.8	95.0
3.	6.0	5.9	98.3
4.	8.0	8.0	100.0
5.	10.0	10.2	102.0

6.3.4 Precision of UV-Vis Data

The data obtained using UV-Vis showed good precision with relative standard deviation (RSD) for calibration measurements found to be between 0.27-1.79 percent (Table 6.2).

6.3.5 UV-Vis Quantitative Data for Paracetamol Tablet Samples

The actual concentration of working solutions for each tablet were calculated by substituting measured absorbance values in the calibration equation. The amount of paracetamol in each tablet was calculated following the steps outlined in section 6.2.3.4.1. The amount of paracetamol in each tablet assessed is recorded in Table 6.4.

Table 6. 4 Summary of UV-Vis quantitative data for paracetamol tablets from around the world (Results are the mean±SD of 3 replicates)

Region	Country (Number of Tablets)	Tablets Analysed	UV Measured Content (mg)	Expected Amount (mg)
Europe (n=14)	UK (n=2)	UK P1T1	532±4	500
		UK P1T2	479±3	
	Cyprus (n=2)	Cyp P1T1	438±6	
		Cyp P1T2	442±6	
	Switzerland (n=2)	Swz P1T1	523±6	
		Swz P1T2	508±5	
	Spain (n=6)	Spn P1T1	529±7	
		Spn P1T2	515±6	
		Spn P2T1	678±8	650
		Spn P2T2	648±5	
		Spn P3T1	1005±13	1000
		Spn P3T2	1008±10	
	Belgium (n=2)	Bel P1T1	1031±11	
		Bel P1T2	1090±11	
Asia & Middle East (n=37)	India (n=19)	Ind P1T1	480±3	500
		Ind P1T2	545±5	
		Ind P2T1	521±5	
		Ind P2T2	539±3	
		Ind P3T1	464±4	
		Ind P3T2	499±4	
		Ind P3T3	528±4	
		Ind P4T1	478±3	
		Ind P4T2	550±5	
		Ind P5T1	504±3	
		Ind P5T2	539±4	
		Ind P5T3	485±3	
		Ind P6T1	487±7	
		Ind P6T2	463±6	
		Ind P7T1	502±7	
		Ind P7T2	442±6	
		Ind P8T1	365±3	325
		Ind P8T2	358±3	
		Ind P9T1	660±6	650
	Pakistan (n=2)	Pak P1T1	449±6	500
		Pak P1T2	453±8	
	Nepal (n=8)	Nep P1T1	501±5	
		Nep P1T2	498±6	
		Nep P2T1	497±7	
		Nep P2T2	475±7	
		Nep P3T1	450±4	
		Nep P3T2	494±3	
		Nep P4T1	517±6	
		Nep P4T2	552±5	

Table 6.4 (Continued)

Region	Country (Number of Tablets)	Tablets Analysed	UV Measured Content (mg)	Expected Amount (mg)
	China (n=4)	Chn P1T1	541±4	
		Chn P1T2	495±3	
		Chn P2T1	488±3	
		Chn P2T2	548±4	
	UAE (n=4)	UAE P1T1	525±7	
		UAE P1T2	513±6	
		UAE P2T1	484±5	
		UAE P2T2	500±6	
Africa and Caribbean Islands (n=18)	Rwanda (n=6)	Rwa P1T1	543±4	500
		Rwa P2T1	476±3	
		Rwa P3T1	511±4	
		Rwa P3T2	581±4	
		Rwa P4T1	503±3	
		Rwa P4T2	519±4	
	Ghana (n=4)	Gha P1T1	476±5	
		Gha P1T2	504±5	
		Gha P2T1	508±6	
		Gha P2T2	479±6	
	Jamaica (n=4)	Jam P1T1	510±5	
		Jam P1T2	515±6	
		Jam P2T1	533±6	
		Jam P2T2	511±7	
	Nigeria (n=4)	Nig P1T1	463±2	
		Nig P1T2	479±4	
		Nig P2T1	516±10	
		Nig P2T2	547±3	

Results shown in Table 6.4 confirm UV quantitative results for all the tablets generally fell within the allowed limits by the British Pharmacopoeia (85-115% of expected amount) (BP, 2017) and well within the global threshold limits (FDA, 2014). These generally agree with the ATR-FTIR data in Chapter 3.
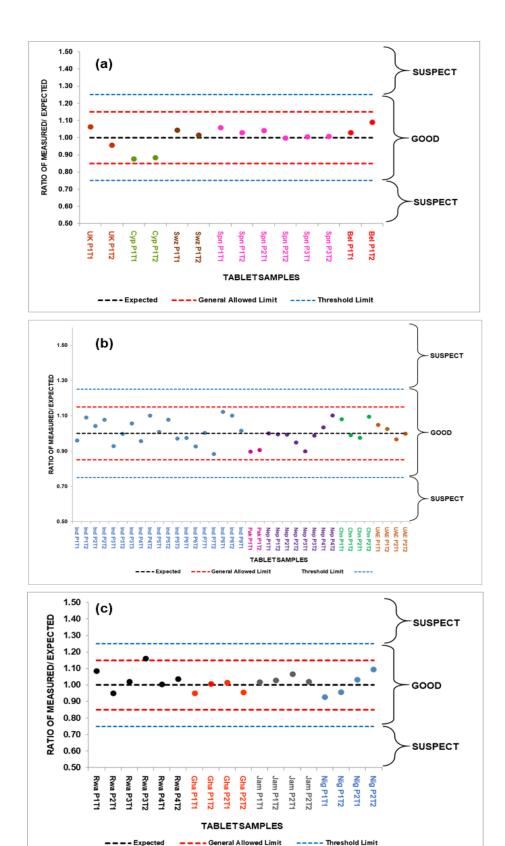
Figure 6. 3 Ratio of measured to expected amounts of Paracetamol in tablet samples from around the world using UV-analysis (a) 14 tablet samples from Europe (b) 37 tablet samples from Asia and the Middle East (c) 18 tablet samples from Africa and the Caribbean Islands

Just like with the ATR-FTIR data, the UV-Vis data is presented as a plot of the ratio of measured to expected amounts of paracetamol versus the tablet sample origin in Fig 6.3(a-c). The diagrams showing paracetamol data from three regions (Europe; Asia and the Middle East; Africa and the Caribbean Islands) clearly indicates that all samples generally fall within the accepted limits as observed in Table 6.4. However, some significant differences were noted. High values were obtained for some tablets from Cyprus (Cyp P1T1, Cyp P1T2) and India (Ind P8T1, Ind P8T2) using ATR-FTIR when compared to UV-Vis data for the same tablets. On the other hand, low paracetamol levels were obtained from both ATR-FTIR and UV-Vis analysis for tablets from Pakistan (Pak P1T1, Pak P1T2). Results based on probe mass spectrometry in Chapter 4 identified a second API (aceclofenac) in addition to the paracetamol in the Ind P8 tablets. Therefore, lower paracetamol values with UV-Vis further suggests that although paracetamol is present in these tablets (Ind P8) as identified by the ATR-FTIR technique, the second API could be the reason for higher paracetamol values in the region of the ATR-FTIR spectra assessed. A detailed comparison of the techniques employed in the study and possible reasons for variation in the data are discussed in Chapter 8. In addition, the significance of the proposed rapid analytical methods in the screening process for FSMs (especially in LMICs) is highlighted.

General agreement between ATR-FTIR further highlights the potential of ATR-FTIR in the screening of medicines.

