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Newly Developed Methods for the Determination of Selected Pharmaceuticals in Water and Vegetable Samples by HPLC and Their Removal

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This is to certify that the Thesis prepared by Bisrtaewongel Tegegne Alemu entitled: *Newly Developed Methods for the Determination of Selected Pharmaceuticals in Water and Vegetable Samples by HPLC and Their Removal* and submitted in fulfillment of the requirement for the Degree of Doctor of Philosophy (Analytical Chemistry) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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ABSTRACT

Pharmaceutical consumptions are now increasing worldwide as it is essential to treat and prevent health issues but at the end of the day, they end up in the environment as a persistent pollutant. However, very little research has been carried out in Africa and there is no study in Ethiopia concerning method development and quantitative analysis of pharmaceuticals in the environment. This research work is aimed to develop analytical methods for the determination of selected pharmaceuticals from water and vegetable samples and it also presents their removal.

An effective liquid chromatography for the determination of thirteen pharmaceuticals (metformin, amoxicillin, chloroquine, theophylline, trimethoprim, caffeine, norfloxacin, ciprofloxacin, acetylsalicylic acid, doxycycline hyclate, metronidazole, albendazole and cloxacillin) was developed and validated. Kromasil C_{18} column, gradient elution with aqueous formic acid (0.1%), methanol and acetonitrile, a UV absorption wavelength of 250 nm and a mobile phase flow rate of 1 mL/min over a 22 min run time was optimized for complete separation. The method was validated and results for: linearity, precision, sensitivity, accuracy, specificity, suitability and method robustness were obtained and met the ICH guidelines. Using the validated chromatographic conditions, solid-phase extraction (SPE) for the extraction of 13 pharmaceutical compounds in water samples was developed. Different types of SPE sorbent, sample solution pH, type of elution solvent, sample load volume and addition of EDTA were evaluated. The LOD and LOQ of the optimized method were in the range 0.1 - 0.8 µg/L and 0.2 - 2.6 µg/L, respectively. The proposed method was applied for water samples from Addis Ababa, Ethiopia. Trimethoprim concentration of 7.8(1.1) µg/L, caffeine 3.2(0.4) µg/L, albendazole 2.1(0.1) µg/L and norfloxacin <LOQ were observed pharmaceuticals in a hospital water sample. Antibiotics trimethoprim and ciprofloxacin in the concentration of 0.5(0.02) µg/L and 0.3(0.01) µg/L, respectively, were found in the sewerage treatment plant influent. As pharmaceuticals are found in very small amounts in the environment, UHPLC-MS was optimized for selected ten pharmaceuticals. The linearity range and the calibration curve were obtained in the concentration range of 0.5 - 1000 µg/L. Limit of LOD and LOQ found ranged from 0.010 - 0.039 µg/L and 0.033 - 0.130 µg/L, respectively. Recovery on spiked river water resulted in > 65% for 70% of the compounds and the rest were found in > 55%.

Amoxicillin was not detected in all sampling points and caffeine was found in high concentration. The concentration ranged from not detected to 48.8 µg/L.

QuEChERS sample preparation was optimized for five selected pharmaceuticals and a combination of d-SPE sorbent, $MgSO_4$, PSA, C_{18} and DE (clean-up sorbent), methanol (extraction solvent), $MgSO_4$ with NaCl (extraction salt) and 5 min extraction time were obtained. The method was successfully applied to different vegetable samples collected from Addis Ababa, Ethiopia and Johannesburg, South Africa (carrot, cabbage and lettuce) and none of the target analytes were found in the sample investigated. The matrix effect study on vegetable samples collected was found to be very high that suppressed the signal during analysis.

For selective removal of pharmaceuticals, molecularly imprinted polymer (MIP) was synthesized and optimized for albendazole, ciprofloxacin, norfloxacin and venlafaxine in an aqueous solution. The polymer was characterized by FT-IR, TGA, XRD, SEM and BET. MIP mass 50 mg, sample pH of 9 and adsorption time of 20 min and 1 mL mixture of formic acid in water and methanol as elution solvent was optimized. The adsorption experimental data fitted with the Freundlich isotherm model and the pseudo-second-order kinetic model best fitted which implied adsorption through chemisorption. MIP reusability was demonstrated for six repeated cycles without significant loss in performance. Lastly, the efficiency of *Moringa oleifera* (seed cake, seed husk and water-soluble protein) was studied for removal of nine pharmaceuticals. The adsorbent was characterized by FT-IR, SEM and points zero charge. Sample solution pH of 5, a contact time of 80 min and 0.5 mg/L initial concentration of the analyte were optimized. Adsorption on the Moringa adsorbent was well defined by Freundlich adsorption isotherm model and the pseudo-second-order kinetic model. The results obtained showed that the use of this plant can be considered as one of the most promising, and low-cost adsorbents for the removal of pharmaceuticals.

Keywords: Pharmaceuticals, Water, Solid-phase extraction, UHPLC-MS, Vegetable, QuECEhERS, Molecularly imprinted polymer, *Moringa oleifera.*

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ABBREVIATIONS

ALB(ABZ)	Albendazole
AMOX	Amoxicillin
ASA	Acetyl salicylic acid
ASTP	Akaki sewerage treatment plant
BET	Brunaue, Emmett and Teller
CAF	Caffeine
CHQ	Chloroquine
CIP	Ciprofloxacin
CLA	Cloxacillin
DOXH	Doxycycline hyclates
EC	Electrical conductivity
EPA	Environmental Protection Agency
ESI	Electrospray ionization
FT-IR	Fourier transforms infrared spectrometry
GC-MS	Gas chromatography mass spectrometry
GWTP	Gefersa water treatment plant
HLB	Hydrophilic lipophilic balance
ICH	International Conference on Harmonization
K_{ow}	Octanol-water partitioning coefficient
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LPME	Liquid phase micro-extraction
MAE	Microwave-assisted extraction
MAX	Mixed-mode anion exchange
MCX	Mixed-mode cation exchange
MET	Metformin
MIP	Molecularly imprinted polymer
MS	Mass spectrometry

MTZ	Metronidazole
NOR	Norfloxacin
NSAIDs	Non steroid anti-inflammatory drugs
QuEChERS	Quick, easy, cheap, effective, rugged and safe
R	Recovery
R^2	Correlation coefficient
RR	Relative recovery
RSD	Relative standard deviation
SD	Standard deviation
SEM	Scanning electron microscope
SPE	Solid phase extraction
SPME	Solid phase microextraction
STP	Sewerage tretement plant
TDS	Total dissolved solids
TGA	Thermogravimetric analysis
THP	Theophylline
TOF	Time of flight
TRM	Trimethoprim
UHPLC	Ultra-high performance liquid chromatography
USEPA	United State Environmental Protection Agency
WHO	World Health Organization
XRD	X-ray diffraction

Chapter 1 - INTRODUCTION

1. Introduction

1.1. Background

Pharmaceuticals are substances or a mixture of substances that contain active ingredients that have been and confer significant benefits to the intended individual. These substances are extensively and increasingly being used in human and veterinary medicines (Al-Qaim et al., 2013; Garcia et al., 2013). They can be used broadly to prevent illnesses and also as growth promoters in livestock and fish farming, as well as in agriculture (Gracia-Lor et al., 2012). Pharmaceuticals can be transformed into more polar and soluble forms referred to as metabolites predominantly intended to work on human and animal bodies. Like other organic pollutants, pharmaceuticals are considered a class of emerging contaminants that have recently attracted the attention of many researchers (Ebele et al., 2017; Khan et al., 2018; WHO, 2012). Emerging pollutants are primarily new or previously undetected compounds lacking regulatory status and whose ecological and human adverse effects are not well established. Pharmaceuticals are known chemicals of environmental concern due to the health risks associated with exposure of aquatic life to these compounds and potentially deleterious effects on human health. It is therefore important to investigate the occurrence and fate of pharmaceuticals in various water bodies. A wide array of sampling regions may be critical in such a study since drug consumption patterns may differ by region, hence the potential pollution intensity may vary (Matongo et al., 2015a).

Pharmaceutical compounds may enter the environment via several pathways which may include, the discharge of treated wastewater, seepage from landfills, septic systems, sewer lines, and surface runoff from animal wastes and land application of manure fertilizers. Concentrations of individual pharmaceutical compounds in wastewater treatment effluents are generally less than 1 μg/L, although concentrations as high as several mg/L have been detected and measured in the effluent from treatment plants receiving waste from pharmaceutical manufacturing facilities. Even though physical and biological processes occurring in aquatic environments may cause attenuation of various pharmaceutical compounds, trace concentrations of human and veterinary

pharmaceutical compounds and metabolites have been detected in surface water, groundwater, and drinking water (Fram and Belitz, 2011). Since the human body can only metabolize a fraction of the administered pharmaceutical, it enters the water cycle as the parent compound and/or its metabolites via excretion, mainly in the urine (55–80%) and to a lesser extent in fecal matter (4–30%) (Bottoni et al., 2010; Verlicchi et al., 2013). The excretion rate is strictly correlated to a specific compound and individual human characteristics, including age, gender, health status and concurrent consumption of other substances. Conventional municipal wastewater treatment plants (WWTPs) are unable to efficiently remove all the different compounds found in sewage. Treated effluents are therefore one of the main sources of pharmaceutical release into the environment (Verlicchi et al., 2013). Liquid wastes from the hospital, improper disposal of expired drugs and unused medication via the toilet; wastewater treatment effluents, intensive farming using veterinary drugs and feed additives in livestock and aquaculture are possible sources of pharmaceutical contamination in the water environment (Aukidy et al., 2014). Moreover, such compounds are often released during production in pharmaceutical processing industries. Studies have revealed that surface waters that are close to such industries tend to have significant concentrations of pharmaceutically active compounds (Che`vre, 2014).

Pharmaceutical origins are often not eliminated during water treatment and are also not biodegraded in the environment even detected in treated drinking water. It has been detected in all aquatic systems like rivers, lakes, seas, and groundwater. The occurrence of pharmaceutically active compounds in the environmental water has been confirmed with the concentrations usually range from the μg/L to ng/L range in surface waters (Tran, 2014). In recent years, pharmaceuticals have received growing attention from environmental and health agencies all over the world owing to recent studies showing the occurrence of pharmaceutical compounds in the environment, especially in water bodies and have become one of the emerging water pollutants (Kumar and Xagoraraki, 2010b). Over the last ten to fifteen years, increasing attention has been paid to the fate and effects of pharmaceuticals in the environment and in particular in surface water bodies. Pharmaceutical concentrations in the different water environments (raw and treated urban wastewater and surface water) have been extensively monitored to assess the removal ability of conventional treatment plants and their occurrence in the final water bodies.

Some compounds were found to cause negative effects on the environment. According to the European Community Directive, the anti-inflammatory diclofenac and the hormones 17β-estradiol and 17α-ethinyl estradiol have been included in the European Watch List, while according to the USEPA, erythromycin, nitroglycerin, and 9 hormones need to be considered a priority (Rechardes, 2003). Nevertheless, pharmaceuticals are still unregulated (emerging) compounds, and there is an ongoing debate within the scientific community regarding which pharmaceutical to include among the substances demanding priority attention (Bottoni et al., 2010; Gaffney et al., 2015).

In a recent study, Martins et al., (2011) found two antimicrobials (sulfamethoxazole and trimethoprim) in hospital effluent at concentrations of 27.8 mg/L and 6.65 mg/L, respectively. But, relatively little information is known about the situation in developing countries where pharmaceuticals are rapidly growing and environmental regulations are not very well established (Tran, 2014). Among various pharmaceuticals, non-steroidal anti-inflammatory drugs (NSAIDs) for treating painful and inflammatory conditions, including ibuprofen (IBP), ketoprofen (KEP), naproxen (NPX), and diclofenac (DFC), as well as clofibric acid (CA), metabolite of blood lipid regulator drug, antibiotics like sulfamethoxazole (SMZ), erythromycin (ERM) have been frequently detected in global water environments (Hernando et al., 2006b; Wen et al., 2014). Besides, because of increasing public concern regarding potential health effects due to the presence of pharmaceuticals, it becomes important to understand and analyze different pharmaceutical residues in the environment. Water consumption is the main pathway for human exposure to pharmaceuticals intake. Drinking water treatment plants are the last step for guaranteeing the safety of drinking water but are not designed to remove pharmaceuticals (Kumar et al., 2010; Wen et al., 2014). Pharmaceuticals are chemicals causing environmental issues due to health risks associated with exposure (Matongo et al., 2015b). The risk to aquatic organisms has been correlated with pharmaceutical residues present in wastewater and continual exposure has also given rise to the presence of multi-drug resistant microorganisms (Mutiyar and Mittal, 2014; Zhang et al., 2012). Human exposure could then occur from the consumption of water or by the consumption of aquatic organisms that have accumulated pharmaceutical residues (Cunningham et al., 2009).

Under the enormous pressure on water supplies, treated water from wastewater treatment plants (WWTPs) appears to be a valuable water resource to supplement agricultural irrigation, although pharmaceuticals may still be absorbed into vegetables and crops (Calderon-Preciado et al., 2012; Wu et al., 2014). A research report by Wu et al., (2014) demonstrated that vegetables irrigated with reclaimed water could accumulate pharmaceuticals in mature edible parts of vegetables. Since pharmaceuticals are widely used, their determination in various environmental compartments requires the development of analytical methods especially for their separation and detection from complex matrices (Fram and Belitz, 2011). The analysis of drugs in a complex matrix from an environmental sample without sample preparation is quite challenging. Generally, sample preparation and concentration of the target analytes are often needed prior to analysis (Al-Qaim et al., 2013; Martín, 2012). Various analytical methods have been developed and applied for the analysis of different groups of pharmaceutically active compounds, according to the properties of the compounds of interest (Nikolaou, 2013). Most pharmaceuticals have acidic and/or basic functionalities; their ionization rate depends on acidic dissociation constants and is controlled by solution pH (Che`vre, 2014; Pavlovic et al., 2007). Pharmaceutical pK_a value enables adjustment of the pH value of the sample solution; log K_{ow} shows the affinity of pharmaceuticals towards water (Pavlovic et al., 2007). Sample treatment and enrichment processes are crucial during environmental analyses since the contaminants in environmental water usually occur in pretty low concentrations in highly complex matrices (Pavlovic et al., 2007).

Liquid-liquid extraction (LLE), solid-phase extraction (SPE), and their micro-extraction approaches, and salting-out liquid-liquid extraction are exhaustive traditional preparation techniques used to extract and pre-concentrate different therapeutic classes of analytes from environmental water samples (Lin et al., 2008; Noche et al., 2011). For pharmaceuticals from an environmental water sample, extraction and pre-concentration using solid-phase extraction (SPE) is reported by many studies (Al-Qaim et al., 2013; Iglesias et al., 2014; Madikizela and Chimuka, 2017; Madikizela et al., 2014; Matongo et al., 2015a; Matongo et al., 2015b). Conventional analytical methods for environmental solid sample preparation include soxhlet, pressurized liquid extraction, ultrasonic-assisted extraction and mostly super critical fluid extraction sample preparation techniques is used (Al-Qaim et al., 2013; Nie et al., 2015; Sabourin et al., 2012; Wu

et al., 2014). The efficiency of the method applied for sample extraction should be optimized based on the physicochemical property of the target pharmaceuticals under consideration. Analytical techniques including high-performance liquid chromatography (HPLC) and gas chromatography (GC) are commonly employed in the pharmaceutical determination of different types of samples (Fatta-Kassinos, 2011). However, liquid chromatography is the most common method used for the separation and determination of compounds because most pharmaceutical compounds are non-volatile (Bendz et al., 2005; Iglesias et al., 2014).

The environmental impact of pharmaceutical residues is evaluated using environmental risk assessment approaches based on the calculation of risk quotients (RQs). This approach uses the predicted environmental concentrations (PECs) or measured environmental concentrations (MECs) of the pollutants with toxicity data using predicted no-effect concentration (PNEC). This approach evaluates a pollutant that is likely to pose a risk for aquatic organisms in the studied water sample (Hernando et al., 2006a; Rivera-Jaimes et al., 2018). Many researchers have tried to develop methods for removing pharmaceuticals from the environment. Different removal methods such as ozone-based advanced oxidation processes (Almomani et al., 2016), nano-filtration (Derakhsheshpoor et al., 2013) and biological activated carbon (Nugroho et al., 2010) have been reported for the treatment of water. However, most of these methods have major disadvantages especially on high operation costs (Akhtar et al., 2015).

For the present study, thirteen different pharmaceutical compounds belonging to seven different therapeutic classes, which included antibiotics (trimethoprim, norfloxacin, ciprofloxacin, doxycycline hyclate, metronidazole, cloxacillin and amoxicillin), anthelmintic (albendazole), nonsteroidal anti-inflammatory (acetylsalicylic acid), antidiabetic (metformin), xanthine (theophylline), antimalarial (chloroquine) and stimulant (caffeine). These pharmaceutical compounds were targeted and selected based on commonly prescribed pharmaceuticals in the country Ethiopia (Bergicho et al ., 2012; Berha and Seyoum, 2018; Jabo et al., 2018). In addition, it is based on the previous trend on their occurrence in environmental samples worldwide and their potential eco-toxicological impact. While many studies have reported the concentration of pharmaceuticals in various environmental compartments in the world, there are still limited studies in Africa and in particular has been no such study in Ethiopia.

1.2. Problem statement and motivation

Several investigations have shown that substances of pharmaceutical origin are often not eliminated during wastewater treatment, and also not completely biodegraded in the environment (Fent, 2006; Li, 2009). Therefore, it is imperative to increase the knowledge about pharmaceuticals and issues associated with them in the environment (Ebele et al., 2017). In most developing countries, there are insufficient health services for their populations and this results in people opting for over-the-counter drugs without an appropriate physician's prescription. This practice leads to misuse and unfitting disposal of pharmaceuticals. Such practices are common and significantly high rates in developing countries compared to developed countries. Also, hospital wastewater treatment facilities are very limited or even absent in most of these countries (Hossain et al., 2018).

Addis Ababa, the capital city of one of the developing countries, Ethiopia, does not have adequate waste management facilities to accommodate the high volumes of solid waste generated by the city from domestic activities. As a result, solid waste is often piled on available open grounds, stream banks and near bridges, where it is washed off into rivers (Mafuta et al., 2011; Yohannes and Elias, 2017). Approximately, 25% of the city's residents do not have a toilet and about 65% of the societies use pit latrines. As a result, the residents use the forest nearby and sometimes deposit their urine and fecal matter directly into nearby rivers. Moreover, houses that are built at the edges of the city's rivers link their toilets directly to the streams resulting in increased pollution in the river systems (Yohannes and Elias, 2017). The Little Akaki River and its tributaries from Addis Ababa River receive the major portion of the waste released in the western part of the city where most of the industries such as tanneries, breweries, wineries, distilleries, pharmaceutical, and national alcohol liquor factories are established (Aschale et al., 2015; Kassegne et al., 2018).