6.4 Conclusion

The quantitative determination of paracetamol in pharmaceutical tablet formulations using UV-Vis spectrophotometry as outlined in this chapter produced some findings worthy of note. UV-Vis results obtained for paracetamol test tablets confirmed the presence of paracetamol in all the tablets analysed via ATR-FTIR in Chapter 3 further validating the data obtained. UV-Vis spectrophotometry involves a lengthy (solvent extraction) sample preparation process and would require adequate training of the technician handling the equipment. Training of the technician or operator is also important because sample specific method development (involving solvent extraction of the analyte) is required in order to obtain reproducible data and this has to be done manually.

With UV-Vis data indicating paracetamol levels for all the tablet samples assessed fell within acceptable limits (and mostly in agreement with ATR-FTIR), it was therefore important to reassess tablets samples where significant differences were observed between the two techniques. Further tests will provide understanding on the reason for the variance in data for the identified tablets. Therefore, investigations based on liquid chromatography, identified as the pharmacopoeia gold standard was necessary. The tablet samples with inconclusive paracetamol data based on UV-Vis and ATR-FTIR formed the basis for liquid chromatography-mass spectrometry described in Chapter 7.

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CHAPTER SEVEN

Confirmatory testing for pharmaceutical ingredient in tablet dosage forms by liquid chromatography- mass spectrometry

7.1 Introduction

As established in previous chapters, the intent of falsifiers of medicine is to deceive and anonymity allows falsifiers of medicines to thrive. As a result, the differences between FSMs and genuine medicines need to be subtle to avoid being detected. Comprehensive data is therefore needed in order to discriminate a falsified or substandard medicine from a genuine one (Dégardin et al, 2015). It is difficult to find one technique that provides all the necessary information when screening for FSMs. As observed with the techniques employed in Chapters three to six, each technique provides unique and valuable information about the medicines being analysed. A good understanding of what information is needed at each stage of the screening process will then inform the approach to be taken (Dégardin et al, 2014). Just as speed of analysis is crucial in the forensic analysis of FSMs, sensitivity cannot be overlooked especially if this information is to form the basis for criminal investigations. It is important that cases of false positive or negative results are ruled out. This is to avoid a situation where either genuine medicines are identified as falsified thereby denying the patients access to these medicines or falsified medicines are identified as genuine posing a threat to public health. Validation of data obtained during preliminary analytical screening of medicines is therefore necessary in order to make conclusive statements about the genuineness of medicines. Identification of an FSM is usually a two stage process where rapid screening tests are used on site followed by confirmatory tests using more sophisticated laboratory based equipment such as the liquid chromatography mass spectrometer combination (LC-MS).

Liquid chromatography is one of those techniques identified as a gold standard for the analysis of medicines (Kovacs et al, 2014; Zou et al, 2017; Montaseri and Forbes, 2018) as explained in detail in chapter two. Prior to LC analysis solvent extraction of medicines from the tablet is required and so sampling time is much longer than with

simpler techniques such as the spectroscopic techniques (in chapters three and four) and probe mass spectrometry (chapter five). However, LC can also be combined with mass spectrometers to provide more information about the medicine in one study. Liquid chromatography- mass spectrometry (LC-MS) combines the separation capabilities of liquid chromatography with the mass detection capabilities of mass spectrometry. Consequently, several studies have used (LC-MS) in the analysis of medicines (Lohmann and Karst, 2006; Van Quekelberghe et al, 2008; Lou et al, 2010). Fenk et al (2010) employed LC-MS in the quantitative analysis of paracetamol, acetylsalicylic acid and caffeine. Fiori and Andrisano (2014) developed LC-MS methods for quantifying six glucocorticoids in pharmaceutical formulations.

The aim of this study was to further validate data for the API (paracetamol) obtained via ATR-FTIR spectroscopy in addition to the UV-Vis data presented in the previous chapter. LC-MS is therefore proposed as a second check or second line validation especially where there is variation in results between initial simple authentication techniques and the first confirmatory techniques used (UV-Vis spectroscopy).

Beyond the validation of ATR-FTIR data for paracetamol, LC-MS results will also allow a comparison of the performance of both the UV-Vis and LC-MS quantitative techniques approved by the British Pharmacopoeia. Details of the performance of different techniques forms the basis for the next Chapter. An assessment of the data provided by different techniques will provide information on the choice of analytical technique to be used during different stages of screening for falsified and substandard medicines.

7.2 Materials and Methods

7.2.1 Materials and Reagents

Reference paracetamol standard was obtained from Sigma-Aldrich Company, Dorset UK. Formic acid and LC-MS grade methanol and water were obtained from Fisher Scientific Ltd, Loughborough, UK.

7.2.2 Test Tablet Samples

Selected tablet formulations containing paracetamol already analysed via ATR-FTIR spectroscopy and UV-Vis Spectroscopy in earlier chapters were subjected to LC-MS analysis in order to further assess the performance of the techniques and validate the results obtained. LC-MS analysis is costly to run both in terms of the instrumentation, the quality of the solvents needed and the time taken for each individual analysis. The initial samples were selected for analysis either because the doses covered the therapeutic range or earlier work had suggested the tablets might be suspect. Details of the tablets analysed including the expected dose of each tablet and country of origin are outlined in Table 7.1.

Table 7. 1 Paracetamol Tablet Samples analysed and their origin

Country (n)	Tablet*	Expected Amount (mg)
UK (2)	UK P1T1, UK P1T2	500
Cyprus (2)	Cyp P1T1, Cyp P1T2	500
Pakistan (2)	Pak P1T1, Pak P1T2	500
Belgium (2)	Bel P1T1, Bel P1T2	1000
India (2)	Ind P8T1, Ind P8T2	325

Note: n = number of samples, *P1T1= Pack 1 Tablet 1 and so on.

7.2.3 Instrumentation

The Agilent 6120 Quadrupole LC/MS system with its accompanying OpenLab CDS Chemstation Edition C.01.09 software for data acquisition and analysis was used. The LC-MS conditions for the separation of paracetamol were as follows:

LC conditions:

Column- Gemini C_{18} (250 x 4.60mm, 5μm) (Phenomenex)

Mobile Phase: (A) 0.1% formic acid in water (B) 0.1% formic acid in Methanol;

A:B = 60:40

Flow rate: 0.7ml/min

Run time: 10mins

Sample injection volume: 20µl

MS conditions:

Ionisation: Atmospheric Pressure Ionisation- Electrospray (API-ES) positive ion mode

m/z range: 100-350

Fragmentor: 100V

Drying gas flow: 11l/min

Nebulizer pressure: 60psig

Drying gas temperature: 310°C

The Agilent 6120 Quadrupole LC/MS system comes with an autosampler that permits the instrument to be set to run multiple injections of all the standard solutions and the test samples in one go with experimental data collected and processed when all analysis is completed. For this analysis, the instrument was programmed to run three injections per sample with a blank run at the start and after each sample. No carry over was observed from one sample to the next.

7.2.4 Methods

As mentioned earlier in the review chapter (Chapter 2), liquid chromatography (LC) is one of the pharmacopoeial gold standards for analysis of medicines. Consequently, already established LC methods for quantifying medicines were adopted and modified for this validation study (Attimarad, 2011; Chandra et al, 2012; Akgeyik et al, 2016).

7.2.4.1 Preparation of Test Tablet Samples for LC-MS analysis

To determine the paracetamol content of the test tablet samples, an amount of powder for each tablet (already crushed and used for ATR-FTIR and UV-Vis analysis)

equivalent to 15.1 mg of paracetamol, based on the stated dose, was accurately weighed out and dissolved in 100ml of diluent in a 100ml volumetric flask. LC-MS grade methanol and water (40:60 v/v) was used as the solvent. The solution was sonicated for 30 minutes to make sure paracetamol in the tablet powder had dissolved completely. Filtration was done with 0.45µm nylon syringe filters and the filtered solution was further diluted to obtain working solutions of 50µM paracetamol for each tablet. These working solutions for each tablet were then subjected to LC-MS analysis along with calibration standard solutions using parameters mentioned earlier in section 7.2.3.