Hazardous health care waste accounts for 10-25% of health care waste, including chemical or pharmaceutical waste (3%), body-part waste (1%), sharps (1%), radioactive and cytotoxic waste as well as broken thermometers (less than 1%). Discharge of such contaminated wastewater

without any treatment poses serious threats to the receiving environment and public health. Such malpractice has already led to the development of multidrug-resistant microbes in the environment (Ashfaq et al., 2017; Debere et al., 2013a). The results of the assessment conducted by the World Health Organization in 22 developing countries showed that the proportion of healthcare facilities that do not use proper waste disposal methods ranges from 18% to 64% (Debere et al., 2013a; Hayleeyesus and Cherinete, 2016). Besides, the previous studies done on waste management in Addis Ababa city revealed that the management of healthcare waste in hospitals was poor (Debere et al., 2013b; Tadesse and Kumie, 2014).

The study shows that rivers, sediments and soils in a vegetable farm in Ethiopia are contaminated with heavy metals due to different industrial waste which is disposed into the rivers because of lack of wastewater treatment plants (Aschale et al., 2015; Mekonnen et al., 2014; Tegegn, 2012). The streams and rivers in Addis Ababa are also a receptacle for a variety of wastes released by the residents. At the same time, the river is used for irrigating vegetables and crops, livestock watering, washing cattle, and other domestic needs for people living in Addis Ababa and nearby (Aschale et al., 2015). The previous study conducted on vegetables irrigated with the river water focused on quantitative analysis of heavy metals (Aschale et al., 2015; Lakew et al., 2018; Weldegebriel et al., 2012) oxalate and phytate (Lakew et al., 2018), and pesticides concentration (Demis et al., 2018). The recent study conducted at Addis Ababa University, Ethiopia (Gezahegn et al., 2019), using salting-out assisted liquid-liquid extraction for ciprofloxacin determination from different water sample results showed a concentration of 0.83 µg/mL from sewage treatment plant was an indication and a motivation to research many potentially contaminated sources.

Several treatment methods have been carried out in recent years regarding the removal of various contaminants from water by using natural adsorbents. However, there is still a significant gap in the investigation of the adsorption efficiency of *Moringa oleifera* seed parts and extracts as adsorbents for the simultaneous removal of multi-class pharmaceuticals from the water sample. The seed of *Moringa oleifera* has coagulating properties and the seeds have been used for various aspects of water treatment such as turbidity, alkalinity, total dissolved solids and hardness and removal of various metal (Shan et al., 2017; Zaid and Ghazali, 2019). The *Moringa*

oleifera (seed cake, seed husk and water-soluble protein) has not been studied as a low-cost adsorbent for multi-class pharmaceutical removal without requiring high technological expertise.

As it is well known that pharmaceutical products are used daily by humans for medical purposes, routine monitoring for the occurrence of their active ingredients in the aquatic environment which includes wastewater, river water, sewerage treatment plant (effluent and influent) and vegetables mostly irrigated with water from those water bodies is required. To the best of our knowledge, information about analytical method development and the occurrence of multi-class pharmaceutical compounds in different water samples and vegetables is by far not done in Ethiopia and there are few reports of such studies in Africa.

Bearing the aforementioned facts in mind, this study, therefore, aimed to provide a brief indication about the analytical methods and concentration of selected pharmaceuticals in different water samples and vegetable samples collected from Addis Ababa, Ethiopia and their removal.

1.3. Objectives

1.3.1. General objective

The general objective of the study was to develop and validate analytical methods for the determination of selective pharmaceutical residues from water and vegetable samples and their removal from water.

1.3.2. Specific objectives

- ✓ To develop and validate HPLC-UV method for simultaneous separation of 13 pharmaceutical compounds.
- ✓ To optimize and use solid-phase extraction for selective extraction of pharmaceuticals from the water sample.
- ✓ To develop UHPLC-MS analytical method for the determination of selected pharmaceutical compounds from water samples.
- ✓ To optimize QuEChERS extraction technique for analysis of pharmaceuticals residues in the vegetable samples.
- ✓ To synthesize and characterize molecularly imprinted polymer for adsorption of selected pharmaceuticals from water samples.
- ✓ To investigate the removal efficiency of *Moringa oleifera* (seed husk, seed cake and water-soluble protein) as a natural adsorbent for multi-class pharmaceuticals from water.

1.4. Significance of the study

This thesis work was done to fill the existing knowledge gap in the analytical sample extraction method for the extraction of selected pharmaceuticals from water and vegetable samples collected from Addis Ababa, Ethiopia. Analytical sample extraction methods were developed and used for water and vegetable sample analysis. For water samples, a solid-phase extraction method was optimized and applied for extraction before using HPLC-UV and UHPLC-MS to determine the level of pharmaceuticals in the Ethiopian environment. The results provide evidence of the persistence of pharmaceuticals in the environment. In addition to this, the removal efficiency of molecularly imprinted polymer and low-cost adsorbents from Moringa seed was assessed which the second one is the plant most used for wastewater treatment. This study will provide input to different bodies such as governmental and non-governmental organizations, policymakers, as well as researchers that are involved in the protection of the environment and people's health from exposure to organic contaminant residues.

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Chapter 2 - LITERATURE REVIEW

2. Literature Review

This section of the dissertation presents a comprehensive detail of concepts related to the findings investigated in this study. This review entails the therapeutic classes, biological health effects and analytical methods used for the determination and adsorption of pharmaceuticals from an environmental sample. Moreover, concepts such as sources and reasons associated with pharmaceuticals pollution, occurrences and fate in several environmental matrices are also covered. Possible analytical extraction methods for sample preparation, analytical instrumentation and methodologies utilized by researchers to successfully investigate pharmaceuticals contamination in the environment were reviewed.

2.1. Pharmacology profiling, biological effects and analytical method used for selected pharmaceuticals analysis

2.1.1. Antibiotics

Antibiotics, also known as antimicrobials, were discovered in 1928 and their unique property was to target and fight infections caused by bacteria. They have been extensively and effectively used in humans and as veterinary medicines. Their benefits have also been recognized in agriculture, aquaculture, beekeeping, and livestock as growth promoters. Antibiotics represent a major source of micro-pollutants as they may form chemical mixtures that exhibit a wide range of mechanisms of action (Elhag et al., 2018). The most common groups of antibiotics with their examples are described below. These pharmaceuticals have played an important role in human development; however, recent speculations have been raised involving their negative impact in the environment.

Amoxicillin (AMOX), chemically known as (2S,5R,6R)-6-[(R)-(-)-2-amino-2-(*p*-hydroxy phenyl) acetamido]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo [3.2.0]heptane-2-carboxylic acid trihydrate is one of the most widely used semisynthetic penicillin in the treatment of acute bacterial sinusitis and community-acquired pneumonia (Prakash et al., 2008).

This pharmaceutical plays an important role in the therapy of infections (Svetlana, 2014). The methods reported for estimation of amoxicillin trihydrate in pure and tablet dosage form include spectrophotometric methods (Naguib et al., 2018; Prakash et al., 2008), kinetics method (Svetlana, 2014) and using RP-HPLC (Sarode et al., 2013).

Ciprofloxacin (CIP) is a synthetic fluoroquinolone derivative which is chemically named as [13-quinoline carboxylic acids, 1-cyclopropyl-6- fluro-1, 4-dilydro-4-oxo-7-(1-piperazinyl)-monohydro chloride. It has demonstrated broad-spectrum activity against many pathogenic gram-positive bacteria such as *Streptococcus, Pneumoniae* and *Enterococcus faecalis*, and gram-negative bacteria including *Salmonella*, *Shigella* (Ali et al., 2011; Palamy and Ruengsitagoon, 2018; Varak and Ebrahimi, 2018). A reported method for ciprofloxacin analysis includes flow injection spectrophotometric method (Palamy and Ruengsitagoon, 2018), spectrophotometric methods (Rekha et al., 2015), HPLC with norfloxacin in pharmaceutical preparation (Kassab et al., 2005), simultaneous extraction with metronidazole using UHPLC-MS/MS (El-bagary et al., 2016).

Cloxacillin (CLA) belongs to the semi-synthetic β-lactam antibiotics and chemically known as monosodium (2S,5R,6R)-6-[o-(2-chlorophenyl)-5-methyl-4-isoxazole carboxamido]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo-[3.2.0]-heptane-2-carboxylatemonohydrate sodium (Patel et al., 2015; Zhi et al., 2012). It is used against *staphylococci* that produce beta-lactamase and also effective against a wide range of gram-negative bacteria and gram-positive bacteria as well as inhibiting synthesis of the bacterial cell wall (Shailesh et al., 2017). A reported analytical method for the estimation of cloxacillin includes a UV-spectrophotometric method (Shailesh et al., 2017) and the HPLC method (Ashnagar and Gharib Naseri, 2007).

Doxycycline Hyclate (DOXH) is a semisynthetic broad-spectrum tetracycline antibiotic, a large range of antibacterial activity, widely used in veterinary medicine and as an animal feed supplement to prevent diseases (Abbas et al., 2018; Kogawa and Salgado, 2013). Some analytical methods have been used for the assessment of doxycycline hyclate in biological and pharmaceutical samples, these include spectrophotometric methods (Abbas et al., 2017) and high-performance liquid chromatography (Kogawa and Salgado, 2013).

Norfloxacin (NOR) is a member of the fluoroquinolones and one of the third-generation members of quinolone antibiotics, chemically named as 1-ethyl-6-fluoro-4-oxo-7-piperazin-1-yl-1H-quinoline-3-carboxylic (Chierentin and Salgado, 2016; Duan et al., 2017). The fluoroquinolones norfloxacin is the first-choice drug for the treatment of diseases caused by *Campylobacter*, *Escherichia coli*, *Shigella*, *Vibrio cholera* and also used to treat gonorrhea, eye infections, as well as urinary tract infections (Zhao et al., 2014). Reported analytical methods have been used for the assessment of norfloxacin which includes chemiluminescence (Shao et al., 2007) and high-performance liquid chromatography with fluorescence detection (Kowalski et al., 2005).

Trimethoprim (TMP) is used as an antibiotic for protozoal infections chemically represented as (5-(3, 4, 5-trimethoxybenzyl) pyrimidine-2, 4-diamine) and has low antimicrobial activity (Amin et al., 2008; Mostafa et al., 2017). Trimethoprim binds dihydrofolate reductase and decreases the level of tetrahydrofolic acid. It structurally resembles pytheridine of di-hydrofolic acid and is a strong inhibitor for dihydrofolate reductase which converts di-hydrofolate into tetra-hydrafolate which in turn blocks purines and finally DNA, RNA and protein synthesis (Mostafa et al., 2017). Reported methods for the determination of trimethoprim include UV–VIS spectrophotometer (Adegoke et al., 2017) and spectrophotometer (Swetha et al., 2018).

Metronidazole (MTZ) (2-methyl-5-nitroimidazole-1-ethanol) belongs to a group of nitroimidazole drugs used in therapeutics mainly in the treatment of susceptible protozoal infections, treatment and prophylaxis of anaerobic bacterial infections and specific bacterial infections (Elkhoudary et al., 2016; Nikodimos and Amare, 2016). Reported analytical methods have been used for the assessment of metronidazole which includes, electrochemical (Nikodimos and Amare, 2016; Yilmaz et al., 2013) and high-performance liquid chromatography techniques (Elkhoudary et al., 2016).

2.1.2. Nonsteroidal anti-inflammatory drugs (NSAIDs)

Non-steroidal anti-inflammatory drugs (NSAIDs) are a diverse group of compounds that reduce or eliminate the erythema, swelling, elevated temperature and pain caused by a variety of inflammatory stimuli. They are one class of pharmaceuticals that are widely used by humans for the treatment of rheumatoid arthritis. It was only until the 19th century when these extracts

became commercialized under the name salicylic and acetylsalicylic acids which are presently known as aspirin (Madikizela and Chimuka, 2017; Narender et al., 2019).

Acetylsalicylic acid (ASA) also known as aspirin with the chemical name 2-acetoxybenzoic acid is one of the most widely used drugs in the world and exerts analgesic, antipyretic and anti-inflammatory effects (de Aguiar et al., 2009; Hobl et al., 2012). Recent studies have shown that aspirin can prevent cardiovascular events and different types of cancer and to reduce even the incidence of Alzheimer's disease occurrence. In contrast, evidence proves that it induces the formation of free radicals which are involved in gastrointestinal damage and the development of gastroduodenal ulcers and it has also antioxidant effects (Cofan and Radovan, 2011). Reported analytical methods developed for the estimation of ASA include reverse-phase liquid chromatography (de Aguiar et al., 2009), an electroanalytical method with a boron-doped diamond electrode in sodium sulfate medium (Cofan and Radovan, 2011), isocratic high-pressure liquid chromatography with post-column hydrolysis and fluorescence detection (Hobl et al., 2012).

2.1.3. Anthelminthic

Anthelminthic is of huge importance for veterinary medicine and human tropical medicine. Anthelminthic are drugs that are used to treat infections associated with parasitic worms through a mechanism that either stuns the worm or simply kills them. These include flat worms, e.g. flukes and tapeworms and roundworms. Anthelmintic pharmaceuticals have a high degree of efficacy, a good margin of safety and versatility of administration (Dolar et al., 2012; Vadav and Singh, 2011).

Albendazole (ALB) is a broad-spectrum benzimidazole parasiticide used for the treatment of parenchymal neuro-cysticercosis and is chemically referred to as methyl-N-[6-(propylsulfanyl)-1H-1,3-benzodiazol-2-yl] carbamate. It is commonly used for the treatment of cystic hydatid disease of the liver, lung, and peritoneum, caused by the larval form of the dog tapeworm, *Echinococcus granulosus* (Li et al., 2014; Sowjanya and Devadasu, 2018). Reported analytical methods have been used for the assessment of metronidazole which includes, voltammetry determination (de Macedo et al., 2016), chromatographic and spectrophotometric methods (Ahmed et al., 2018).

2.1.4. Anti-malaria

Chloroquine (CHQ) is chemically known as 7-chloro-4[[4-(diethylamino)-1-methylbutyl] amino] quinolone, it is in the class of anti-malaria used in treating malaria fever and it is also prescribed to decrease the symptoms of rheumatoid arthritis and to treat systemic and discoid lupus erythematosus in adults. Chloroquine has the tendency especially in high dosages to cause blurring of one's vision, bleaching of hair and mild skin eruptions. The observed adverse effects are attributed to its cumulative effect, and drug binding to melanin and drug retention in pigment cells for prolonged periods (Baravkar, 2014; Qarah As et al., 2017). Recently reported analytical methods used for the estimation of chloroquine include, UHPLC-DAD simultaneously with primaquine (Miranda et al., 2015) and spectrometric assay (Kanakapura et al., 2016).

2.1.5. Central nervous system stimulant

Central nervous system (CNS) stimulants were originally used by athletes to improve performance on the day of competition. It is a class of stimulants that includes psychomotor stimulants, sympathomimetics, and miscellaneous CNS stimulants including, caffeine, amfetamines, ephedrine and cocaine. These substances are either prohibited or monitored, previously by the International Olympic Committee (IOC) and now by the World Anti-Doping Agency (WADA) and are screened on daily basis by accredited laboratories (Avois et al., 2006).

Caffeine (CAF) is a commonly named as 1,3,7-trimethylxanthine chemically described as 1,3,7-trimethyl-1H-purine-2,6(3H,7H)-dione. It is an alkaloid of the methylxanthine family, present in many foods, which acts as a stimulant, psychotropic and a mild diuretic (Chowdhury et al., 2012). Caffeine is a naturally occurring alkaloid that is found in varying quantities in beans, leaves, and fruits of more than 60 plants. Some common sources of caffeine are the kola nut (*Cola acuminate*), cacao bean (*Theobroma cacao*), yerba mate (*Ilexparaguariensis*), and guarana berries (*Paullinia cupana*); however, roasted coffee beans (*Coffea arabica* and *Coffea robusta*), and tea leaves (*Camelia siniensis*) are the world's primary sources of dietary caffeine. It is found in common beverages and most widely consumed active food ingredients throughout the world and in a variety of medications (Heckman et al., 2010). Reported analytical methods for caffeine include simple and rapid RP-HPLC (Chowdhury et al., 2012; Radi et al., 2016), simultaneously with paracetamol, aspirin, ibuprofen and codeine in commercial dosage form using UV-spectroscopic method (Saeed and Ahmed, 2017).

2.1.6. Anti-diabetic

Anti-diabetic pharmaceutical is medicine for diabetes treatment. Diabetes mellitus refers to a group of chronic metabolic diseases that are generally characterized by hyperglycemia, which eventually leads to damage of multiple body systems. There are two types of diabetes, type 1 (T1DM) and type 2 (T2DM) diabetes mellitus. T1DM is referred to as insulin-dependent diabetes mellitus (IDDM) and is caused by the impaired production of insulin. T2DM, however, is commonly associated with the inability of cells to respond to insulin (insulin resistance) and hence referred to as non-insulin-dependent diabetes mellitus (NIDDM) (Gupta et al., 2017; Habte et al., 2017).

Metformin (MET) is used in the treatment of diabetes mellitus II with a chemical name 1, 1-dimethylbiguanides. It is a biguanide class of anti-diabetic drug that has suppressive action on the production of hepatic glucose and improves hyperglycemia (Silpavathi and Das, 2015). It is reported that spectrophotometric methods may be used for the determination of metformin hydrochloride from pure and dosage forms (Ashour and Kabbani, 2003). Another study also reported the determination of metformin hydrochloride from pharmaceutical dosage using nuclear magnetic resonance (Gadape and Parikh, 2011), UV spectrophotometry and HPLC (Umapathi et al., 2012).

2.1.7. Xanthine

Another group of pharmaceuticals included in this study is the theophylline class of xanthine pharmaceuticals. Theophylline (THP), with a chemical name 3,7-dihydro-1,3-dimethyl purine-2, 6(1H)-dione is a xanthine derivative used for the management of asthma and chronic obstructive pulmonary disease. It is also shown to exhibit some anti-inflammatory properties, inhibiting the activity of CD4 lymphocytes in vitro and mediator release from mast cells (Panda et al., 2013; Sayeed et al., 2012). The analytical method used for the estimation of theophylline from pharmaceutical dosage reported was using high-performance liquid chromatography (Kanakal et al., 2014; Maithani and Singh, 2011).

2.2. Pharmaceutical regulation and utilization in Ethiopia

In one of the sub-Saharan African countries, Ethiopia, the pharmaceutical sector is being guided by a national drug policy. "The Pharmacists and Druggists Proclamation No 43/1942" was the idea for pharmaceutical regulation for both pharmacists and druggists together with the facilities where they practiced. Comprehensive regulation of the pharmaceutical sector was started in the early stages by a regulation called "Pharmacy Regulation No. 288/ 1964", which formed the legal basis for the official establishment of drug regulation in the history of Ethiopia. The Pharmacy and Laboratory Department under the Ministry of Health was responsible for medicines regulation until June 1999 when a new regulation called the "Drug Administration and Control Proclamation No. 176/1999" was promulgated on 29 June 1999. Following this proclamation, the regulatory component of the Pharmacy Department was transformed into an independent Drug Administration and Control Authority (DACA) of Ethiopia in September 2001 (Suleman et al., 2016). DACA was re-structured as Food, Medicine and Health Care Administration and Control Authority (EFMHACA) of Ethiopia by "Proclamation No. 661/2009" in 2010 bearing additional responsibilities like regulation of food, health care personnel and settings (EFMHACA, 2013).

The rapid growth and development of the pharmaceutical sector after the downfall of the Dergue regime in Ethiopia has led to the majority of pharmaceuticals and medical supplies being provided by both the public and private sectors. Currently, there are 32 plants (small and large scale) involved in the manufacturing of pharmaceuticals and related products of which only 12 are manufacturers of generic finished pharmaceutical dosage forms. The remaining are involved in the small-scale manufacturing of medical devices, supplies, laboratory reagents, cosmetics, and disinfectants (EFMHACA, 2013). According to EFMHACA, there are 133 importers, 272 wholesalers, 377 pharmacies, 1699 drug shops and 1392 rural drug vendors currently existing in Ethiopia (Suleman et al., 2016).

Pharmaceutical expenditure is more than 70% of total healthcare expenditure in low and middle-income countries but irrational use has been primarily observed in health care systems of developing countries. World Health Organization estimates that more than half of all pharmaceuticals are irrationally prescribed or dispensed with more than half of the patients

failing to adhere to the prescribed regimens. Common reasons for irrational use of medicines include lack of adequate information about the prescribed drugs, faulty and inadequate training of medical graduates, poor communication between health care providers and patients, lack of diagnostic facilities and defective supply systems (Sisay et al., 2017). This is a common practice in developing countries like Ethiopia and it is enormous in terms of both inadequate resources and the adverse clinical consequences of therapies that may have health risks (Wubetu et al., 2018).

In Ethiopia, although the practice is not legal, the counter sale of antibiotics at partial doses and without prescription is common. This practice coupled with weak regulatory mechanisms contributed to increasing antimicrobial resistance (Gebretekle and Serbessa, 2016).

2.3. Pharmaceuticals as emerging contaminants in the environment

Organic compounds previously not known to be significant in the environment, in terms of distribution and occurrence, are now being more widely detected as analytical techniques improve. These compounds, which have the potential to cause known and/or suspected adverse ecological or human health effects, are often collectively referred to as emerging contaminants. Emerging contaminants include newly synthesized substances as well as different compounds and their transformation products such as pharmaceuticals, personal care products, pesticides, veterinary products, industrial compounds/by-products, food additives, and engineered nanomaterials (Lei et al., 2015; Sorensen et al., 2014).

Pharmaceuticals, however, are constantly in use and are discharged by individuals, hospitals, and agro-based and pharmaceutical industries, making their entrance into the environment more continuous (Agunbiade and Moodley, 2016). In recent years, pharmaceuticals have received growing attention from environmental and health agencies all over the world owing to recent studies showing the occurrence of pharmaceutical compounds in the environment, especially in water bodies and have become one of the emerging water pollutants (Houtman et al., 2014; Kumar and Xagoraraki, 2010b).

Nevertheless, pharmaceuticals are still unregulated (emerging) compounds, and there is an ongoing debate within the scientific community regarding which to include among the substances demanding priority attention (Bottoni et al., 2010). Indeed, according to the European

Community Directive, the anti-inflammatory diclofenac and the hormones 17β-estradiol and 17α-ethinyl estradiol have been included in the European Watch List, while according to the USEPA, erythromycin, nitroglycerin, and 9 hormones need to be considered a priority (Rechardes, 2003). Figure 2.3.1 shows pharmaceuticals mostly detected in different environmental water (Cizmas et al., 2015).

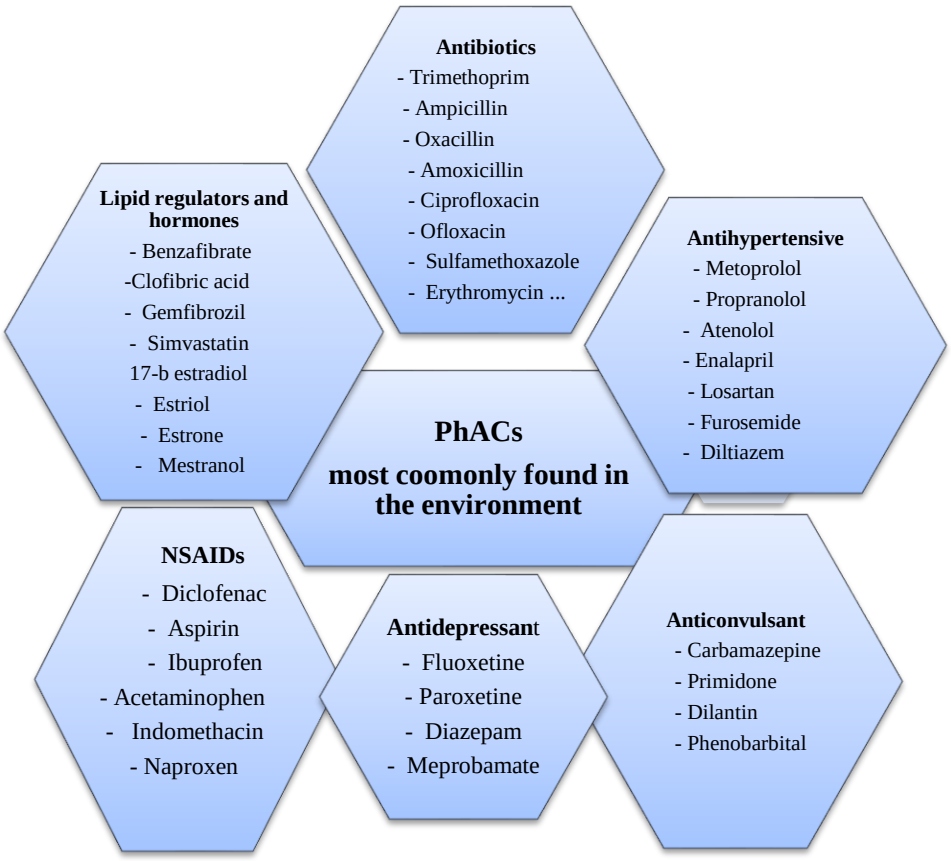


Figure 2.3. 1. Pharmaceutical compound frequently detected in the environment water.

To date, many research papers have indicated the widespread presence of pharmaceuticals in the environment. However, many of these scientific papers emerged from developed and middle–income countries, while African countries are still lagging behind in terms of identifying and quantifying pharmaceuticals in environmental samples. Furthermore, the capability of sewage treatment processes for the removal of pharmaceutical constituents in Africa is not fully achievable (Agunbiade and Moodley, 2014; Madikizela et al., 2017b; Ngumba et al., 2016a). The detection of pharmaceuticals in wastewater was first reported in Kansas City in 1976 and

1985, the presence of 25 pharmaceuticals in the river Lee (UK) with concentrations of 1000 ng/L was investigated (Fent, 2006; Richardson and Bowron, 1985). Since 2017, there has been a steady increase in the availability of information on the contamination of water resources caused by pharmaceuticals in some African countries. However, out of African countries, only 9 have researchers (Figure 2.3.2) who were active in investigating the occurrence of pharmaceuticals in different water bodies reported over the last three years (2017–2019 (Madikizela et al., 2020).

Figure 2.3. 2. Map of African countries (circle) where pharmaceuticals have been detected in water bodies.

2.4. Sources of pharmaceuticals in the environment

Pharmaceutical compounds may enter the environment through various pathways (Fram and Belitz, 2011). They end up in the environment through human consumption of medicines and disposal of unused drugs via the drain, excreted through urine or feces as a mixture of their original or biologically active forms and enter aquatic systems in different ways. The main pathway from humans is via ingestion followed by excretion and disposal of wastewater. Figure 2.4.1 shows the representative pathway of pharmaceuticals in the body after administration to

excretion (Ayanda, 2015; Celiz et al., 2009). Pharmaceuticals that are consumed by humans are excreted either in their original form or as metabolites. Pharmaceuticals absorption from the site of administration permits entry of the compound into the bloodstream. Once absorbed, the drug may then leave the bloodstream and disperse into the tissues and intracellular fluids where it can reversibly bind to receptors. This dispersal is called distribution, while some drug molecules are binding to receptors, others may be released from the receptors and be picked up again by the blood stream. Drug particles in the blood stream are available to undergo biochemical changes, referred to as metabolism, in the liver or other tissues. Finally, the drug and its metabolites are excreted from the body in urine and or feces. Metabolism and excretion are both pathways of drug elimination from the body. They join the sewage system and the WWTP and maybe finally released to the receiving aquatic system since they may be only partially decontaminated in the treatment plant (Che`vre, 2014; Wolf et al., 2012). Municipal wastewater is, therefore, the main route that brings human pharmaceuticals after normal use and disposal of unused medicines into the environment (Fent, 2006; Tran, 2014; Ying et al., 2009). Pharmaceuticals that are not readily degraded in the sewage treatment plant (STP) are being discharged in treated effluents resulting in the contamination of rivers, lakes, estuaries and rarely, groundwater and drinking water. Where sewage sludge is applied to agricultural fields, contamination of soil, runoff into surface water and also drainage may occur. Moreover, veterinary pharmaceuticals may enter aquatic systems via manure application to fields and subsequent runoff, but also via direct application in aquaculture like fish farming (Fent, 2006; Li, 2009). Pathways for the entrance of pharmaceuticals to the environment, and subsequently to the human food web, are the application of manure, use of sewage sludge as soil fertilizer and conditioner and irrigation of fields with wastewater containing pharmaceutical residues (Eggen et al., 2011; Eggen and Lillo, 2012).

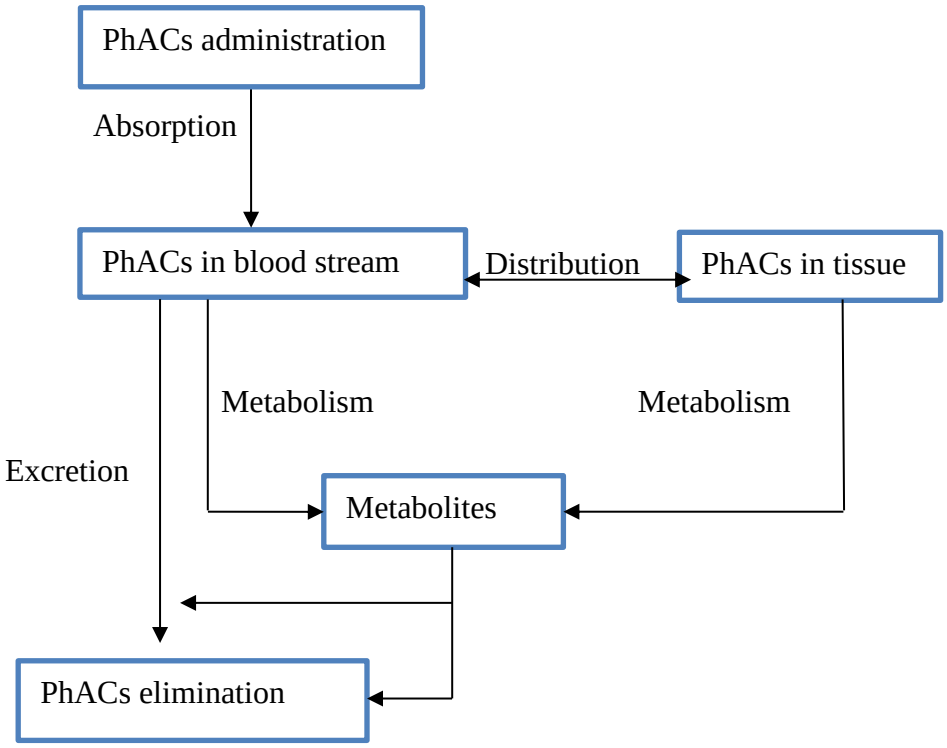


Figure 2.4. 1. Fundamental pathway of pharmaceutical movement and modification in the body

PhACs bloodstream

Possible sources and destinations of pharmaceutical residues in the aquatic environment are was reported by Al-Farsi et al., (2017) (Figure 2.4.2). Generally, three major pathways are observed for pharmaceuticals pollution in the environment which includes the release via human pathways, veterinary pathways, and industry release during production (Williams, 2005). Pharmaceuticals administered for animals are also present in manure and dung or urine on pastures. They can reach the soil environment and be transported to water by surface runoff or erosion (Hamscher et al., 2005). The other pathway of pharmaceuticals contamination in the environment is directly from the production industry (Che`vre, 2014).

Several studies argue that wastewater treatment plants are regarded as the main route of entry of pharmaceuticals into the environment because they often do not eliminate them efficiently (Kosma et al., 2014; Pereira et al., 2016). The risk to aquatic organisms has been correlated with pharmaceutical residues present in wastewater and continual exposure has also given rise to the

presence of multi-drug resistance microorganisms (Mutiyar and Mittal, 2014; Zhang et al., 2012). Pharmaceuticals could be taken up by crop/vegetable produce from agricultural lands, and enter humans and animals through food chains (Chuang et al., 2015). Aquatic organisms are exposed via wastewater residues of pharmaceuticals over their whole life and human exposure could also occur from the consumption of water or aquatic organisms that have accumulated pharmaceutical residues (Cunningham et al., 2009).

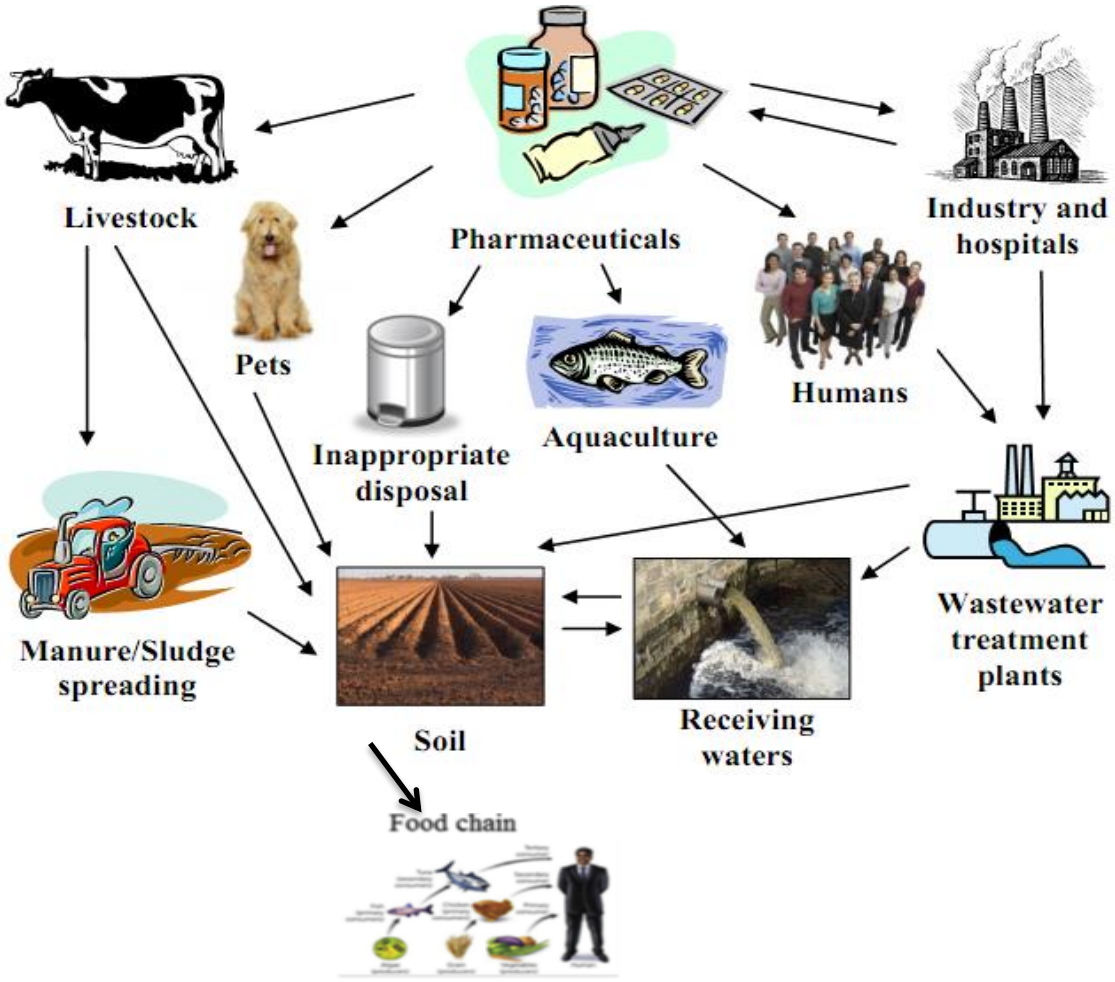


Figure 2.4. 2. Source and pathway of pharmaceuticals contamination

2.5. Pharmaceutical effect on the environment and human health

Pharmaceuticals in the environment can be fatal and are a potential threat to humans, plants, and animals. It is well known that acute toxicity for aquatic organisms is unlikely to occur at the lower measured environmental concentrations. However, due to toxic effects caused by prolonged exposure to low concentrations, evaluation of the chronic potential of pollutants is crucial (Olaitan, 2014). Once inside the organism, the residue may induce a variety of adverse effects, ranging from cellular impairment to the death of the organism (Wagil et al., 2014). The most widely studied pharmaceutical that affects the aquatic environment is the active ingredient of contraceptive tablets which is the 17α-ethinylestradiol (EE2) that has the potential to adversely affect the sensitive hormone pathways that regulate reproductive functions (Khan et al., 2018; Olaitan, 2014). In the environment, the antibiotics may either inhibit functions among the microorganisms or the microorganisms will develop resistance to the antibiotics. Both of these effects can lead to a change in ecosystem functioning and threaten biodiversity. The consequence of resistant bacteria spreading is, of course, the risk of humans getting infected with resistant bacteria resulting in sicknesses that cannot be cured by antibiotics. Due to their long-term presence in water bodies, drug-resistant bacteria may be produced (Khan et al., 2018).