7.2.4.2 Preparation of paracetamol standard solutions and calibration graphs

A stock solution was prepared by dissolving 15.1mg of paracetamol reference standard in 40ml methanol in a 100ml volumetric flask. This was shaken properly and made up to the mark with water to obtain a 1mM solution of paracetamol. The solution was then filtered with a 0.45µm nylon syringe filter. Serial dilutions of the 1mM stock solution were made to obtain concentrations in the range of 5-80µM (5, 7.5, 10, 25, 50, 60, 75 and 80 µM) for paracetamol.

7.2.4.3 Chromatograms and Mass spectra- Qualitative analyses

All chromatograms and spectral data were collected for each sample in positive ionisation mode using parameters as outlined in the instrumentation section. Characteristic paracetamol peak was present and reproducibility of data in terms of retention time of paracetamol was assessed. Data was collected in both scan mode and single ion monitoring (SIM) mode for the protonated molecular ion [MH]+ of paracetamol at *m/z* 152. In scan mode, characteristic ions for paracetamol were identified from the mass spectra recorded.

7.2.4.4 Quantitative analysis of test tablet samples and calculations

All quantitative data was processed using Microsoft Excel 2016. Measured levels of paracetamol in working solution were obtained by substituting peak area values for the samples in the calibration equation. The measured levels were indicative of the amount of the API in 50µM working solution of the test sample. Since working solution was a 20 fold dilution of the 1mM stock, actual concentration of paracetamol in stock was 20 times the amount in the 50µM solution. Test tablet sample equivalent of 15.1mg was dissolved to obtain 1mM (1000µM) stock solution. Therefore,

If 15.1mg …………. 1000µM

Amount of API dissolved (mg) = (15.1 x Measured concentration (µM) x 20) /1000

Consequently,

Actual dosage of API in tablet (mg) = (W/AT) x AP

Where W = Total weight of the tablet; AT = Amount of tablet powder dissolved in solvent and AP = Amount of API in tablet powder dissolved (mg).

7.2.4.5 Validation of method

The adopted method was validated in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy and precision.

7.2.4.5.1 Linearity

Linearity was assessed based on the correlation coefficient (R^2 - value) of the calibration curve for the mean peak area versus the paracetamol standard concentrations. LOD and LOQ were determined based on the 3.3 σ/s and 10 σ/s criteria, respectively; where σ refers to the standard deviation of peak area and 's' is the slope of the calibration curve. Paracetamol was found to obey Beer Lambert's law for these concentrations as it showed good linearity.

7.2.4.5.2 Accuracy

To check accuracy of the method, recovery for paracetamol standards based on the calibration curve was assessed using an incurred sample analysis protocol. Peaks areas, for test samples, were substituted into the calibration curve equation to obtain measured concentrations of paracetamol standards and these were compared to expected concentrations of the paracetamol standards.

7.2.4.5.3 Precision

Precision of the method was assessed based on repeatability and variation in terms of the level of the expected API shown by the relative standard deviation (%RSD).

7.3 Results and Discussion

7.3.1 Qualitative analysis - Chromatograms
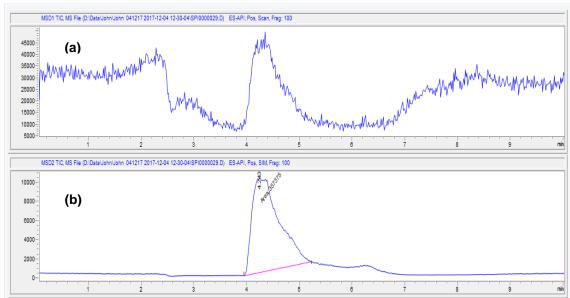
Figure 7. 1 LC-MS traces for reference paracetamol (a) TIC scan mode (b) SIM mode at *m/z* 152
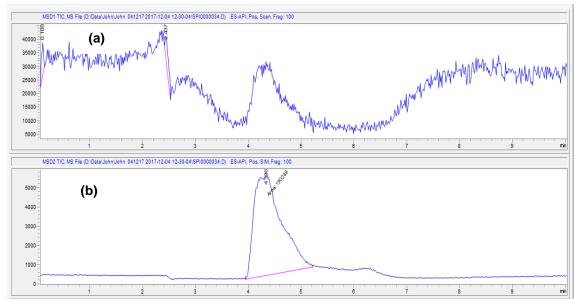
Figure 7. 2 LC-MS traces for paracetamol tablet (a) TIC scan mode (b) SIM mode at *m/z* 152

Figures 7.1 and 7.2 show the total ion chromatogram for a paracetamol standard and test tablet sample respectively. Chromatograms for each sample were recorded in both scan and SIM m/z 152 as shown. The presence of the single peak with similar retention times (4mins) in both the standard and the test sample suggests the presence of the API (paracetamol) in the tablets. In all test tablets analysed, paracetamol was found to be present.
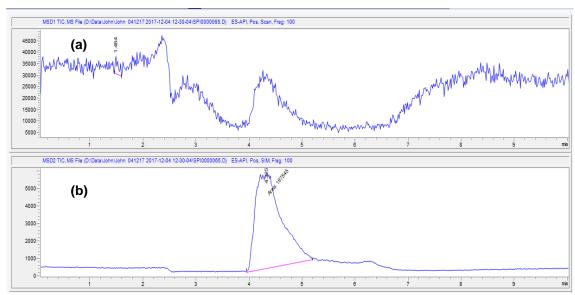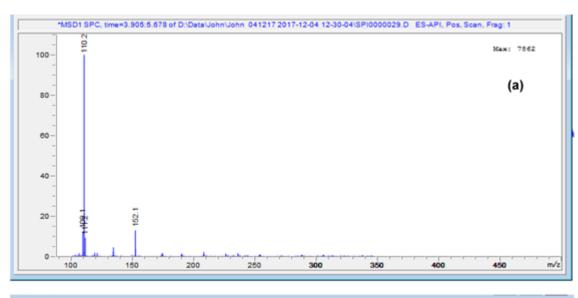
Figure 7. 3 LC-MS traces for paracetamol tablet (Ind P8) (a) TIC scan mode (b) SIM mode at *m/z* 152

Figure 7.3 is the chromatogram for the paracetamol tablet sample identified as suspect and needing further analysis based on ATR-FTIR data in Chapter three (Ind P8). There was no obvious difference in the chromatograms with Fig 7.3 showing similarities with Fig 7.1 and 7.2. The mass spectra were then considered for extra information about the tablets.