2.5.1. Uptake of pharmaceuticals by plants

Using treated wastewater and river water to irrigate crops is already widespread in different countries particularly where freshwater is limited (Al-Farsi et al., 2017). A number of studies have recently reported that plants can take up pharmaceutical compounds from the growth media via their roots (Azanu et al., 2016). Pharmaceutically active compounds of human and veterinary origin are applied to land via the use of reclaimed wastewater and biosolids as well as the application of animal manures. The uptake of organic compounds into plants mostly depends on the properties of the compounds including the octanol/water partition coefficient (K_{ow}) and molecular size. These would influence their diffusion and uptake into plants (Boonsaner and Hawker, 2012). The phytotoxicity of the plant through exposure via soil depends on sorption kinetics, pH and soil organic matter. These factors affect the bioavailability and the uptake of pharmaceuticals by plants (Carter et al., 2015; Carvalho et al., 2014). Pharmaceuticals with lower pKa values are relatively stronger and ionize easily under normal environmental conditions.

Furthermore, it is elaborated that compounds of molecular mass below 1000 and high K_{OW} values are easily absorbed by plant roots (Zhang et al., 2017). This becomes important in the understanding of absorption and translocation of pharmaceuticals from the soil into the plant system (Al-Farsi et al., 2017).

These pharmaceuticals widely found in agricultural production fields originate primarily from land application of animal manures and sewage biosolids, as well as crop irrigation with reclaimed water from wastewater treatment plants (WWTPs)(Chuang et al., 2015). Human pharmaceuticals commonly present in treated wastewater and biosolids can be actively taken up by various species of plants grown using a nutrient solution fortified with pharmaceuticals under ideal hydroponics conditions (Herklotz et al., 2010). A recent study also reported vegetables could uptake antibiotics from contaminated water (Azanu et al., 2016) and from soil (Chitescu et al., 2013). The results by Wu et al., (2014) reported that vegetables irrigated with reclaimed water could accumulate 0.8 ng/g of caffeine, 1.4 ng/g of carbamazepine, and 0.26 ng/g of naproxen in edible parts of vegetables.

2.5.2. Human intake of pharmaceuticals

The fact that pharmaceuticals are manufactured to cause biological effects has raised concerns about the impacts of unintentional pharmaceutical exposure on the health of human and ecological communities (Kumar et al., 2010). This is especially the case in countries that depend strongly on surface waters as a source of drinking water (Houtman et al., 2014). Commonly, treated wastewater referred to as reclaimed or recycled water is a valuable water source in arid and semi-arid areas for irrigation, where freshwater sources are becoming increasingly scarce due to urbanization and climate change (Dodgen et al., 2013). Human exposure could also occur in the following scenarios: (1) Indirect ingestion of food crops and/or vegetables irrigated with reclaimed wastewater or grown on sewage-sludge-amended soil, (2) Inhalation of pharmaceuticals during application of reclaimed wastewater for irrigation purposes, and (3) Dermal exposure (Kumar et al., 2010; Kumar and Xagoraraki, 2010a). Humane exposure to pharmaceutical residues from water (Figure 2.5.1) shows that pharmaceutical detected in reclaimed wastewater, highly persistent in soil, and taken up by crops that healthy individuals

consuming reclaimed wastewater-irrigated vegetables and excreted the compound in their urine (Kumar et al., 2010; Paltiel et al., 2016,).

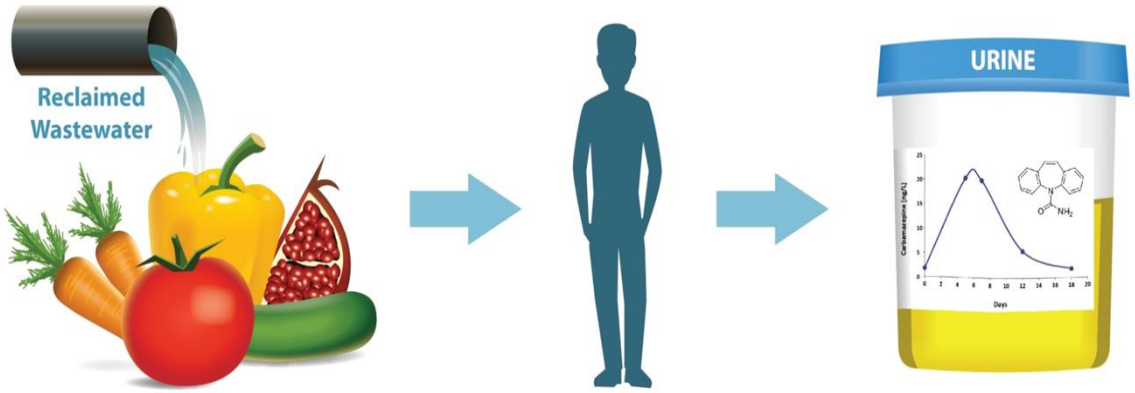


Figure 2.5. 1. Human exposure to pharmaceutical residues from water

2.6. Environmental risk assessment

To evaluate the adverse environmental effects of the pharmaceuticals risks were estimated based on risk quotient that comprises a useful tool to characterize the potential ecological risk of many contaminants in aquatic ecosystems. This method is valuable to conduct a screening-level risk assessment for the selected pharmaceuticals. The risk quotient (RQ) can be calculated as the ratio between Measured Environmental Concentration (MEC) and Predicted No-Effect Concentration (PNEC) (Hernando et al., 2006a). MEC is the measured environmental concentration, which corresponds to the highest detected concentration, and PNEC was estimated using the lowest values of acute EC50 or LC50 or the chronic NOEC, divided by a default assessment factor (AF). EC50 or LC50 is the lowest median concentration to aquatic organisms at different trophic levels of the ecosystem including daphnia manga, algae and fish. AF is the safety factor set at 1000 following the guidelines of the Water Framework Directive (Directive 2000/60/EC). Three different species from different trophic levels namely fish, Daphnia Magna and algae that represent the aquatic ecosystem are used to obtain PNEC values (Aydin et al., 2018). In this study, the ecotoxicity data is obtained from (ECOSAR v 2.0) predictive model software from U.S. Environmental Protection Agency adopting the approach reported by Mansour et al., (2016) for each of the three species using an assessment factor of 1000. The risk quotient was evaluated

according to the commonly used risk ranking criterion. RQ < 0.1 is considered insignificant risk, 0.1 < RQ < 1 is considered low risk, 1 < RQ < 10 is considered moderate risk, and RQ > 10 is considered high risk to aquatic organisms (Aydin et al., 2019; Mansour et al., 2016; Verlicchi et al., 2012).

2.7 Sample extraction techniques

2.7.1. Environmental liquid sample extraction

Sample preparation is always the major bottleneck in any analytical procedure for the determination of chemical residues in the environmental sample (Stubbings and Bigwood, 2009). The residues of pharmaceutical compounds are usually present at very low concentrations in the environmental water, therefore sample preparation and pre-concentration steps are necessary before analysis (Pavlovic et al., 2007; Stubbings and Bigwood, 2009; Wille et al., 2012). A researcher reported a typical procedure for the analysis of a broad group of pharmaceuticals in aqueous matrices. This procedure included filtration, extraction, an additional clean-up step (if necessary), derivatization in the case of detection with gas chromatography (GC), and, finally, detection with GC or liquid chromatography (LC) in combination with mass spectrometry (MS) (Fatta et al., 2007; Wille et al., 2012). As stated by Baranoloska et al. (2011), the use of a novel MS detector allows increasing confidence in the identification of an analyte. Figure 2.6.1 shows the common procedure used by different researchers for the determination of pharmaceutically active compounds in water samples.

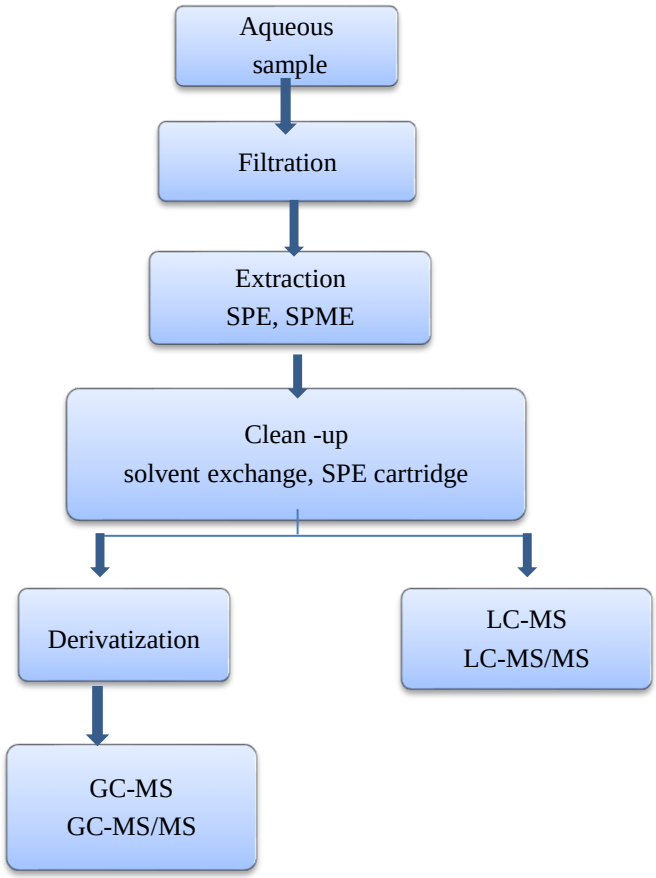


Figure 2.6. 1. A typical procedure for the analysis of pharmaceuticals in aqueous matrices

In previous studies, several procedures have been developed for the pre-concentration of pharmaceuticals from water sample matrices including solid-phase extraction (SPE) (Al-Qaim et al., 2013; Iglesias et al., 2014), liquid-liquid extraction (LLE) (Padrón, 2014), magnetic solid-phase extraction (MSPE) (Tolmacheva et al., 2016), hollow fiber liquid phase micro-extraction (Sharifi et al., 2016) and salting-out assisted liquid-liquid extraction (Alshishania et al., 2018; Gezahegn et al., 2019; Noche et al., 2011; Xia et al., 2012) as well as hallow fiber silicon membrane (Amdany et al., 2015). Very recently molecularly imprinted polymer solid-phase extractions (MISPE) have been employed for pharmaceutical analysis from water samples (Madikizela et al., 2017a; Zunngu et al., 2017).

2.7.1.1. Liquid-liquid extraction

Liquid-liquid extraction (LLE) is the traditional sample preparation system that has been used extensively for the extraction and pre-concentration of organic compounds from aqueous samples (Ye et al., 2007). In a typical liquid-liquid operation, two or more immiscible liquid phases that are not in equilibrium are brought in contact to produce an exchange of materials across the interfacial area, where the mass transfer is driven by the chemical potential gradients established between the phases. The transfer process for a compound continues until its chemical potential adopts the same value in each of the different co-existing phases (Lopez-Montilla et al., 2005).

The extraction efficiency in LLE is usually enhanced with the increase of the salt concentration in sample solution due to an effect referred to as the salting-out effect (Ye et al., 2007). It is characterized by long analytical time, manual manipulation of the extracts, large consumption of sample and reagents and generation of large amounts of waste. In recent years, various sample preparation methods, based on solvent micro-extraction approaches, such as liquid-phase micro-extraction (LPME) have been developed (Zhang et al., 2013). However, solid-phase extraction (SPE) as a sample pretreatment technique has been considered a good alternative to liquid-liquid extraction, due to convenience, time-saving and cost-effective attributes among others (Guo et al., 2020).

2.7.1.2. Solid-phase extraction

Solid-phase extraction (SPE) is an analytical sample preparation method based on the distribution of analytes between solid sorbent packed in a cartridge and liquid sample which moves through the solid phase. Solid-phase usually consists of small porous particles of silica with or without bonded organic phase, organic polymers and ion exchangers. Mechanisms of extractions are based on adsorption, partitioning, or ion exchange according to the kind of solid phase used. The applicability of SPE is mainly determined by the sorbent used in the extraction process. Nowadays a large number of sorbents are available, and the most frequently used group of sorbents include chemically modified silica gel, polymer sorbents, graphitized or porous carbon-containing and molecularly imprinted polymers (Al-Qaim et al., 2013; Madrakian et al., 2013; Pavlovic et al., 2007).

The basic approach in this sample extraction method involves the analyte of interest being retained with the matrix interferences washed out. Steps in the use of SPE are presented below in (Figure 2.6.2) (Pavlovic et al., 2007). Sorbents packed in a cartridge are placed on the manifold, and the appropriate extraction solvent is used in the four-step extraction process. These steps include (1) conditioning of sorbent; (2) loading of the sample; (3) washing of impurities; and (4) elution of the analyte (Faleye et al., 2017). In most cases, SPE is selected due to minimum solvent usage and additional clean-up, a performance which is important for purification of intended pharmaceuticals from the matrix (Iglesias et al., 2014; Padrón, 2014; Wille et al., 2012). In the application of SPE, parameters such as a different solvent for elution, different types of adsorbents used, the volume of the sample solution, and the pH of the solution with selected pharmaceuticals need to be adjusted (Al-Qaim et al., 2013; Lin et al., 2008; Madikizela et al., 2018d).

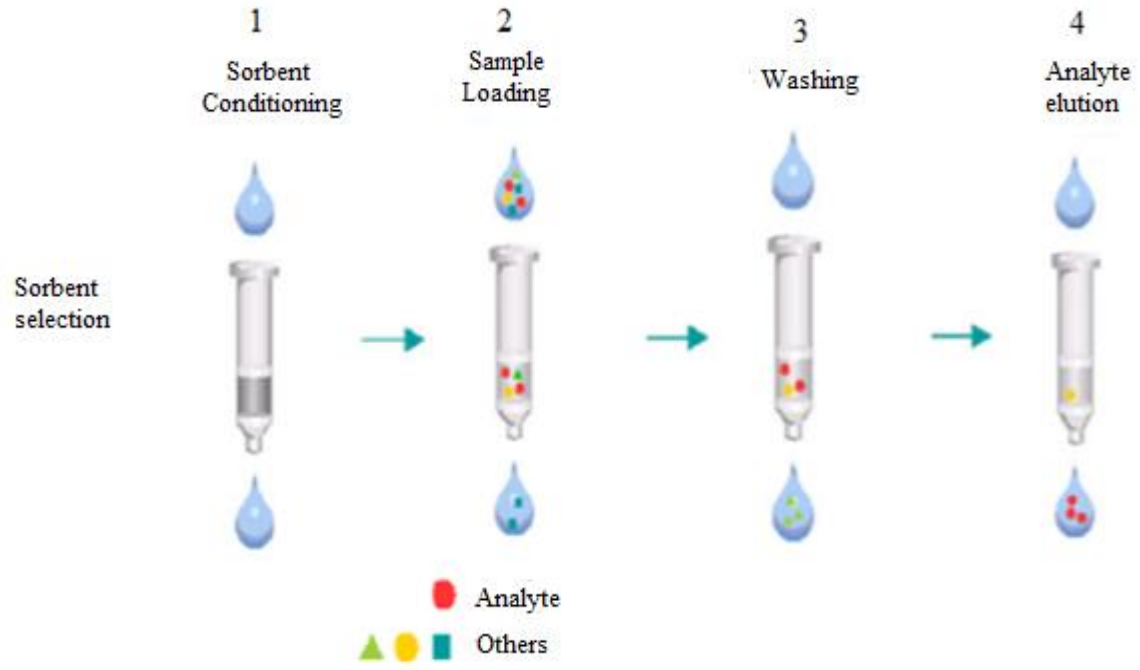


Figure 2.6. 2. Solid-phase extraction steps

SPE cartridges are available in a wide variety of chemistries, packing materials, and sizes. Selecting the most suitable product for each application and sample is crucial. As a rough guide, the phases are categorized by the primary interaction mechanism with the analyte of interest: (i)

reversed-phase: extraction of hydrophobic analytes from the aqueous matrix, (ii) normal phase: extraction of polar analytes from nonpolar organic solvents, (iii) ion exchange: extraction of charged analytes from aqueous or non-polar organic samples, and (iv) mixed-mode phases: phases with combined multiple interaction mechanisms.

Solid-phase extraction is a selective method that offers countless diverse choices of sorbents, ranging from the traditional reversed-phase sorbents (C18, C8), normal phase (silica, alumina), and ion exchange, to mixed-mode (ion exchange + reversed-phase) and functionalized resins based on styrene-divinylbenzene (SDVB) polymers. Among them, the most frequently used groups are silica and bonded silica sorbents, polymer sorbents, graphitized or porous carbon, and new extraction sorbents including molecularly imprinted polymers and immunosorbents (Buszewski and Szultka, 2012). Different SPE sorbents used for method development and analysis of pharmaceutical compounds in this study are listed in (Figure 2.6.3) together with the structure (Matuszewski et al., 2003).

The solid-phase extraction sorbent, hydrophilic-lipophilic balance (HLB) contains two groups, a divinylbenzene monomer that offers lipophilic interactions and an N-vinylpyrrolidone monomer offering hydrophilic interactions. Lipophilic interactions enable reverse-phase retention whereas hydrophilic interactions enable retention of polar compounds. These interactions give HLB its unique property to simultaneously retain acids, bases, and neutrals irrespective of whether they are polar or non-polar and are referred to as universal SPE cartridges for neutral compounds (Carmona and Pico, 2018; Deng et al., 2018a). The sorbent preserves analyte retention even if the bed dries out, which makes it suitable for application in auto-mated (SPE) systems (Buszewski and Szultka, 2012).

The mixed-mode cation exchanger (MCX) cartridge operates through two modes, strong cation exchange (dominant interaction) and reverse phase, which allows for the extraction of basic and neutral compounds with low molecular weight (Deng et al., 2018a). The other common SPE sorbent is a mixed-mode anion exchanger (MAX). It is made of a mixed-mode polymer sorbent with both reversed-phase and anion-exchange functionalities and is mostly used in acidic pharmaceutical extraction (Lee et al., 2005; Madikizela et al., 2018d).

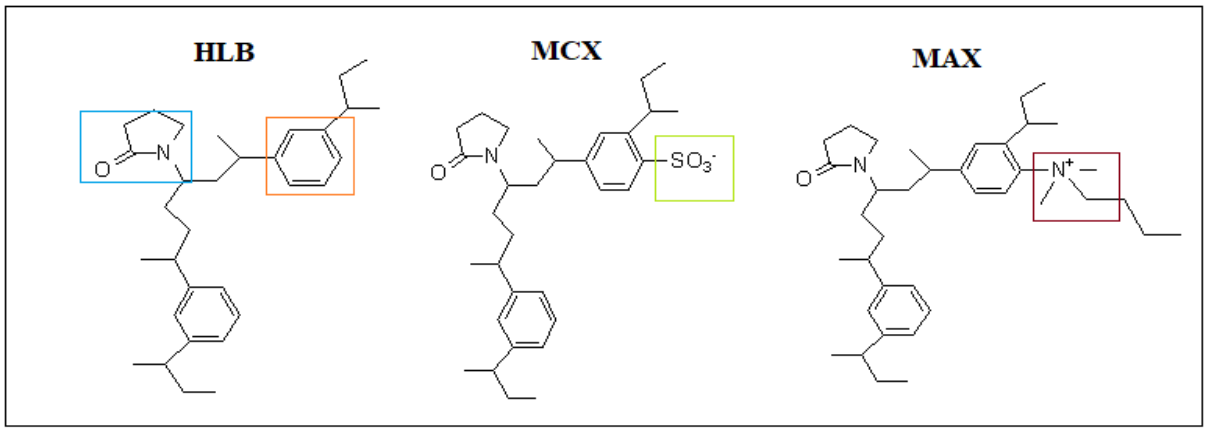


Figure 2.6. 3. Solid-phase extraction sorbent used in the study

2.7.1.3. Solid-phase microextraction (SPME)

Solid-phase micro-extraction (SPME) was introduced by Pawliszyn and co-workers, in 1989, representing a significantly advanced technique concerning simplification and miniaturization of the analytical process. It employs a fused silica fiber coated on the outside with an appropriate stationary phase. SPME can integrate sampling, extraction, pre-concentration and sample introduction into a single step. The technique is very simple, fast, solvent-free, inexpensive, easily automated, and reliable, and it has been applied to both headspace and direct aqueous sample analysis with excellent sensitivity and good selectivity (Sarafraz-Yazdi and Razavi, 2015). SPME represents a solvent-free sampling technique, often used in the extraction of organic micro-pollutants from water samples. SPME fibers are commercially available in a "syringe-like" format, which allows for its protection during septum puncture. Volatile analytes can be emitted from the sample and isolated from the headspace or adsorbed by direct immersion into the liquid sample and concentrated in the fiber coating (Mottaleb et al., 2014; Vas and Vekey, 2004).

2.7.2. Environmental solid sample preparation

2.7.2.1. Pressurized hot water extraction (PHWE)

The use of pressurized hot water extraction (PHWE) in analytical chemistry started with the work in environmental analysis in the mid-1990s by Hawthorne and colleagues (Plaza and Turner, 2015). This is an extraction technique that uses liquid water as an extractant (extraction solvent) at temperatures above the atmospheric boiling point of water (100 °C/273 K, 0.1 MPa), but below the critical point of water (374 °C/647 K, 22.1 MPa) (Cam et al., 2019; Plaza and Turner, 2015).

Hence, traditional water is not considered a suitable extraction fluid for non-polar or organic compounds at room temperature. The dramatic changes in the physical-chemical properties of water, especially in its dielectric constant (ε), at elevated temperatures and pressures enhance its usefulness as an extraction solvent. When the temperature of the water is raised, there is a steady decrease in its permittivity, viscosity and surface tension but an increase in its diffusivity characteristics. With enough pressure to maintain water in the liquid phase at elevated temperature, the initial value of the dielectric constant of 80 at 25 ℃ decreases to 27 at 250 ℃ and 50 bar, which falls between that of methanol (ε = 33) and ethanol (ε = 24) at 25 ℃. Under these conditions, water behaves like certain organic solvents which can dissolve a wide range of medium and low polarity analytes (Fernandez-Gonzalez et al., 2008; Teo et al., 2010).

Water is easily available, non-toxic and can be recycled or disposed of to the environment with minimal problems. This is the green advantage of PHWE as alternative sample preparation is the reduction in the consumption of organic solvents as well as solvent-free extractions of the analytes for analytical determination (Fernandez-Gonzalez et al., 2008; Teo et al., 2009). Hence, PHWE has steadily become an efficient and low-cost method of extraction for less-polar organic components from environmental soil, sediments and plant materials. Figure 2.6.4 presents the schematic diagram of the extraction techniques (Teo et al., 2010). Recent studies report the use of PHWE for the extraction of pharmaceuticals from environmental samples (Jelic et al., 2009; Vazquez-Roig et al., 2010). However, a major setback to this ingenious system has been the thermal degradation phenomenon observed at elevated temperatures for certain compounds (Gbashi et al., 2016).

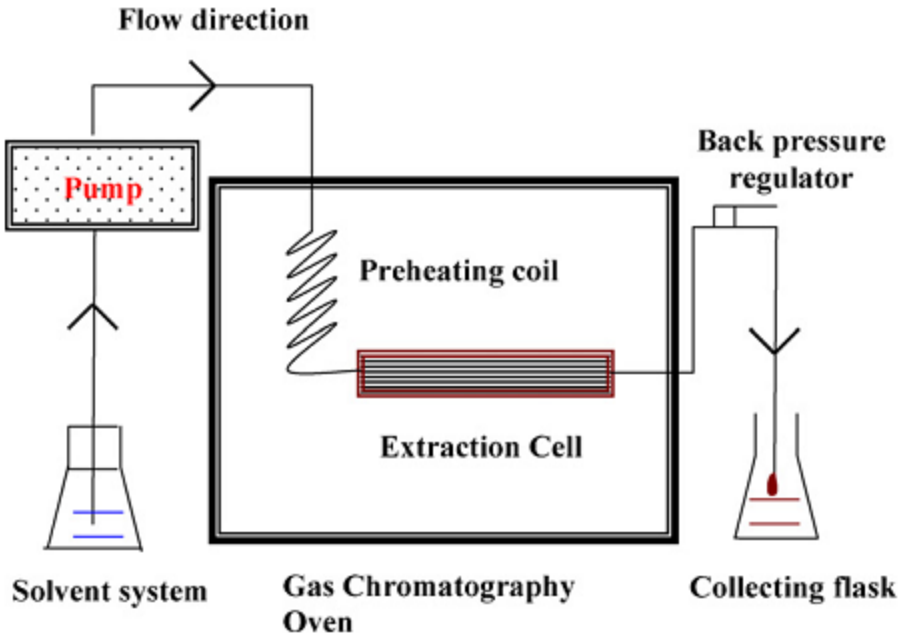


Figure 2.6.4. A schematic diagram of a pressurized hot water extraction system

2.7.2.2. Microwave extraction technique

The use of microwave energy in sample preparation first emerged in the early 1970s (Sanchez-Prado, 2011), specifically for the extraction of organic compounds used by Ganzler (Ganzler, 1986). Microwaves are a form of non-ionizing electromagnetic energy at frequencies ranging from 300 MHz to 300 GHz and positioned between the infrared and radio wave in the electromagnetic spectrum. This energy is transmitted as waves, which can penetrate biomaterials and interact with polar molecules transferring them into materials, such as water to generate heat (Mandal et al., 2007; Sadeghi et al., 2017).

Compared to traditional extraction techniques, microwave extraction has several advantages such as reduction of extraction time and solvent consumption as well as the possibility of running multiple samples simultaneously (Merdassa et al., 2014). Several studies have investigated the microwave-assisted solvent extraction technique for the separation of certain target pharmaceutical and personal care products from solid matrices, since its extraction efficiency is supreme to traditional Soxhlet methods, and requires less time and solvent volume (Merdassa et al., 2014; Rice and Mitra, 2007) as reported by different researchers (Sanchez-Prado et al.,

2015). This method of extraction was used to extract endocrine-disrupting chemicals and pharmaceuticals from the environmental sample (Dobor et al., 2010; Liu et al., 2004; Varga et al., 2010). However, these sample preparation methods are effective, yet very expensive (Vera et al., 2013). A good alternative is QuEChERS (quick, easy, cheap, effective, rugged and safe) multi-residue procedure simplifies and reduces the time taken to complete the extraction and clean-up processes (Stubbings and Bigwood, 2009).

2.7.2.3. Quick, Easy, Cheap, Effective, Rugged, and Safe (QuECEhERS) method of sample preparation

QuEChERS analytical sample extraction method was developed by Anastassiades and Lehotay for the first time and used for multi-class pesticide residues extraction from fruits and vegetables. It is based on an extraction step with acetonitrile followed by a partition step with salts and dispersive solid-phase extraction (d-SPE) clean-up using small quantities of sorbents (Anastassiades et al., 2003). It is based on liquid-liquid partitioning of the analytes between water and organic solvents using salts to produce the effect of salting out, followed by a dispersive solid-phase extraction (d-SPE). This procedure offers many advantages including basic instrumentation for application, small sample amounts, rapid extraction, and low waste volume, which results in a time-efficient method (Pena-Herrera et al., 2019; Rejczak and Tuzimski, 2015). This extraction method is simple, rapid and requires low solvent consumption, which is an advantage in the area of green chemistry (Salvia et al., 2014). It is a type of sample preparation method which has attracted substantial attention because of its advantages including safety, ease of operation, high recovery, high reproducibility, selectivity and cost-effectiveness (Deng et al., 2018b). The method is effective for the analysis of several groups of compounds, including pharmaceuticals, mycotoxins, and polycyclic aromatic hydrocarbons, in a wide variety of complex matrices (Anastassiades et al., 2003; González-Curbelo et al., 2015; Westland and Dorman, 2013).

Extraction solvent

The choice of extraction solvent is one of the most important decisions in any extraction. Many aspects have to be considered, including the ability to cover the desired analytical spectrum (ranging from polar to non-polar compounds); the selectivity that can be reached during extraction; partitioning and clean-up; achieving separation from water; amenability to chromatographic separation techniques; cost; safety; environmental impact; and, handling concerns. Acetonitrile (ACN) is the most commonly used extraction solvent due to its ability to separate easily from water when an appropriate mixture of salts (magnesium sulphate ($MgSO_4$) and sodium chloride (NaCl)) is added (Anastassiades et al., 2003; Vera et al., 2013). However, if ACN does not provide adequate recoveries, other solvents can be employed, namely: ethyl acetate, acetone and methanol (Rejczak and Tuzimski, 2015; Vera et al., 2013). A recent study reported using the QuEChERS method for the determination of pharmaceuticals from vegetable samples using a mixture of acetonitrile, methanol and water as extraction solvent (Chuang et al., 2015).

Salt for phase separation

Sodium chloride addition helps to control the polarity of the extraction solvents and thus increases the selectivity of extraction(Rejczak and Tuzimski, 2015). The addition of NaCl typically leads to increased recoveries of polar compounds, but this also depends on the nature of the solvents involved in the partitioning step and allows the control of the percentage of water in the organic phase. The use of $MgSO_4$ may bind large amounts of water and thus significantly reduce the water phase. This also promotes the partitioning of analytes into the organic layer. Nevertheless, to bind a significant fraction of water, $MgSO_4$ should be added at amounts well exceeding its saturation in water (Vera et al., 2013). However, the use of $MgSO_4$ sometimes has drawbacks in reducing the extraction efficiency of pharmaceuticals like tetracycline and macrolides and alternatively, Na_2SO_4 may be used (Bourdat-Deschamps et al., 2014; Kang et al., 2014). Another study used QuEChERS for purifying the matrix effect during the analysis of pharmaceuticals from environmental samples using sodium acetate (CH_3COONa) and sodium sulfate (Na_2SO_4) as an extraction salt (Bourdat-Deschamps et al., 2014).

Clean-up in QuEChERS

In a QuEChERS sample extraction process, the cleanup protocols refer to removal of the matrix-matched materials such as polar pigment, fatty acids, organic compounds, and sugar (Al-Thaiban et al., 2018; Beos et al., 2015). The dispersive solid-phase extraction (d-SPE) technique has been applied with the QuEChERS method to promote the removal of co-extractives from the extract (Barci et al., 2020). Dispersive SPE shows advantages over classical solid-phase extraction like no need of use and follow SPE steps and manifold with vacuum, no dilution of extract and therefore no evaporation needed, less sorbent expenditure, faster, cheaper and no experience needed to perform (Rejczak and Tuzimski, 2015). To achieve the goal of purification, sorbents play a vital role in QuEChERS for cleanup. Different sorbents are usually used in d-SPE clean-up, like primary secondary amine (PSA), octadecylsilane (C18) and graphitized carbon (GCB), diatomaceous earth, Florisil®, and the bio-sorbent chitosan (Barci et al., 2020; Deng et al., 2018b). Magnesium sulfate is added simultaneously with the d-SPE sorbent to remove the majority of the undesirable water and improve analyte partitioning to provide better clean-up (Rejczak and Tuzimski, 2015; Vera et al., 2013).

The sorbent PSA is normally used to remove fatty acids, sugars, organic acids, lipids, and some pigments, whereas C18 is effective for the removal of lipids. GCB is used to remove pigments like carotenoids and chlorophyll, typically from highly pigmented matrices, like green leafy vegetables (Tankiewicz, 2019). However, GCB should be carefully used because it can interact with planar structures resulting in low recoveries (Barci et al., 2020; González-Curbelo et al., 2015; Li et al., 2008). Most parts of the modifications were focused on the clean-up step and different sorbents have been used besides common sorbent PSA and C_{18} (Volpatto et al., 2016). In the study reported by (Stubbings and Bigwood, 2009) on the determination of veterinary drug residues in animal tissue using a QuEChERS approach, amine (NH_2) was used as a cleanup SPE sorbent. Another study used QuEChERS for sample preparation; solid-phase extraction (SPE) purification was carried out in normal phase on silica solid phase for removal of interference (Baugros et al., 2009).

Addition of ethylene diamine tetraacetic acid (Na_2EDTA)

Several studies reported the use of Na_2EDTA to increase the extraction recovery of pharmaceuticals from environmental samples to facilitate the extraction of bound compounds by avoiding the complexation of analytes with cations (Aguilera-Luiz et al., 2008; Bourdat-Deschamps et al., 2014). The study reported by Choi et al., (2009) depicts the addition of Na_2EDTA, during the extraction of environmental samples for analysis of pharmaceuticals like ciprofloxacin, improves the extraction recovery upon addition.

2.8. Analytical methods for the determination of pharmaceutical residues in the environment

2.8.1. Chromatography analysis of pharmaceutical residues

Most of the chromatographic methods recently developed pursue the simultaneous determination of multi-class compounds since various compounds from different therapeutic classes are found when monitoring environmental samples. Multi-class methods provide more information about the occurrence of pharmaceuticals than single-group analysis, with reduced analysis time and cost. However, the development of these methods involves a compromise in the selection of experimental conditions (Gracia-Lor et al., 2011).

The detection of pharmaceuticals is influenced by different properties, hence specific and suitable methods need to be developed to identify and quantify them from the environment. The analytical methods employed for the analysis of pharmaceuticals are also varied and the sample preparation procedure generally involves the use of large sample volumes, multiple extractions, sophisticated sample clean up, and/or derivatization before analysis. The selection of methods is dependent on the physical and chemical properties of the target compound selected for qualitative and quantitative analysis (Shraim, 2017; WHO, 2012). Analytical techniques including high-performance liquid chromatography (HPLC) and gas chromatography (GC) with various detectors allow for the analysis of multiple class analytes with improved efficiency when coupled with a mass spectrometer (MS) which is sensitive and selective. These chromatographic techniques are commonly employed in environmental sample analyses (Fatta-Kassinos, 2011). For the separation and determination of pharmaceuticals, high-performance liquid

chromatography (HPLC) is the most commonly used method compared to gas chromatography (GC) (Salgado et al., 2010). In a recent research report, the determination of pharmaceuticals from different samples was performed by various chromatographic techniques, including high-performance liquid chromatography with UV and diode array detection (HPLC-DAD) (Baranowska and Kowalski, 2011; Madureira et al., 2010), (HPLC-UV) (Silva and Oliveira, 2018), liquid chromatography-mass spectrometer (LC-MS) (Iglesias et al., 2014; Nannou et al., 2015), liquid chromatography-tandem mass spectrometer (LC-MS/MS) (Chen et al., 2016; Ort, 2010) and gas chromatography-mass spectrometer (GC-MS) (Farré et al., 2007a; Qureshi et al., 2014) for non-volatiles and electrochemical sensors (Bendz et al., 2005; Sanghavi et al., 2015). As it is presented in (Figure 2.8.1) ultrahigh-performance liquid chromatography is used frequently compared to simple liquid chromatography for the analysis of such pollutants from the environment (Carmona and Pico, 2018). This may be explained by the fact that liquid chromatography requires minimal effort to operate and is relatively selective hence aiding efficient compound detection and analysis. With ultra-high pressure liquid chromatography (UHPLC) the efficiency increases with the pump pressure. The use of UHPLC results in better conditions including, better resolution, increased peak height and a significant reduction in sample analysis time and mobile phase consumption when it is compared with traditional liquid chromatography (LC) (Oliveira et al., 2015).

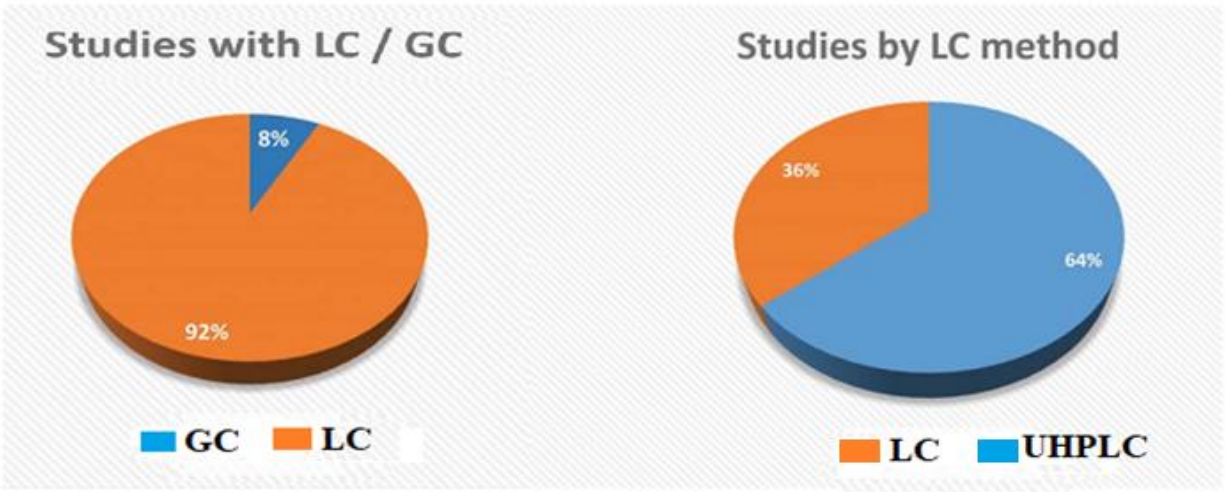


Figure 2.8. 1. The proportion of studies by gas chromatography and liquid chromatography technique

2.8.1.1. Gas chromatography

The chromatographic technique is used for the analysis of pharmaceutically active compounds in the aquatic environment. Gas chromatography is a very sensitive, rapid and reliable analytical instrument used for the separation and identification of volatile organic compounds (Sahil, 2011). However, a derivatization step must be performed prior to gas chromatography analysis to increase the volatility, decrease the polarity of these analytes, and increase thermal stability and to protect the analyte from thermal degradation and in some cases to improve their detection. The common derivatization reactions commonly used for gas chromatography applications are silylation, acylation, alkylation and esterification. Among the available alkylating reagents, diazomethane is the most common for analysis of acidic pharmaceuticals because of its high reactivity toward most acidic groups (Farré et al., 2007b). As mentioned by Fatta et al., (2007) gas chromatography-mass spectrometry (GC-MS) can be successfully applied for nonpolar and volatile pharmaceutical compounds, however, the time-consuming derivatization step, during which there are risks of analyte losses and also, most of the chemicals used for derivatization process are toxic, for instance, diazomethane is carcinogenic may form as drawbacks for this technique.