7.3.2 Qualitative analysis - Mass spectra
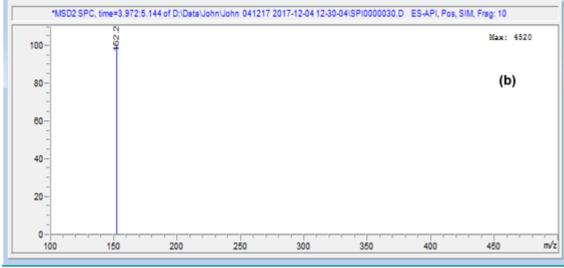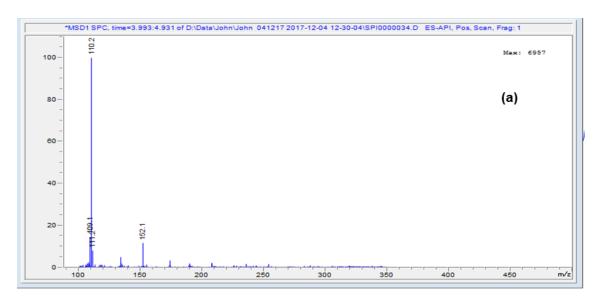
Figure 7. 4 Mass spectrum for paracetamol standard (a) scan mode (b) SIM mode at *m/z* 152
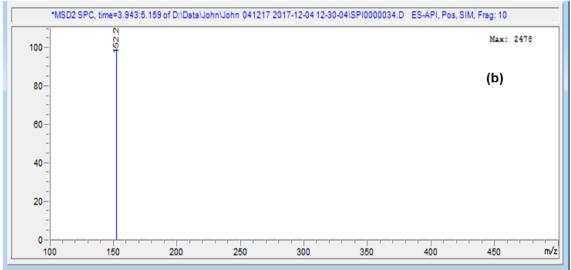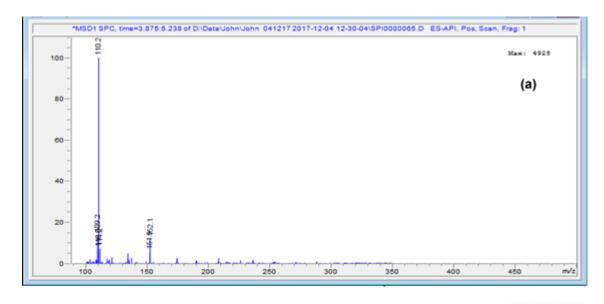
Figure 7. 5 Mass spectrum for paracetamol tablet (a) scan mode (b) SIM mode at *m/z* 152

In Figs 7.4 and 7.5, the $[M + H]^+$ ions of analytes are highlighted. Ions at *m/z* 152 and 110 confirm the presence of paracetamol in both the standard and the paracetamol tablet. These results are same as those observed with the ASAP probe MS in Chapter five further validating the probe MS data.

Based on the mass spectral data, the presence of paracetamol was also confirmed for all selected tablets assessed.

The mass spectra for the sample (Ind P8) identified as suspect using ATR-FTIR was studied in scan mode. Paracetamol ions were confirmed at *m/z* 152 and 110 as shown

in Fig 7.6a. In addition, ions at *m/z* 289 and 215, identified as fragment ions for aceclofenac, were also detected at a retention time of 2.586min (Fig 7.6b). This result also agrees with probe MS data in Chapter five confirming the presence of another API (aceclofenac) in the Ind P8 tablets assessed.
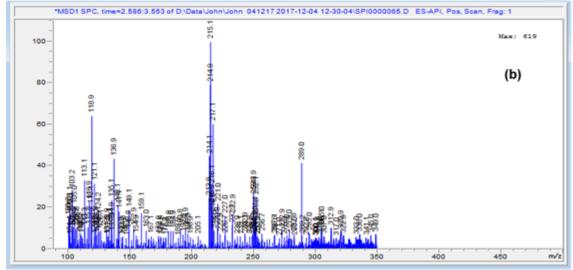
Figure 7. 6 Mass spectrum for suspect paracetamol tablet using scan mode at (a) 3.875minutes retention time (b) 2.586 minutes retention time

7.3.3 Quantification data: Linearity, Limit of Detection (LOD) and Limit of Quantification (LOQ)

Calibration curves were plotted for paracetamol based on data shown in Table 7.2. Relative standard deviation (RSD) values are also highlighted in addition to the mean

and standard deviation (SD). Linearity of paracetamol data obtained using the method is highlighted by correlation coefficients ≥ 0.997. Fig 7.7 shows the calibration curve with all data points (including replicate readings for each concentration) and correlation coefficient of the mean reading.

Table 7. 2 Paracetamol standard concentrations and peaks areas for LC-MS calibration curve

Concentration (µM)	Replicate 1	Replicate 2	Replicate 3	Mean±SD	RSD
5	24087	24079.5	23877.3	24014.6±119	0.50
7.5	32979.8	32246.6	32802.5	32676.3±383	1.17
10	36145.8	36511	36108	36254.9±227	0.61
25	74944.5	75757.8	75734.6	75479.0±463	0.61
40	125417	122499	125657	124524.3±1758	1.41
50	138961	138978	138311	138750.0±380	0.27
60	166498	165109	166399	166002.0±775	0.47
75	202910	201497	203973	202793.3±1242	0.61
80	212561	216584	214618	214587.7±2012	0.94
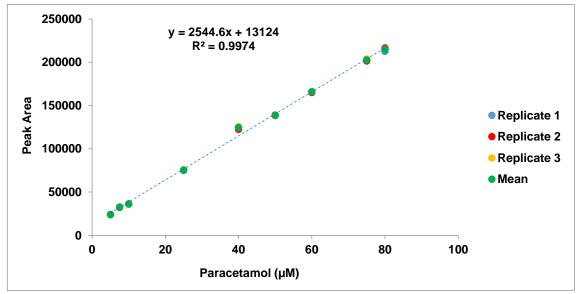
Figure 7. 7 LC-MS Calibration curve for paracetamol standards over the range 5-80µM

All three replicates per paracetamol standard analysed are displayed in Fig 7.7 indicating reproducibility of the data. Using the equations mentioned earlier, LOD and LOQ for the method were calculated to be 3.0µM and 9.1µM respectively.

7.3.4 Accuracy/ Incurred sample analysis

Reference standards were analysed using the developed protocol. Mean peak area values obtained were resubstituted in the calibration equation to check closeness of the measured concentration values based on the method to the expected concentrations as outlined in section 7.2.4.5.2. Results obtained were satisfactory as shown by the data in Table 7.3. Mean recovery of measured concentration values compared to expected concentrations was 98±7%.

Table 7. 3 Comparison of measured versus expected concentrations of paracetamol in standard calibration solutions

Standard	Expected concentration (µM)	Measured concentration (µM)	Recovery (%)
1.	5	4.3	85.6
2.	7.5	7.7	102.5
3.	10	9.1	90.9
4.	25	24.5	98.0
5.	40	43.8	109.4
6.	50	49.4	98.7
7.	60	60.1	100.1
8.	75	74.5	99.4
9.	80	79.2	99.0

7.3.5 Precision of Data

The data obtained using LC-MS showed good precision with relative standard deviation (RSD) for calibration measurements found to be between 0.27-1.41percent (Table 7.2).

7.3.6 Quantitative Data for Test Tablet Samples

Actual concentration of working solution for test tablets (expected to be 50µM) was deduced by substituting the peak area for each sample in the calibration equation. Actual amount of paracetamol in tablet mixture dissolved and total paracetamol dosage in the whole tablet were calculated using details explained in section 7.2.4.6. Total paracetamol dosages for all test tablets assessed are shown in Table 7.4.

Table 7. 4 Quantitative results for paracetamol tablet samples analysed and their origin

Country (n)	Tablet*	Measured Amount (mg)**	Expected Amount (mg)
UK (2)	UK P1T1	499.2±9	500
	UK P1T2	502.3±3	
Cyprus (2)	Cyp P1T1	494.6±9	
	Cyp P1T2	520.1±13	
Pakistan (2)	Pak P1T1	496.9±8	
	Pak P1T2	499.0±6	
Belgium (2)	Bel P1T1	1001.9±7	1000
	Bel P1T2	989.4±19	
India (2)	Ind P8T1	329.4±3	325
	Ind P8T2	324.8±5	

** Results are the mean±SD of three replicates

Results shown in Table 7.4 confirm results for all the ten tablets assessed to be well within allowed limits by the British Pharmacopoeia (85-115% of expected amount). Tablets which were initially identified as questionable or at the threshold limit (using the quick spectroscopic technique - ATR-FTIR) and needing further analysis, were found to have passed screening based on the quantitative LC-MS paracetamol data obtained. These were also in agreement with UV-Vis data obtained in chapter six.