2.8.1.2. Liquid chromatography

Liquid chromatography is the most common method used for the separation and determination of pharmaceuticals since, most pharmaceutically active compounds are non-volatile (Bendz et al., 2005; Iglesias et al., 2014). The most common mode of liquid chromatography is reverse-phase separation characterized by having a less polar stationary phase than the mobile phase. The most common columns are those consisting of a stationary phase with long carbonaceous aliphatic chains bound to silica with pores in the micrometer range. The most used silica columns are the named C_8 and C_{18}, corresponding to the length of the carbon chain (hydrocarbons). This is due to the robustness offered by the C_{18} column, which can retain both polar and non-polar analytes that are sequentially eluted by the mobile phase (Carmona and Pico, 2018). As per the ICH method validation can be defined as “Establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics”. Method validation studies include system suitability, linearity, precision, accuracy, specificity, robustness, the limit of detection,

the limit of quantification and stability of samples (ICH, 2005). The detection of pharmaceuticals in different environmental matrices is a great challenge due to the complexity of matrices and low concentration (~ng/L). Liquid chromatography with mass spectrometry is preferable for this purpose due to its high sensitivity the representative schematic diagram is given below in (Figure 2.8.2) (Ashfaq et al., 2017).

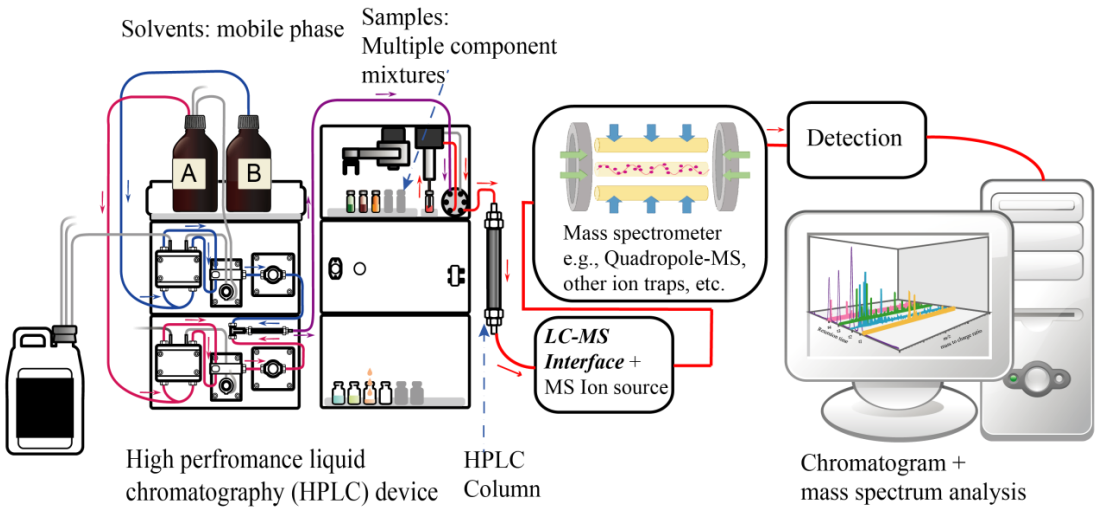


Figure 2.8. 2 Schematic diagram of HPLC with mass spectrometry.

Mass-spectrometry (MS)

Mass spectrometry is an instrumental technique based on the separation of ions according to their mass-to-charge ratios (m/z), in a vacuum, in the gas phase. After ionization, the molecular ions formed can undergo further fragmentation and all these formed ions are then separated in the mass spectrometer according to their m/z. A mass spectrometer is composed of three fundamental parts: an ion source, a mass analyzer and a detector (Cajka et al., 2008). Several ionization methods are available. Hence, the fitness of the ion source will depend on the polarity, molecular weight and thermal lability of the analytes, as well as the complexity of the sample to be analyzed (Cajka et al., 2008).

Ionization source

The ionization source plays an important role in mass spectrometry as it is responsible for generating detectable ions from the sample solution. A number of ionization sources for solid and liquid samples are available. The most common techniques for transferring compounds from

liquids into the gas phase are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). As they are soft ionization techniques they are widely used for the analysis of pharmaceuticals (Jakob-Rodamer, 2014). Electrospray ionization (ESI) is the most common ionization technique for pharmaceuticals (Figure 2.8.3) (Cajka et al., 2008). The analyte solution from the LC is sprayed (vaporized) through a capillary with a voltage of ±2-5 kV to produce gas-phase ions to enter the mass analyzer. ESI requires ions already in solution, protonated $[M+H]^+$ or deprotonated $[M-H]^-$ molecular ions (Jakob-Rodamer, 2014).

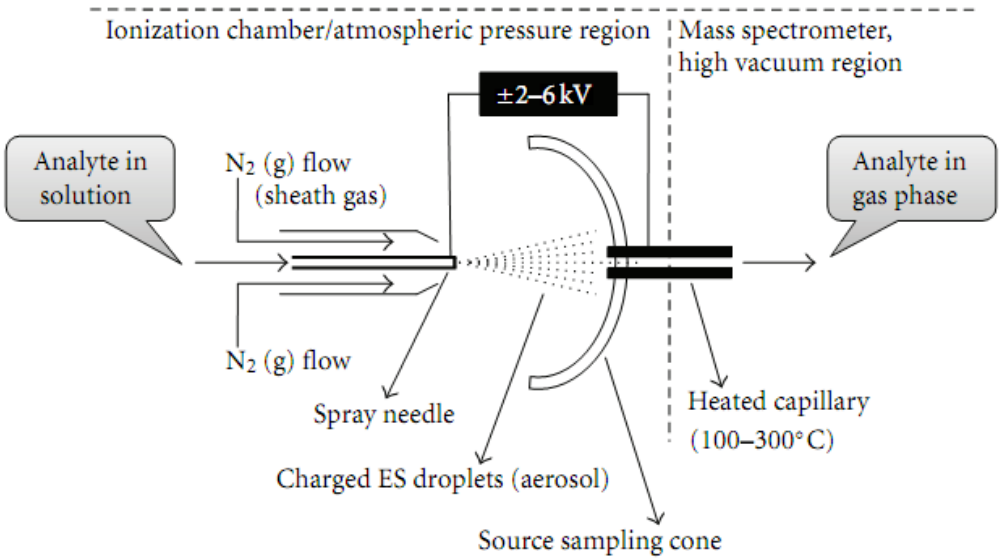


Figure 2.8. 3 A schematic representation of the ESI-ion source.

Mass analyzer time of flight (TOF)

Time-of-flight mass analyzer enables the mass to charge ratio of detected ions to be determined by measuring their time of flight from the specimen surface to the detector (Pareige et al., 2016). Concerning mass analyzers, they can vary from quadrupole, quadrupole ion trap, linear quadrupole ion trap and magnetic sector, representing scanning instruments, to other types of mass analyzers, such as time-of-flight (TOF) (Figure 2.8.4) Fourier-transform ion cyclotron resonance analyzer and orbitrap, which belong to the group of non-scanning instruments. After ions are separated in the mas analyzer they move to the detector which monitor of the ion current and finally records the data as mass spectra (Cajka et al., 2008). The role of each level: (1) The

pulse of ions from the orthogonal accelerator (spatial focusing); (2) separation of ions according to their flight times; (3) focusing of the kinetic energy of ions; (4) separation of focused ions according to their flight times, dependent on their weight (adapted from (Cajka et al., 2008).

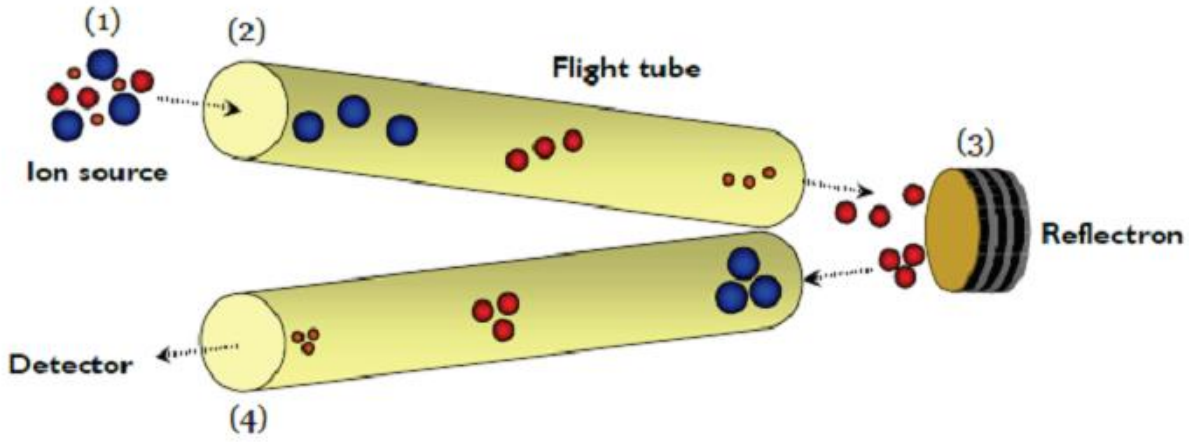


Figure 2.8. 4. Time-of-flight mass analyzer

2.9. Molecularly imprinted polymer (MIP)

Molecularly-imprinted polymers (MIPs) are materials prepared in the presence of a template that serves as a mold for the formation of a template-complementary binding site (Hu et al., 2013). Molecular imprinting is a rapidly developing technique for the preparation of polymers with excellent molecular recognition properties, which has been found effective in a variety of extraction techniques (Sun et al., 2014). The molecularly imprinting technique is a burgeoning field that provides a high selectivity towards the selected molecules (Roland and Bhawani, 2016; Soleimani et al., 2019). In molecular imprinting, the formation of interaction complexes between the molecule of interest (template) and the functional monomers in the presence of a crosslinker and a porogen solvent, enables the production of specific sites within the polymeric material that is physically and chemically complementary to the template. After removing the template, the cavity can selectively bind the molecule in the final MIP application (Anirudhan et al., 2017; Hu et al., 2013; Viveiros et al., 2018). Advantages of MIPs, such as ease of preparation, stability at extremes of pH and temperature, high selectivity and low cost has led to the development of various MIP applications in chromatographic separation, solid-phase extraction, chemical

sensors and catalysis (Chen and Li, 2013; Madikizela et al., 2018b; Vasapollo et al., 2011). As it is reported by different scholars, MIPs are highly selective towards pharmaceuticals and are also re-usable in analytical applications which is an appreciable attribute in green chemistry (Madikizela et al., 2018a; Madikizela et al., 2018c).

Nowadays, molecularly imprinted polymers (MIPs) are being designed to improve the selectivity of analytical methods due to the complexity of environmental samples and interact selectively with a specific target compound (Dorko et al., 2018; Madikizela et al., 2018d). Various MIPs have been recently developed and applied for selective determination of residual pesticides, drugs, organic dyes, mycotoxins and persistent organic pollutants in food and environmental matrices (Speltini et al., 2017). They are specially designed sorbents that display enhanced selectivity towards certain structures or to a very closely related structure and they have been widely used in various applications (Beltran et al., 2010). As reported in the literature, multi-template molecularly imprinted polymers using acidic pharmaceuticals mixture ibuprofen, naproxen, ketoprofen, diclofenac, and clofibric acid as the template, has been prepared for the removal of acidic pharmaceuticals from contaminated water (Dai et al., 2012). The report presented by Madikizela (Madikizela and Chimuka, 2016) also uses multi-template molecularly imprinted polymers using naproxen, ibuprofen and diclofenac as a template for adsorption and removal studies from aqueous solutions. Another study reported by Lu et al., (2019) the use of dual-template molecular imprinted polymer using norfloxacin and enrofloxacin as templates and a novel dummy molecularly imprinted polymers used daidzein as a template reported by Sun (Sun et al., 2014).

2.9.1. The component in the molecularly imprinted polymer

MIPs synthesis protocols must contain the following essential ingredients: template, functional monomer, cross-linker, solvent (porogen) and an initiator (Wu et al., 2018). Depending on the nature of the template molecule, the elements for the polymerization mixture will be selected. Each ingredient added within the polymerization blend has its particular influence over the properties and performances of the final imprinted polymer (Adumitrăchioaie, 2018). The schematic diagram (Figure 2.9.1) shows a general overview of MIP preparation (Hu et al., 2013).

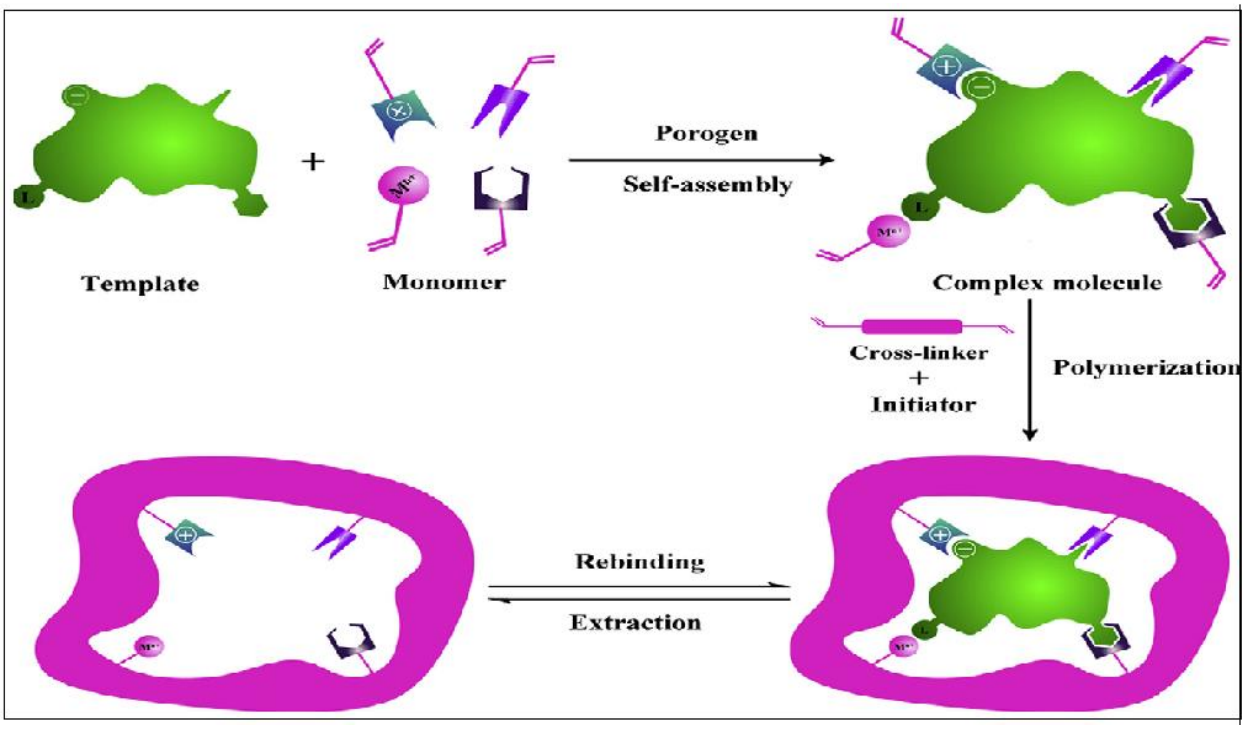


Figure 2.9. 1. The schematic diagram for the synthesis of MIPs.

Initiator

Polymerization is a chain reaction that starts with the activation of a single monomer molecule which becomes the active center of the entire reaction. The triggering of the reactive species is generally due to the presence of an initiator in the polymerization mixture (Adumitrăchioaie, 2018). Several chemical initiators with different chemical properties can be used as the radical source in free radical polymerization (Figure 2.9.2)(Yan and Row, 2006).

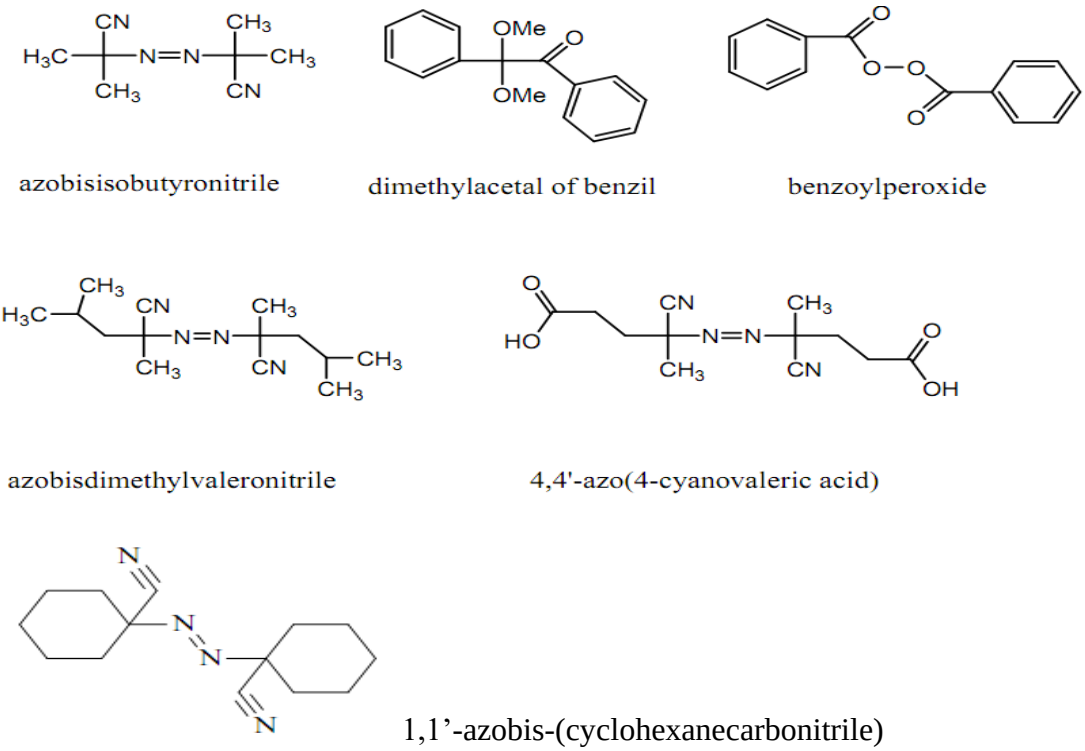


Figure 2.9. 2. Chemical structure of common initiators used in non-covalent molecular imprinting.