7.4 Conclusion

In screening for falsified and substandard medicines, time of analysis is very crucial but sensitivity of the technique employed is also important in order to obtain correct information. In this work sample extraction and the analytical run can take up-to 20 minutes per sample and a balance between speed of analysis and sensitivity is therefore necessary to facilitate efficient screening for falsified and substandard medicines.

Results obtained for selected paracetamol tablets using LC-MS clarifies data obtained via spectroscopic techniques by confirming paracetamol was present in the right amounts. However, it does not provide information about possible contaminants or additional APIs present. The LC-MS results further validate data obtained using MS

and solvent extraction techniques. Data based on LC-MS analysis together with data already acquired using other techniques (in Chapters three to six) also support the call for a sequential approach to screening medicines highlighted by the authentication pyramid in Chapter two. Simpler techniques can be used identify suspect samples in the field thereby cutting down the number of samples that need to be assessed in the laboratories. LC-MS is therefore very useful as a confirmatory technique in reassessing questionable samples identified based on simpler authentication techniques while screening for FSMs.

Variation in data between the techniques can be linked to several factors including the sensitivity of the technique which will be discussed in more detail in the next chapter (Chapter eight). A more efficient approach to screening for falsified and substandard medicines is also proposed in Chapter eight.

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CHAPTER EIGHT

Analytical Strategy for Identification of Falsified and Substandard Medicines

8.1 Introduction

In previous chapters, analytical methods were assessed individually for their potential in the investigation of FSMs. Each analytical method employed provided unique and relevant information about the test tablets analysed. Therefore, cumulative assessment of these analytical methods and the corresponding data obtained will allow for an in-depth understanding of the tablets samples and the analytical strategy required for the control of FSMs (Mackey et al, 2015; Shore, 2015; Rebiere et al, 2017). As mentioned in Chapter 2, the analytical strategy employed in the control of FSMs depends on several factors. The principal factor will be the country in which the analysis takes place, in this work, low and middle income countries (LMICs) are the concern. Within these countries other factors include, time and location of analysis, availability of funds, the purpose for the testing and the expertise of the operator (Martino et al, 2010; Dégardin et al, 2014; Rebiere et al, 2017).

Based on the factors mentioned above, this chapter assesses the performance of the different analytical methods employed and the context within which each one can be applied to facilitate an efficient approach to the screening of FSMs. Furthermore, the development of an analytical strategy combining data from several techniques to arrive at conclusive decision about a suspect medicine is also outlined.

8.2 Cost of Technique/Analysis

The overall cost of using a particular analytical technique plays a major role in its feasibility in the screening of FSMs (Davison, 2011; Hamilton et al, 2016; Zou et al, 2017). The gold standard confirmatory technique LC-MS (highlighted in Chapter 7) is a pharmacopoeia approved method for the qualitative and quantitative analysis of pharmaceutical formulations but is expensive. UV-Vis provides pharmacopoeia approved quantitative data but both these techniques require solvent extraction and trained technical staff and access to these confirmatory techniques will therefore be a

challenge in areas where funds are not readily available like the LMICs. In such regions these techniques might only be used in one or two regulatory or quality control laboratories located centrally. In countries like the UK, such instruments are available at the county analyst. There is also the high cost of analyses due to the need for trained staff, the use of solvents and the maintenance of the instrument since they are laboratory based equipment requiring a controlled environment (Dégardin et al, 2014). On the other hand, with IR based spectroscopic techniques like the ATR-FTIR and Raman (in Chapters 3 and 4) overall cost of analyses is much less since the equipment is considerably cheaper and test tablet samples can be analysed directly without solvent extraction thereby eliminating the cost of solvents (Nuhu, 2011; Custers et al, 2016). They are also handy and easy to maintain making them good options for the analysis of test tablet samples in regions with limited funds. Furthermore, techniques such as Probe MS (Chapter 5) are cheaper than the LC-MS systems and comparable to the IR based systems but with better detection abilities than the spectroscopic techniques. Detection ability of the DIP-MS is discussed in section 8.6. Cost of analysis is also reduced compared to LC-MS because samples are analysed more quickly without solvent extraction. Table 8.1 summarizes estimated costs in pound sterling (GBP) of the equipment used in this study for the analysis of tablet medicines.

Table 8. 1 Equipment costs – new purchase

Type	Lab based	Portable	Cost (GBP)
UV-Vis	Yes	-	5,000-10,000
Raman	Yes	-	30,000-100,000
Raman	-	Yes	30,000-60,000
ATR-FTIR	Yes	-	30,000-60,000
ATR-FTIR	-	Yes	15,000-30,000
ASAP-MS	Yes	-	60,000
Probe MS	Yes	-	70,000-120,000
LC-MS	Yes	-	80,000-120,000

8.3 Sampling time and sample throughput

As established earlier, time is of essence in the detection of FSMs since unlike any other falsified goods; they pose a huge risk to public health. Analytical strategies for the detection of FSMs must therefore be able to spot these medicines within the shortest possible time in order to facilitate their immediate withdrawal from the market and improve patient safety (Fernandez et al, 2011).

Considering the techniques employed, the ATR-FTIR and Raman techniques allowed rapid analysis of test tablet samples with results obtained in less than five minutes. ASAP Probe MS results were also obtained within the same time frame as with ATR-FTIR and Raman spectroscopy. These techniques therefore provide an avenue for quick pass/fail tests on tablet samples and so many samples can be assessed within a short period of time. The requirements for these rapid techniques are similar to the airport scanners where the aim is to get as many individuals through the system retaining only those who are suspect.

However, the pharmacopoeia approved techniques (UV-Vis and LC-MS) took much longer to obtain results which involved solvent extraction of the API before analysis of the sample to obtain data. These techniques were therefore more useful as second line confirmation for tablet samples identified as suspect by the IR based techniques (Chapters 6 and 7). Rapid pass/fail tests reduce the number of samples requiring analysis via solvent extraction techniques thereby saving time and the cost of running all samples available.

8.4 Ease of use

The ease of use determines the where and by whom the technique can be used in the detection of FSMs. This cuts across the sample preparation phase to the analysis of the test tablet sample. The rapid techniques employed in Chapters 3 -5 (ATR-FTIR, Raman spectroscopy and probe MS) are considered easy to use since tablet samples

(whole or crushed) are analysed directly without any need for solvent extraction. Eliminating the solvent extraction phase also helps in maintaining the integrity of the original tablet sample (non-destructive). The simplicity of the rapid techniques implies that they can be run by operators with minimal training if adequate software based identification is incorporated into the software. Quantitative UV-Vis and LC-MS on the other hand requires operators with advanced scientific training to correctly solvent extract APIs for analysis and also ensure analysis proceeds under the right experimental conditions.

8.5 Complexity of Data Output

Beyond the ease of use of the device, interpretation of experimental data is also important in the analysis of FSMs. Results obtained showed that though the spectroscopic techniques were quick and easy to use, data obtained using these techniques were more challenging to interpret. Spectra obtained for samples using ATR-FTIR and Raman spectroscopy (Chapters 3 and 4) had several peaks for the same analyte providing a fingerprint which could be used to identify the API as long as there was only one API present in the tablet. Multiple APIs produced complex overlapping spectra difficult to interpret.