Functional monomer

A monomer in MIP synthesis is one component that plays a great role during the polymerization process. The functional monomer provides functional groups that can form a complex with the template by covalent or non-covalent interactions. The choice of the monomer is very important to create highly specific cavities designed for the template molecule. The strength of the interaction between the template and the monomer affects the affinity of MIPs and determines the accuracy and selectivity of recognition sites. In the non-covalent approach, they are normally used in excess compared to the template to favor the formation of template-monomer assemblies (Vasapollo et al., 2011). Some typical functional monomers commonly used for the polymerization of molecularly imprinted polymers are presented in (Figure 2.9.3) (Yan and Row, 2006).

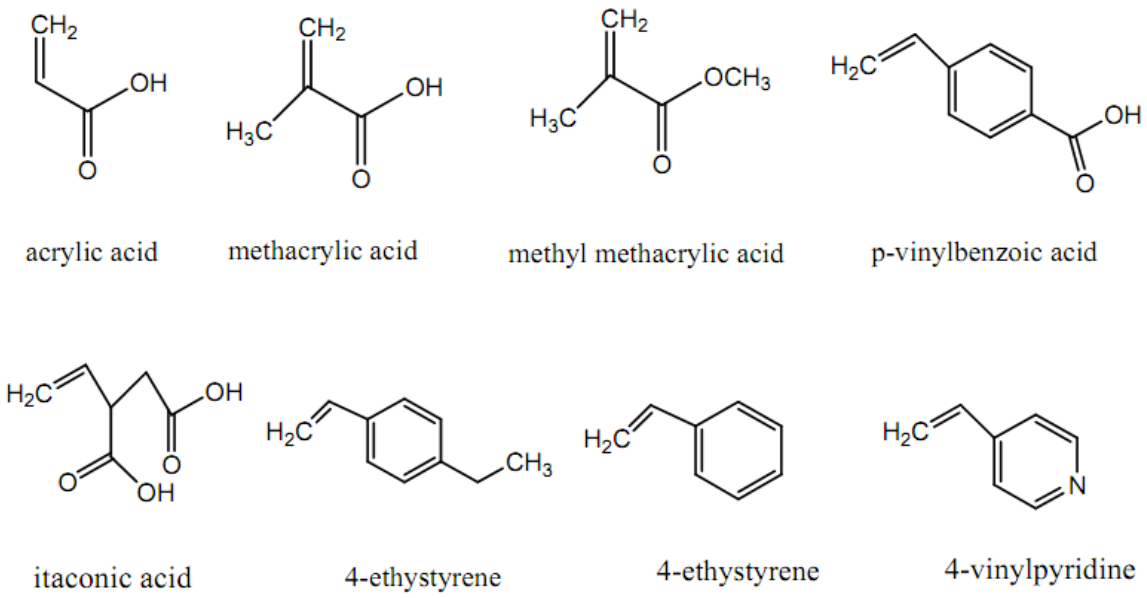


Figure 2.9. 3 Structure of the most common functional monomers used for molecular imprinting.

Solvent (porogen)

The solvent in MIP preparation has a function in dissolving all the components in the polymerization. It serves as the second important function in creating the pores in the macroporous polymer (Hui Lee and Doong, 2016). Moreover, the nature and volume of the solvent play an important role in the molecular imprinting process. The solvent should produce large pores to ensure good flow through and increasing the volume of the solvents, enlarges polymer pore volume. For this reason, it is quite common to refer to the solvent as the "porogen". The porogen is a non-covalent imprinting polymerization that should also be chosen considering its role in promoting template-functional monomer complex formation. Less polar solvents such as chloroform or toluene increase the complex formation facilitating polar non-covalent interactions such as hydrogen bonds. Moreover, more polar solvents tend to dissociate the non-covalent interactions in the prepolymer complex, especially protic solvents that afford a high degree of disruption of the hydrogen bonds (Hui Lee and Doong, 2016; Vasapollo et al., 2011).

Template

In molecularly imprinted polymer preparation, the template is one component that should be able to form stable complexes with the functional monomer. In principle, the target compound is chosen as the template molecule, but often it can be replaced by a structural-related molecule (dummy templating, also named surrogate), as much as possible resembling the analyte in terms of size and chemical groups (Speltini et al., 2017). The usage of organic compounds as template molecules cannot be easily avoided in molecular imprinting. In the concept of green synthesis, the major challenge with the formation of MIPs is the need to use one mole equivalent of the template molecule to generate the complementary polymer cavities that selectively bind the analyte molecule. Besides, molecular imprinting could be improved by a selection of a template that can lead to the production of a MIP with high cross selectivity to a particular group of organic compounds (Ncube et al., 2017). The requirements of a template in molecular imprinting are: templates should be chemically inert during the polymerization and they should be stable at moderately elevated temperatures (around 60 ^{o}C) or upon exposure to ultraviolet light (Cormack and Elorza, 2004).

Cross-linker

The final component in MIP preparation is a cross-linker that is expected to fulfill three major functions in MIP polymerization: First of all, the cross-linker is important in controlling the morphology of the polymer matrix, whether it is gel-type, macroporous, or a microgel powder. Secondly, it serves to stabilize the imprinted binding site. Finally, it imparts mechanical stability to the polymer matrix. From a polymerization point of view, high cross-link ratios are generally preferred to access permanently porous (macroporous) materials and to be able to generate materials with adequate mechanical stability. So the amount of cross-linker should be high enough to maintain the stability of the recognition sites (Bakhtiar et al., 2019; Yan and Row, 2006). The high cross-linker ratio is generally used to access permanently porous (macroporous) materials with adequate mechanical stability (Vasapollo et al., 2011). Commonly used cross-linkers in imprinted polymers preparation are given in Figure 2.9.4 (Vasapollo et al., 2011; Yan and Row, 2006).

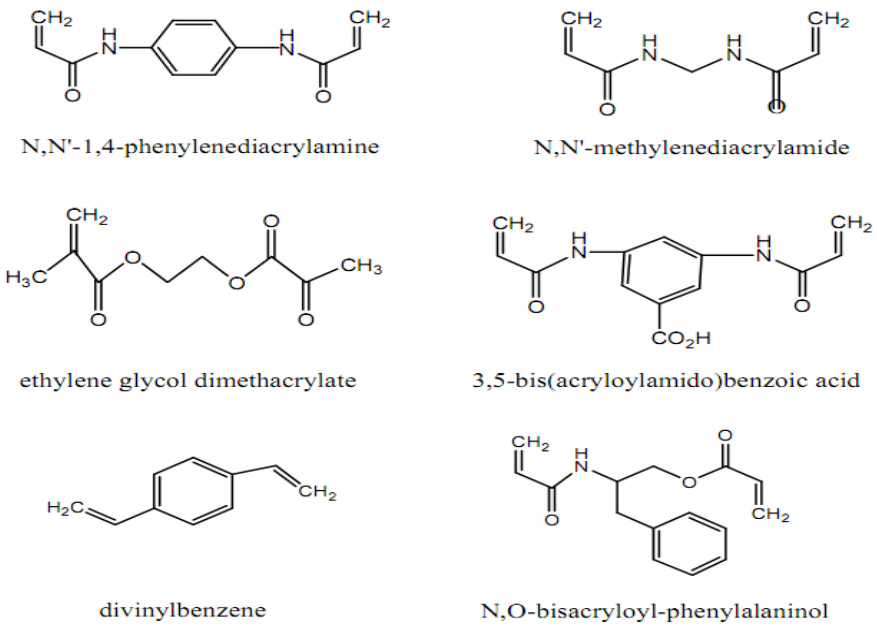


Figure 2.9. 4. Structure of the most common cross-linkers used for molecular imprinting.

2.9.2. Molecular imprinting strategies

Molecular imprinting strategies have been established based on covalent or non-covalent interactions between the template and functional monomers (Wu et al., 2018) (Figure 2.9.5). In both cases, the functional monomers, chosen to allow interactions with the functional groups of the imprinted molecule are polymerized in the presence of the imprinted molecule. The binding sites are formed by covalent or non-covalent interaction between the functional group of the imprint template and the monomer, followed by a crosslinked copolymerization (Yan and Row, 2006).

Covalent approach

The covalent approach was introduced by Wulff and Sarchan which involves the formation of reversible covalent bonds between the template and monomers before polymerization (Martín-Esteban, 2016). In this imprinting strategy, a stable monomer template molecule is formed by utilizing the reversible covalent bonds between the template molecule and the functional monomer which possess functional groups alcohol, diol, aldehyde, ketone, primary amine, or carboxylic acid functional. The covalent bond is then cleaved after polymerization resulting in the removal of the template from the polymer matrix. When the template is reintroduced into the

polymer matrix, again the covalent bond will be formed within the binding sites (Shah et al., 2012; Speltini et al., 2017; Yan and Row, 2006). Moreover, the requirements of covalent imprinting are different than those for non-covalent imprinting, particularly for the ratios of functional monomer, cross-linker and template. However, since the choice of reversible covalent interactions and the number of potential templates are substantially limited, reversible covalent interactions with polymerizable monomers are fewer in number and often require an acid hydrolysis procedure to cleave the covalent bonds between the template and the functional monomer (Shah et al., 2012; Yan and Row, 2006).

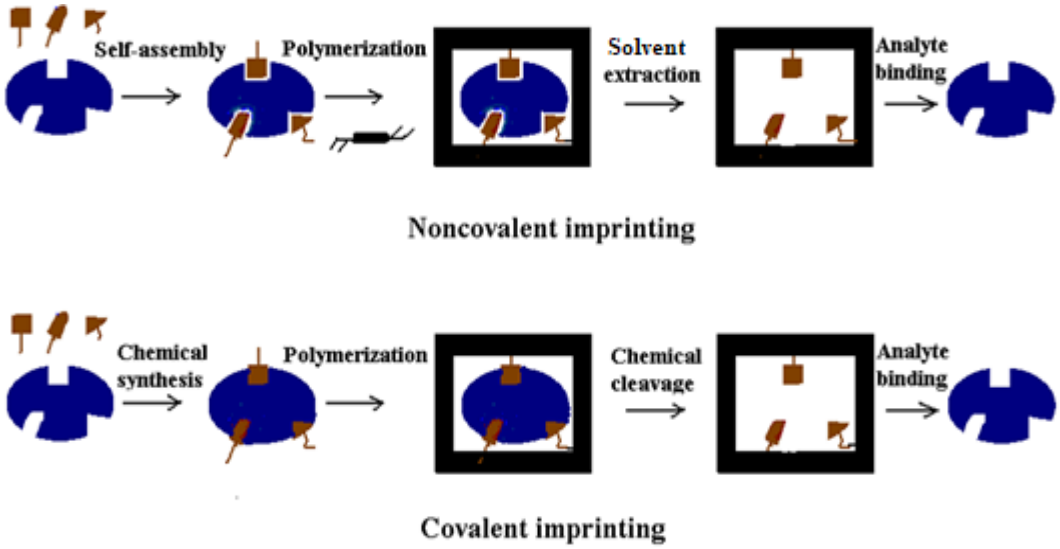


Figure 2.9. 5. Schematic representation of covalent and non-covalent molecular imprinting procedures

Non - covalent approach

The noncovalent approach was introduced by Mosbach and a co-worker and nowadays it is by far the most used for the preparation of MIPs due to its simplicity (Martín-Esteban, 2016; Speltini et al., 2017). In the non-covalent approach, the special binding sites are formed by the self-assembly between the template and monomer, followed by a crosslinked copolymerization. The imprinted molecules interact, during both the imprinting procedure and the rebinding, with the polymer via non-covalent interactions, like ionic, hydrophobic and hydrogen bonding. Knowing the basic optimization of non-covalent methods is important for two reasons: the

methodology is far easier than covalent methods, and it produces higher affinity binding sites that are not in the covalent method (Martín-Esteban, 2016; Shah et al., 2012; Yan and Row, 2006)

2.9.3. Polymerization methods of MIP

The polymerization process in MIP synthesis includes not only the optimal composition of the polymerization mixture but also the selection of the polymerization technique (Adumitrăchioaie, 2018). Molecularly imprinted technique is based on a process where functional monomer and cross-linker are copolymerized in the presence of the template molecules, involving the formation of cavities in which the template is arranged. In the first step, the template interacts with a functional monomer that contains complementary functional groups through hydrogen bonding, reversible covalent bonds, electrostatic interactions and van der Waals forces. In a second step, the monomer-template complex is polymerized in the presence of a large excess of the cross-linking agent. The chemical bonds between the monomer and the cross-linker establish the position of the functional monomer around the template. Finally, after polymerization, the template can be removed from the polymeric structure revealing binding sites with shape, size and chemical functionality complementary to it (Anirudhan et al., 2017; Scorrano et al., 2011).
The polymerization method used for MIP synthesis includes bulk polymerization, suspension polymerization, multi-step swelling polymerization, precipitation polymerization, surface polymerization and in-situ polymerization (Table 2.8.1) (Speltini et al., 2017). The conventional technique is bulk polymerization, the most immediate procedure that requires the final product to be crushed, ground and sieved from a proper solvent before use. These steps are potentially detrimental for the binding sites and lead to partial recovery of useful solid phase and large distribution of the beads particle size, thus limiting the applications to off-line (Speltini et al., 2017; Yan and Row, 2006). The three well-applied polymerization techniques are bulk, precipitation and suspension polymerization. Bulk polymerization results in the total conversion of all the reagents into a solid polymer. The polymer is then dried by evaporating the excess solvent. In both precipitation and suspension polymerization, the excess amount of the porogenic solvent is used in synthesis. Therefore, the two polymerization techniques (precipitation and suspension) lead to the generation of the solid polymer in a large volume of the organic solvent.

In this viewing platform, both precipitation and suspension polymerization methods result in the large generation of liquid waste; hence, the bulk polymerization approach is relatively greener. However, some drawbacks associated with the bulk polymerization results from the need for milling of the polymer that often leads to the formation of irregularly shaped particles and demolishing of the binding sites. Well defined shaped particles or improved shapes are obtained when other polymerization techniques such as precipitation or suspension methods are used (Ncube et al., 2017; Xu et al., 2011).

Table 2.9. 1. Common polymerization methods used for MIP synthesis

Polymerization method	Advantage	Limitation
Bulk polymerization	Polymerization simplicity and universality, No required particular skills or sophisticated instrumentation	Tedious procedures of grinding, sieving, and column packing, Irregular particle in size and shape, low performance.
Suspension polymerization	Spherical particles, Highly reproducible results, Large scale possible	Phase partitioning of complicates system, Water is incompatible with most imprinted procedures, Specialist surfactant polymers required
Multi-step swelling polymerization	Monodisperse beads of controlled diameter, Excellent particle for HPLC	Complicated procedures and reaction conditions, Need for aqueous emulsions,
Precipitation polymerization	Imprinted microspheres, Uniform size and high yields	Large amount of template High dilution factor
Surface polymerization	Monodisperse product, Thin imprinted layers	Complicated system, Time-consuming

In-situ polymerization	One-step, in-situ preparation, Cost-efficient, good porosity	Extensive optimization required for each new template system

2.9.4. MIP Characterization

MIPs are solids so they are selectively characterized by methods that do not require solutions (Spivak, 2005). Infrared analysis is an important chemical characterization method to detect the functional groups present in a compound (Roland and Bhawani, 2016). Moreover, nitrogen sorption porosimetry using BET (Brunauer, Emmett and Teller) analysis is the main technique used to determine specific surface area, specific pore volume, pore size distribution and average pore diameter values of the polymer particles while mercury intrusion porosimetry is sometimes used and is more suitable for larger pores characterization (Vasapollo et al., 2011). Studies have shown that morphology obtained by scanning electron microscopy (SEM) and structural properties of MIPs are important factors that affect the binding behavior of the polymers which can be controlled by manipulating polymerization conditions (Roland and Bhawani, 2016; Sundar et al., 2010).

2.9.5 Application of molecularly imprinted polymer (MIP)

Advantages of MIP such as ease of preparation, stability at the extreme range of pH and temperature and low cost have led to the development of the polymer for different applications (Chen and Li, 2013; Manzo et al., 2015). It is also re-usable in analytical applications which is good in green chemistry (Madikizela et al., 2018c). Such properties of MIPs have made them a highly interesting tool for different application areas, including separation sciences and purification, sensors and biosensors, catalysis and drug delivery (Vasapollo et al., 2011; Yusof et al., 2013). The MIPs have received tremendous importance due to their vast applications in different fields. Some of the most promising areas in which MIPs have potential commercial applications are given in (Shah et al., 2012) (Figure 2.9.6).

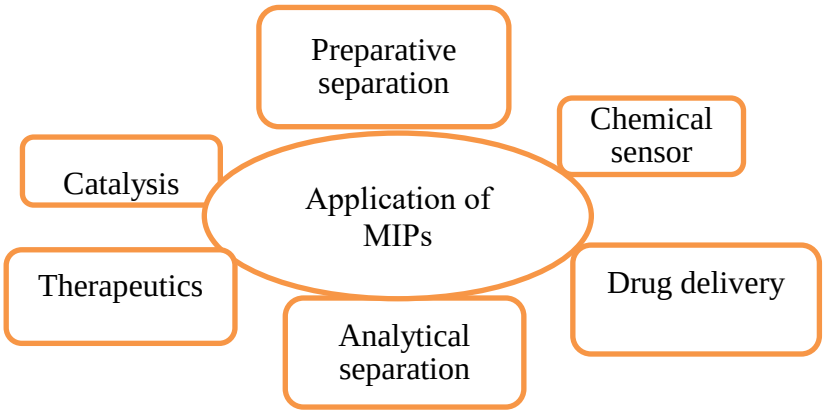


Figure 2.9. 6. Schematic representation of major applications of MIPs in various fields

2.10 Pharmaceutical removal from water

Biologically active chemical pharmaceuticals are widely used to prevent and treat diseases in humans and as veterinary drugs. However, they are regarded as emerging contaminants due to their persistence and potential deleterious effect on the aquatic ecosystem (Tiwari et al., 2017). Nowadays, rigorous research has been carried out to find innovative and cost-effective methods to remove such contaminants. Amongst the adsorbents, bio-materials have received much attention and have recently become an extensive area of interest due to their effectiveness and versatility in the removal of different kinds of pollutants from water (Kebede et al., 2019a). However, the application of these adsorbents is limited due to the high costs associated with their use, while the removal efficiency of others is inadequate. Many studies have focused on developing or finding low-cost sorbents which include peat, bentonite, steel-plant slag, fly ash and maize cob. Yet, these low-cost sorbents generally have low uptake, which means that large amounts of sorbents are needed and none of them has been used for the removal of pharmaceuticals (Kebede et al., 2019b).