Conversely, Probe MS, and LC-MS were more specific with results showing peaks unique to the API being assessed.

8.6 Detection Capability

It is one thing for a technique to be able to identify the target API. It is another thing for that same technique to detect the API in the concentration at which it is present in the test tablet or indeed the presence of multiple APIs.

Given the complexity of the spectral data obtained using IR based techniques (as discussed above), analyses of tablet medicines was most feasible with simpler single

API tablets (as with paracetamol). The difficulty in quantifying chloroquine using ATR-FTIR PCA algorithm highlights the challenges that abound in the analysis of such APIs in tablets with spectral peaks that are not well defined or separated. In Chapter 4, Raman spectroscopy with PCA was employed in successfully discriminating paracetamol and chloroquine tablet samples into different groups depending on their formulation. However, the inability to discriminate the paracetamol tablets, Ind P4, (coloured and containing a second API), suggests that there might be a limit in the degree of difference detectable by the PCA algorithm. Due to the specificity of the probe MS, and LC-MS techniques, they are able to analyse complex/multiple API samples more effectively (Jonahnsson et al, 2014; Rebiere et al, 2017). The specificity of probe MS is evidenced by its use in this study for the characterisation of suspect tablet samples identified using ATR-FTIR (Ind P8T1 and Ind P8T2) and confirming the presence of another API in addition to paracetamol. However, there were challenges using probe MS for the direct quantification of paracetamol (Chapter 5) due to the negligible amounts of crushed tablet powder required for analysis.

Figure 8.1 assesses the performance of the rapid ATR-FTIR method developed in the determination of paracetamol content of tablets versus the pharmacopoeia approved UV-Vis analysis.
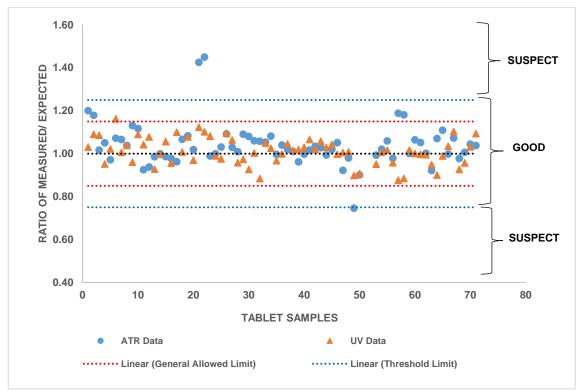
Figure 8. 1 Comparing the ratio of measured to expected amounts of paracetamol in tablet samples based on ATR-FTIR and UV-Vis showing agreement data from both techniques and the ability of the ATR-FTIR to spot suspect samples.

Results based on ATR-FTIR and UV-Vis confirmed the presence of paracetamol in all tablets assessed. Quantitative results indicate two paracetamol tablet samples which were identified as suspect using ATR-FTIR but found to be within the accepted range using UV-Vis. These are the same tablets from India (Ind P8T1 and Ind P8T2) found to contain a second and undeclared API, aceclofenac. Aceclofenac could therefore be responsible for the overestimation of paracetamol concentration using ATR-FTIR compared to the UV-Vis data for the same tablets. Therefore, the novel ATR-FTIR method was able to identify these as suspect paracetamol tablets. Other techniques were used to identify the API (aceclofenac) which was not declared on the blister pack. Quantitative ATR-FTIR data also indicated that five other tablets were outside the general allowed limits (85-115% of expected) but within the threshold limits (75-125% of expected). These five tablets in addition to the IndP8 tablets and UK P1 tablets (used as control) formed the basis for further LC-MS analysis discussed in Chapter 7.

Table 8.2 and Fig 8.2 assess the performance of ATR-FTIR, UV-Vis and LC-MS techniques based on the quantitative data for the selected paracetamol tablets.

Table 8. 2 Comparing quantitative results for a selected range of paracetamol tablets based on ATR-FTIR, UV-Vis and LC-MS

Country	Tablet	ATR-FTIR (mg)	UV-Vis (mg)	LC-MS (mg)	Expected Amount (mg)
UK	UK P1T1	514±15	532±4	499±9	500
UK	UK P1T2	505±15	479±3	502±3	500
Cyprus	Cyp P1T1	594±14	438±6	495±9	500
Cyprus	Cyp P1T2	591±5	442±6	520±13	500
Pakistan	Pak P1T1	373±14	449±6	497±8	500
Pakistan	Pak P1T2	451±8	453±8	499±6	500
Belgium	Bel P1T1	1196±55	1031±11	1002±7	1000
Belgium	Bel P1T2	1178±24	1090±11	989±19	1000
India	Ind P8T1	464±12	365±3	329±3	325
India	Ind P8T2	472±4	358±3	325±5	325

The results in Figure 8.2 are intriguing demonstrating that the most compound selective methods confirms the care taken to deliver the correct dosage level. The scatter observed from the other two techniques is derived from the decreasing specificity of the chosen quantification methodologies. UV-Vis requires solvent extraction, providing limited specificity, followed by measurement at a single wavelength whilst ATR-FTIR requires a crushed sample and measurement at a single wavelength.
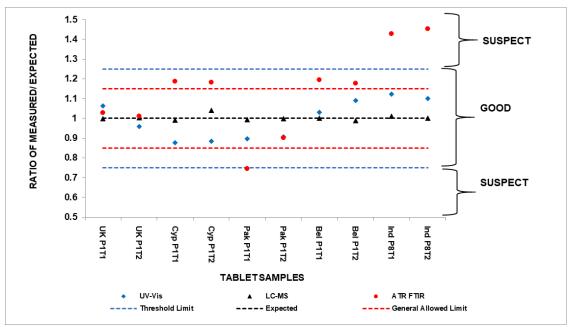
Figure 8. 2 Comparing the performance of ATR-FTIR, UV-Vis and LC-MS in the quantitative analysis of selected paracetamol tablets (showing increasing selectivity of the techniques).

LC-MS and UV-Vis results for the selected tablets suggest paracetamol content falls within the expected range for all tablets analysed. Although ATR-FTIR overestimation of paracetamol in Ind P8 tablets could be linked to the presence of a second API, the reason for variation of quantitative data in Cyp P1, Pak P1 and Bel P1 is not clear. Rebiere et al (2017) highlights the use of PCA in checking for homogeneity in batches of tablet samples. Since PCA was used for ATR-FTIR quantification of test tablets, the variation in the data for Cyp P1, Pak P1 and Bel P1 might be due to differences in homogeneity of the samples. Unlike the suspect samples which showed variation in quantitative data using the three techniques, UK P1 tablets (used as control) showed close agreement in quantitative data based on ATR-FTIR, UV-Vis and LC-MS (Fig 8.2). This could be an indication of differences in the formulation of these tablets collected from different parts of the world.

8.7 Site where analyses takes place

Finally, a crucial question to be answered when developing a strategy for rapid detection of FSMs is "do you take the sample to the machine or the machine to the

sample?" In other words, it is important to decide if in-field or laboratory analysis of medicines would provide the required information. This will inform the technique of choice for analysis (Dégardin et al, 2015; Fadlallah et al, 2016).

Spectroscopic techniques like ATR-FTIR and Raman can be manufactured as portable devices that can easily be moved around making them good choices for in-field analyses. The speed of analysis with the spectroscopic techniques implies that valuable information about tablet samples can be obtained within a short time and in the field. This will enhance the availability of data at every stage of the authentication process rather than waiting for data obtained in batches after test tablet samples must have been taken to laboratories for analysis. For instance, there are only ten WHO prequalified quality control laboratories in Africa (WHO, 2018). This implies that these ten quality control laboratories serve the 54 countries in the continent. If all samples for authentication in these countries are sent to these quality control laboratories to obtain primary data for the test samples, a backlog of samples would mean long waiting times in order to obtain any valuable information about the samples.

There is also the question of whether bench-top or handheld devices should be used. This is also dependent on the data required. Zheng et al (2014) in their comparative study, assert that bench-top Raman spectrometers were found to be ten times more sensitive than the handheld devices.

Techniques like probe MS, UV-Vis and LC-MS require a controlled environment to work effectively and so are laboratory based. Therefore, samples to be assessed using these techniques have to be taken to the laboratory for analyses since the devices are not rugged enough to be used on the field. A constant smooth electricity supply is also needed for these devices to operate whereas some of the portable spectroscopic techniques can be battery powered.

In deciding whether to analyse tablet samples on field or take them to the laboratory, some important factors need to be considered such as:

- Likelihood of damaging samples while in transit

- Storage conditions for samples

- The kind of analysis required

- Time frame during which results need to be delivered

An analytical strategy can then be chosen after reviewing the requirements for the test tablet samples to be analysed. The analytical strategy outlines successive analytical steps required to arrive at the conclusion that a test tablet sample could be falsified or substandard. The analytical strategy could also be a standalone test that provides the information needed at the time. For instance, in a situation where there is adverse reaction due to FSMs and the patients' life is at risk, conclusive results need to be delivered within the shortest time possible. Consequently, the analytical strategy suggested in this study is highlighted in Fig 8.3.

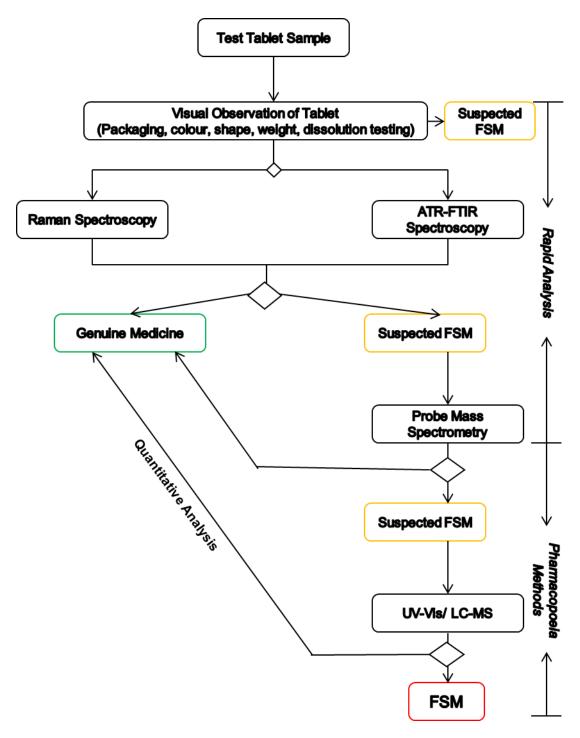**Figure 8. 3** Analytical Strategy for the detection of Falsified and Substandard Medicines (FSMs)

As mentioned earlier, simple and rapid analytical methods used for initial screening do not negate the use of the laboratory based pharmacopoeia approved methods since each method provides unique data. Analysis based on the rapid and pharmacopoeia methods identified in the analytical strategy can proceed simultaneously. It is important

to note that the analytical strategy can be re-adjusted or modified at each step to ensure valuable data is obtained. If a particular analysis does not provide enough information needed to help characterise the test tablet samples in question, an additional method may be employed to help validate initial results.

8.8 Conclusion

A good and efficient analytical strategy is required to effectively control FSMs. As such, a proper assessment of the situation has to be made for this to be achieved since there is difficulty in defining a general methodology for the rapid detection of FSMs (Rebiere et al, 2017; Zou et al, 2017). Based on data obtained from previous experimental Chapters (3-7), this chapter highlights scenarios in which the analytical techniques employed are most feasible. As mentioned earlier, this study is targeted at LMICs where facilities are not readily available. Rapid analytical methods developed in the study hold great potential in the quick screening and identification of FSMs especially in LMICs for a number of reasons:

- They are cheaper than the solvent extraction LC-MS method which is the gold standard for analysis of medicines since funds spent in purchasing solvents and training technicians are saved.

- They are quicker which implies that more tablet samples can be assessed within a short period of time with suspect samples sent for further analysis.

- Data produced by the ATR-FTIR and Raman spectroscopy might be complex but these can be addressed by software programs for data analysis being part of the equipment set up. This will allow operators with little or no experience to run quick test on tablet samples with the equipment producing the data and the analysis of the data in one go. They are therefore, simple and easy to use.

- Although some of the rapid techniques (ATR-FTIR and Raman) may not be as selective as the UV-Vis and LC-MS, they provide quick YES/NO answers which is most important in the first line screening of medicines.

- Portable versions of the ATR-FTIR and Raman equipment exist making these techniques suitable for in-field analysis of medicines especially in LMICs where rural areas are not easily accessible. A summary of major findings based on the rapid techniques employed are highlighted in Table 8.3.

Table 8. 3 Key features and findings from rapid analytical techniques employed in the screening of tablet medicines

Key feature/findings	ATR-FTIR spectroscopy	Raman Spectroscopy	DIP Mass Spectrometry
Tablet sample analysed directly	x	✓	x
Crushed Tablet sample	✓	✓	✓
Identification of Single API	✓	✓	✓
Identification of Multiple APIs	✓	x	✓
Quantification of API	✓	x	x

Development of the analytical strategy used in this research that combines valuable data obtained from different techniques employed in order to produce robust analytical results in the authentication of medicines is also discussed. Analytical strategies for the detection of FSMs are therefore dependent on the context of the analyses and as such can be approached in different ways. This can be observed in the approach adopted in this study focusing on identification of FSMs in LMICs.

A summary of the main findings in this research and future directions of the study are contained in the next chapter.

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CHAPTER NINE

Conclusion and Future Perspectives

9.1 Conclusion

Falsified and substandard medicines are a menace which if not curbed could lead to devastating public health issues. It could occur at any point in the pharmaceutical distribution chain; from the manufacturers of medicines until just before it gets to the final consumer. Therefore, safeguarding of medicines is important in ensuring medicines dispensed or sold to patients are what they say on the pack. Hence, authentication of medicines is becoming an important area of research, especially with the recent increase in reports of all kinds of FSMs across the globe.

Pharmacopoeia approved analytical methods like the HPLC have generally been used for the assessment of medicines but these methods are expensive, involve lengthy solvent extraction procedures and operate optimally in a controlled environment (laboratory). Control of FSMs is dependent on their early detection and withdrawal from the market. Thus, the speed of analyses and the availability of these analytical techniques at every point in the pharmaceutical distribution chain are important for effective screening of medicines. The high cost of these techniques implies that they might not be feasible in LMICs.

For many years the 'mini-lab' which is a combination of simple liquid chromatography and colorimetric analyses has been used in LMICs for the identification of FSMs. These investigations did however require the use of solvent extraction prior to analysis.

The work reported here demonstrated the novel use of ATR-FTIR spectroscopy as a simple, rapid and solvent free method for the determination of API content in pharmaceutical tablet formulations. Previous research in this area considered the use of conventional FTIR spectroscopy for the determination of API content. Conventional analyses using FTIR involve a rigorous and lengthy sample preparation protocol (Chapter 3) where KBr discs, containing the sample, are made for analysis. Maintaining

spectral reproducibility between disc samples is a huge challenge requiring a certain skill level from the analyst. Most studies using IR spectroscopy have also required manipulation of raw spectral data via baseline correction or normalisation.

The novel ATR-FTIR method in this study proposed a more simplified process; directly using raw spectral data for analysis. The performance of this method was investigated using paracetamol and chloroquine test tablets from around the world with a local reference library created consisting a range of APIs and excipients for identification of samples. Paracetamol identification and quantification was feasible with the method identifying suspect samples needing further investigation. Based on the characteristic peak identified, paracetamol could be detected in tablets down to 5% w/w. The whole process from crushing each test tablet to spectral analysis was done within five minutes. Paracetamol concentrations down to 10%w/w were quantified using a partial least square (PLS) regression approach and were found to be dependent on the excipient used in the preparation of standard paracetamol/excipient mixtures for calibration. Chloroquine on the other hand could be identified but performed poorly using the quantitative methods. Nevertheless, the ATR-FTIR method could still identify suspect chloroquine tablet by spectral comparison. Difficulty in quantifying chloroquine could be attributed to complexity in spectral fingerprint of the antimalarial API where peaks were less intense and not as well-separated/defined as with paracetamol.

Raman spectroscopy is another rapid IR spectroscopic technique and one of the most commonly used for analysis of FSMs. However, just as with FTIR, most Raman studies have been based on manipulated spectra. Following the successful application of raw ATR-FTIR spectra for analyses of test tablets, raw Raman spectra were also used for the characterisation of paracetamol and chloroquine tablet samples. This work demonstrates that Raman spectroscopy with PCA is able to discriminate between different groups of tablet medicines without any manipulation of the raw spectra. This

novel Raman method could potentially be used in forensic/criminal investigations for discriminating tablet samples from the same origin or source and acquisition of data that is tenable in a law court.

Furthermore, as mentioned in Chapter 2 and 5, there are no known studies on the use of probe MS for screening FSMs in the literature. This research highlights the use of probe MS for the rapid analysis of tablet dosage forms. Due to its specificity probe MS was used as a second-line rapid screening technique for further assessment of paracetamol and antimalarial tablet samples. Probe MS techniques were applied to tablets identified as suspect by the spectroscopic techniques and also to those with API levels lower than the limit of the detection for the IR based methods. Probe MS was able to confirm the presence of multiple APIs and also detect and identify a compound (API-aceclofenac) not declared on the blister pack of some suspect tablets identified by IR spectroscopy. However there was difficulty in the direct quantification of paracetamol with probe MS since calibration was non-linear.

The use of UV-Vis spectroscopy and LC-MS for validation of ATR-FTIR quantitative data in this research suggests individual analytical techniques provide unique data which contributes towards detection of FSMs. However, the context of the analysis will determine what technique(s) should be used. Furthermore, this research also demonstrates that a combination of data acquired via different analytical techniques allows the investigator to arrive at a more informed decision about the tablet sample in question. Therefore the investigator is able to develop an efficient analytical strategy using techniques which when combined provide more conclusive data about the tablet sample being investigated within the shortest time possible.

Finally, the potential use of the simple, rapid and solvent free analytical methods developed in this research, in addition to the pharmacopoeia approved methods, could

facilitate identification of FSMs on the field and even in the LMICs where sophisticated analytical facilities are not readily available.

9.2 Recommendations and Future work

Based on the findings of this study, the following recommendations for further work on the rapid identification of FSMs are suggested:
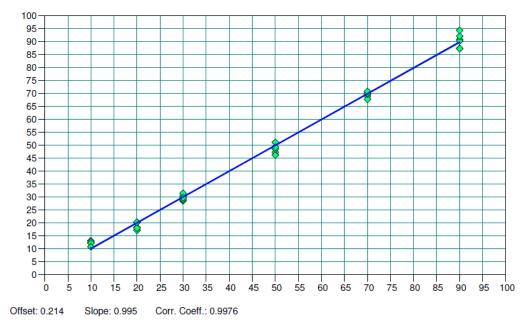
- With respect to quantitative screening of APIs using ATR-FTIR spectroscopy, the method applied was successful for simpler API tablets with well-defined spectra such as paracetamol. However, there was difficulty in the direct quantification of chloroquine using the method developed. Therefore it is recommended the ATR-FTIR quantitative methods be modified to account for more complex/multiple API tablets. Modification of the method can be done by using more complex API/excipient mixtures for calibration. For example, the API can be mixed with more than one excipient in order to prepare a mixture that is even more representative of the pharmaceutical tablet formulation rather than the binary API/ excipient mixtures used in the current method. In addition, the potential of ATR-FTIR in the discrimination of chiral compounds via shifts in the IR band could also be investigated. This will enable the detection of falsified medicines where an enantiomer is used instead of the required API.

- Raman spectroscopy in combination with PCA was successful in the discrimination of tablet samples into groups without spectral manipulation. It will therefore be interesting to consider the potential of Raman spectroscopy and PCA in the quantitative analyses of tablets samples using their corresponding raw spectra and not the manipulated spectra which is more common in the literature.

- Direct quantification of the API (paracetamol) was a challenge using both probe MS analyses. This is because calibration was not linear for higher concentrations of standard paracetamol/excipient mixtures. Non-linearity of calibration data could be due to inconsistency in amount of tablet powder used for analysis since minimal (µg) amounts were used for each run. Nevertheless, some linearity was observed for paracetamol in excipient concentrations less than or equal to 10% w/w. Paracetamol in excipient concentrations less than or equal to 10% w/w does not cover the range in which this API generally occurs in tablet medicines. This is, however, a common level for many APIs in tablet formulations and a proper understanding of the linearity observed at lower concentrations might provide a basis for improving the quantitative capabilities of the probe MS to cover this concentration range. Another way forward might be dispersing exact amounts of tablet powder in a matrix to eliminate slight variations between each run.

In the grand scheme of things, efforts towards serialisation and traceability of medicines will go a long way in addressing FSMs. A central database providing real time monitoring of medicines from the manufacturer to the final consumer (including results of screening tests conducted while in transit) will help provide more valuable data about FSMs worldwide. The use of digital health in patient engagement could be useful in the fight against FSMs. Miniaturisation of analytical methods for screening medicines such that they can be incorporated in mobile phone applications, will allow the patient a final check on the medicine at the point of purchase. This will in turn improve the confidence of the patients on the medicine administered.

APPENDIX 3

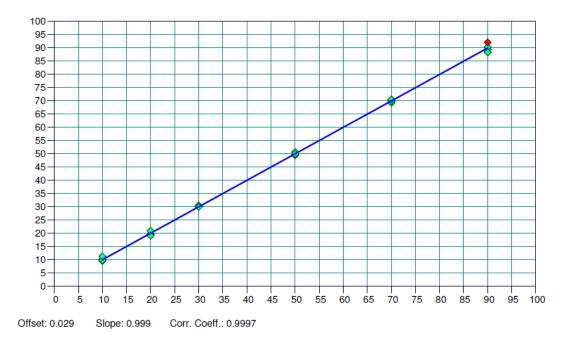**Fit vs True / Paracetamol [% w/w] / Calibration**

Offset: 0.214   Slope: 0.995   Corr. Coeff.: 0.9976

A3.1 Calibration curve for Paracetamol in Maize starch for the range 1236 - 1210cm^{-1} and peak centred at 1225cm^{-1}

Fit vs True / Paracetamol [% w/w] / Calibration

Offset: 0.027   Slope: 0.999   Corr. Coeff.: 0.9997

A3.2 Calibration curve for Paracetamol in Maize starch for the range 1524 – 1210cm^{-1}

Offset: 0.029   Slope: 0.999   Corr. Coeff.: 0.9997

A3.3 Calibration curve for Paracetamol in Maize starch for the full spectral range 4000 – 400cm^{-1}