Therefore, new, economical, easily available, environmentally friendly and highly effective sorbents are in demand for the removal of pharmaceuticals. Adsorption study for removal of contaminants is a common and simple method. However, the preparation of adsorbents is the key factor, because morphological properties such as particle size and shape, binding surface

area and overall removal capacity depend on it. Simple to prepare, hazard-free and environment-friendly treatments are the major requirements for sustainable preparation of adsorbents for removal of contaminants. Adsorptive removal of pharmaceuticals from water sources has been the subject of research interest over the last two decades. Many studies have assessed the capability of adsorption processes in removing pharmaceuticals from water (Akhtar et al., 2015). *Moringa* has previously been used for water treatment such as flocculation, coagulation, and removal of heavy metals and the seeds of *Moringa* extract, which has coagulation properties similar to those of alum and synthetic cationic polymers (Kebede et al., 2019a; Ndabigengesere et al., 1995).

2.10.1. Adsorption isotherms and kinetics

2.10.1.1. Adsorption isotherms

Adsorption isotherms in adsorption kinetics study are used to determine the mechanism of adsorption which describes how molecules or ions of adsorbate interact with adsorbent surface sites (Battas et al., 2019; Özacar et al., 2008). Adsorption isotherms in adsorption studies are used to ascertain the mechanism of adsorption. It describes the equilibrium at a given temperature between the adsorbed solute concentration and the un-adsorbed concentration (Battas et al., 2019). Two adsorption isotherm models namely, Langmuir and Freundlich are commonly used to evaluate the adsorption mechanism (Ayawei et al., 2017; Battas et al., 2019; Inyinbor et al., 2016)

The Langmuir isotherm which assumes a surface with homogeneous binding sites, equivalent sorption energies, and no interactions between adsorbed species is expressed by the mathematical relation using Eq. (1)

$$\frac{C_e}{qe} = \frac{C_e}{q_{max}} + \frac{1}{q_{max}K_L} \quad (1)$$

where;- C_e is the equilibrium concentration of the target molecule (mg/L), q_e is the amount of the adsorbed molecule at equilibrium (mg/g), q_{max} is the maximum adsorption capacity (mg/g) and K_L is the Langmuir adsorption equilibrium constant calculated from the slopes and intercepts of the linear plot of C_e/q_e versus C_e, K_L is an important tool in the calculation

of the dimensionless equilibrium parameters (R_L) which explains the favorability of adsorption process; R_L is calculated using Eq. (2).

$$R_L = \frac{1}{(1+K_L C_o)} \quad (2)$$

where;- K_L is Langmuir constant (mg/g) and C_o is the initial concentration of adsorbate. R_L values indicate the adsorption to be unfavorable when $R_L > 1$, linear when $R_L = 1$, favorable when $0 < R_L < 1$, and irreversible when $R_L = 0$.

The Freundlich isotherm is an empirical model not limited to monolayer coverage alone but also describes multilayer adsorption. This isotherm gives an expression that defines the surface heterogeneity and the exponential distribution of active sites and their energies. It is expressed mathematically using Eq. (3).

$$\log q_e = \log k_f + \frac{1}{n} \log C_e \quad (3)$$

where: C_e is the equilibrium concentration of the target molecule (mg/L), q_e is the amount of the adsorbed molecule at equilibrium (mg/g), K_f a constant related to the adsorbent capacity and $\frac{1}{n}$ is an empirical parameter related to the adsorption intensity of the adsorbent. Typically, 1/n values range from 1 downwards. A value of $1/n \leq 1$ signifies that the adsorption is favorable. When the value of $1/n > 1$, K_f decreases with concentration which means unfavorable adsorption indicative of saturation of adsorption sites available to the adsorbate, resulting in relatively less adsorption. The Freundlich isotherm constants (n, K_f) are calculated from the slopes and intercepts of the linear plot of $\log q_e$ versus $\log Ce$ (Kuczajowska-Zadrożna et al., 2020). Moreover, the n-value which is greater than 1 and a higher k_f value indicates adsorption is favourable (Madikizela et al., 2018d).

2.10.1.2. Adsorption kinetics

The rate of adsorption and the controlling mechanism of the process can be described by the adsorption kinetics. The kinetic study is important to an adsorption process because it depicts the uptake rate of adsorbate, and controls the residual time of the whole adsorption process

(Demirbas et al., 2009; Mironyuk et al., 2019). The pseudo-first-order rate expression of Lagergren is expressed as follows (Aydin et al., 2008).

The pseudo-first-order kinetic rate equation is expressed as:

$$\log(qe - q_t) = \log qe - \frac{k_1 t}{2.303} \quad (4)$$

The pseudo-second-order kinetic rate equation is expressed as:

$$\frac{t}{q_t} = \frac{1}{k_2 q^2_e} + \frac{1}{q_e} t \quad (5)$$

where, t is the rebinding time (min), q_t (mg/g) is the adsorption capacity of a different time, qe (mg/g) is the equilibrium rebinding capacity, k_1 (min^{-1}) is the first-order rate constant and k_2 is the rate constant of second-order adsorption ($g(mg\ min)^{-1}$). Log ($qe-q_t$) rebinding time t (min) resulting in a straight line from a plot versus rebinding time t (min) resulting in a straight line with a coefficient of determination, R^2 and k_1 were calculated from the slope of the linear plot. According to the pseudo-second-order model, t/q_t was plotted versus t (min) and the coefficient of determination; R^2 and k_2 were calculated from the intercept of the linear plot. The results show that the second-order mechanism is applicable which suggests that chemisorption might be the rate-limiting step that controls the adsorption process (Inyinbor et al., 2016).

The kinetic models (pseudo-first order and pseudo-second-order) were validated for the adsorption and the applicable fit were verified using the sum of squared error (SSE, percentage) given in Eq. (6),

$$SSE = \frac{\sqrt{(qe,exp - qe,cal)^2}}{N} \quad (6)$$

where N is the number of data points used for each model. The validation values are used to compare the models which can fit better with the experimental data. The lower value of SSE, % indicates the best fit of the model.

Chapter 3 - EXPERIMENTAL

3. Experimental

This section of the dissertation presents the experimental design that briefly explains how each experiment was conducted which include, chemicals and reagents used, instruments and equipment and experimental procedures that were clearly stated. This part includes six sub-sections each explaining briefly the experimental procedures undertaken to fulfill the objectives of the study.

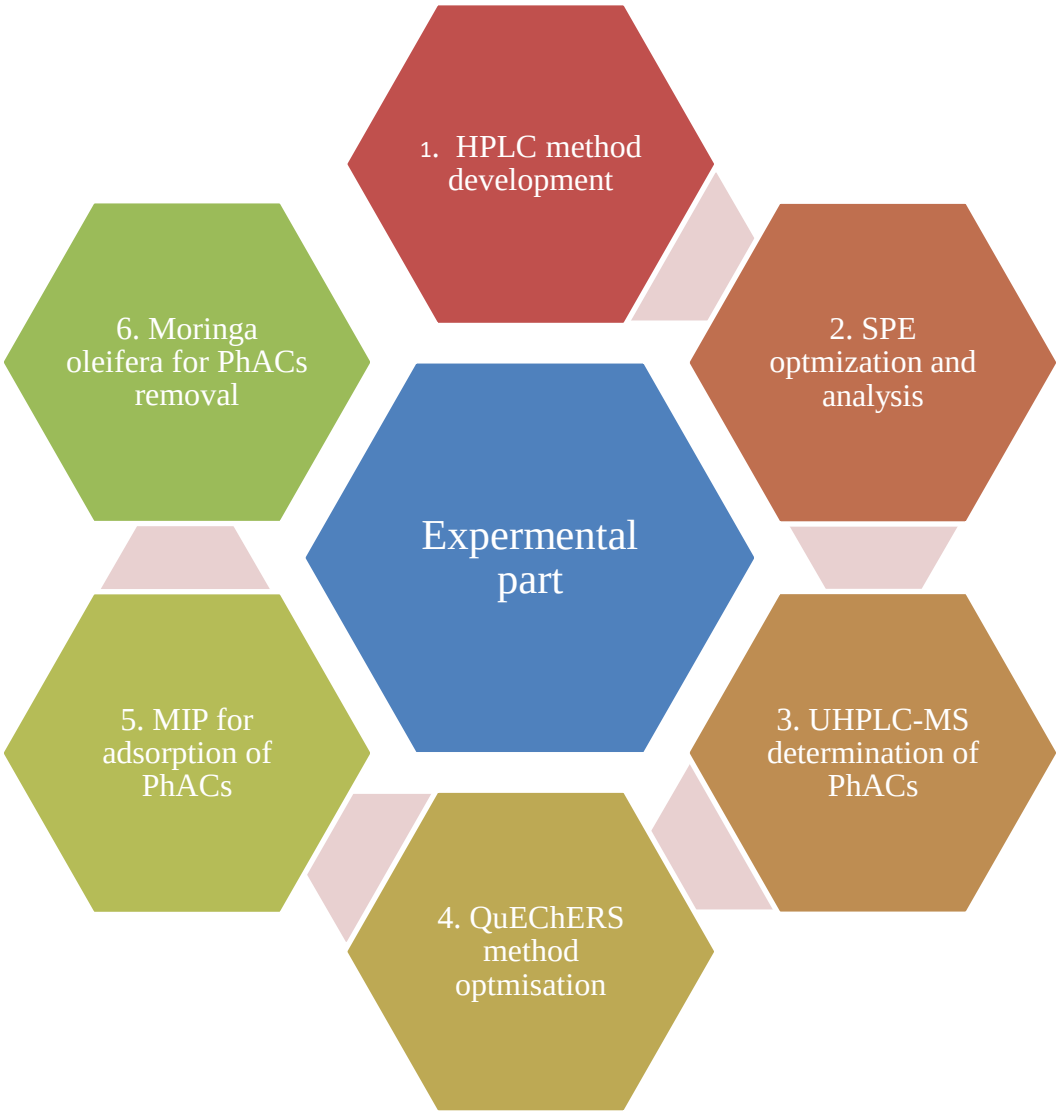


Figure 3. 1. Flow diagram showing the experimental part covered in this study.

3.1. Chemicals, reagents and instrumentation used in this study

3.1.1. Chemicals and reagents

All the chemicals used in this study were analytical and HPLC-grade solvents. Methanol (>99%), acetonitrile (>99.9%) and acetic acid (99%) were from Fisher Scientific (London, UK), formic acid (>96%) from Sigma Aldrich (Darmstadt, Germany) and ultra-pure water was prepared by using Direct-Q 3 UV Millipore system (Massachusset, UK). Disodium ethylene diamine tetraacetic acid (Na_2EDTA) from Sigma Aldrich (Netherlands) was used to study the effect during extraction, Sodium hydroxide pellets were purchased from Associated Chemical Enterprises (Johannesburg, South Africa) and used for pH adjustment. Extraction salts; magnesium sulfate anhydrate obtained from Sigma Aldrich (Gmbh, Japan), sodium chloride from Associated Chemical Enterprise (Johannesburg, South Africa) and ammonium acetate and sodium acetate from Sigma Aldrich (Netherlands) were used. Extraction cleanup, primary secondary amine (PSA) and octadecyl (C_{18}) and diatomaceous earth were obtained from Sigma Aldrich (USA). Nylon syringe filters, 0.22 µm (Agela technology, New York) was used for filtration of the sample extract prior to injection into the HPLC. Dimethyl sulfoxide (DMSO), ethylene glycol dimethyl acrylate (EGDMA) from Sigma Aldrich (Germany) 1, 1-azobis-(cyclohexanecaranitrile) (98%) from Sigma Aldrich (India) and 4-vinyl benzoic acid Sigma Aldrich (Japan) were used. Mettler Toledo, Easy FE 20 (China) was used for pH measurement. Water-soluble protein extraction solvent, petroleum ether (37% w/v) was obtained from an associated chemical enterprise (Johannesburg, South Africa). Precipitating salt ammonium sulfate $(NH_4)_2SO_4$ was from Analytic PLC (Johannesburg, South Africa). Dialysis tubing, high retention seamless cellulose tubing was from Sigma Aldrich (Johannesburg, South Africa). A pharmaceutical-grade standard (MET, AMOX, CHQ, ASA, THP, TMP, CAF, NOR, CIP, DOX, MTZ, CLA and ABZ) (Table 3.1.1) (assigned purity >99 %) was obtained as a gift from Addis Pharmaceuticals (Adigrat, Ethiopia). Venlafaxine (98%), a pharmaceutical compound used as a template were purchased from Sigma-Aldrich (Johannesburg, South Africa). Tablets containing the target active compounds were purchased from the local market Addis Ababa, Ethiopia and Johannesburg, South Africa. The physical-chemical properties of the studied compounds are shown in Table 3.1.1.

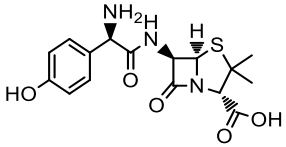
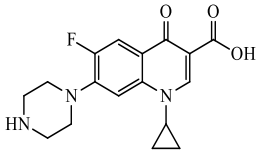
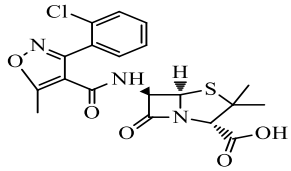
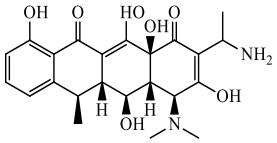
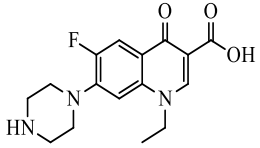
3.1.2. Instrumentation

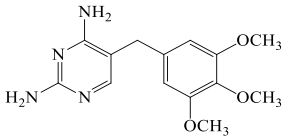
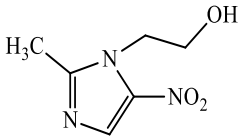
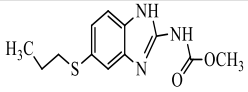
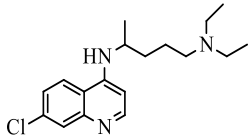
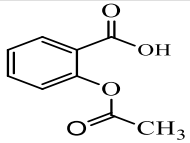
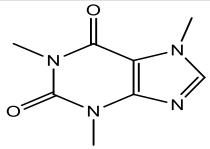
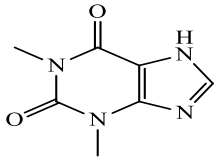
Chromatographic separation of (thirteen PhACs) pharmaceutical compounds, QuEChERS extraction of (five PhACs) and molecularly imprinted polymer adsorption study (four PhACs) were performed on an HPLC model of Agilent 1260 Series equipped with Quaternary Pump, Agilent 1260 Series Vacuum Degasser, Agilent 1260 Series autosampler and Agilent 1260 Series UV detector purchased from Agilent Technologies (Singapore, Germany). Data acquisition and processing were accomplished with LC Chemstation software (Agilent Technologies). Kromasil C_{18}, 5 µm (4.6 mm x 150 mm) column was used for separation (Stockholm, Sweden). Analytical balance (OHAUS, Europe, GmbH Langacher 44.8606, and Switzerland) was used to weigh the standard for solution preparation. Digital ultrasonic cleaner model: PS-10A (China) used for sonication of mobile phase before use. SPE cartridge Supel^{TM} -Select (HLB, MAX and MCX) 500 mg and 12 mL was from Sigma Aldrich (South Africa, Johannesburg). A pH meter (Mettler Toledo, Five Easy FE20, China) was used for adjusting the pH of the sample water before extraction. Syringe filters (PVDF) 0.22 µm (Agilent Technologies, New York) were used for filtration of the standard and extracted sample prior to injection into the HPLC for analysis. In the extraction of pharmaceuticals from the vegetable sample using QuEChERS, centrfiguator, (Hettich Zentrifugen, Rotofix 32A, Germany) and vortex mixer (VELP.SCIENTIFICA, Italy) was used.

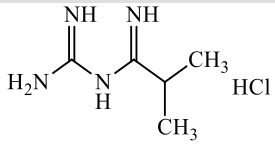
Polymer characterization was done using, FT-IR equipped with attenuated total reflection (Perkin Elmer, Llantrisant, United Kingdom). A scanning electron microscopy (SEM), JOEL model JSM 6700F (Tokyo, Japan) was used to study the polymer morphology. The BET analysis was evaluated using the Flow Prep 060 instrument from Micromeritics (Aachen, Germany). For XRD analysis, the instrument from Bruker AXS (Karlsruhe, Germany) was equipped with an XRD commander for data collection and Eva software for processing.

A Dionex Ultimate 3000 UHPLC-DAD (Thermo Fisher Scientific, Bremen, Germany) instrument interfaced to a Bruker Maxis Impact II electrospray high-resolution time-of-flight mass spectrometer (ESI-TOF-MS) (Bruker Daltonics, Bremen, Germany) with Bruker HyStar acquisition software (rev. 3.2) was used for the determination and removal of ten pharmaceutical compounds.

Table 3.1. 1. Selected pharmaceutical in different therapeutic class analysed in this study

Therapeutic Class	Molecular formula	Molecular weight (g/mol)	Structure of the compound	Wavelength (nm)	pKa	Log P	Water solubility mg/mL	References
Antibiotics								
Amoxicillin	$C_{16}H_{25}N_3O_7S$	365.5		335	3.23	0.75	0.96	(Faleye et al., 2017)
Ciprofloxacin	$C_{17}H_{18}FN_3O_3$	331.3		280	5.9/8.9	0.28	1.01	(Faleye et al., 2017; Vazquez-Roig et al., 2010)
Cloxacillin	$C_{19}H_{19}ClN_3NaO_6S$	435.8		254	2.78	2.48	0.0532	(Drugbank, 2021)
Doxycycline hyclates	$C_{22}H_{24}N_2O_8$	444.4		273	3.27	-0.72	0.63	(Faleye et al., 2017)
Norfloxacin	$C_{16}H_{18}FN_3O_3$	319.3		273	6.2/8.2	-0.1	1,350	(Faleye et al., 2017; Vazquez-Roig et al., 2010)

Trimethoprim	$C_{14}H_{18}N_4O_3$	290.3		271	7.2	0.91	0.4	(Ngumba et al., 2016a)
Metronidazole	$C_6H_9N_3O_3$	171.2		340	15.44	-0.15	5.92	(Faleye et al., 2017)
Anthelminthic								
Albendazole	$C_{12}H_{15}N_3O_2S$	265.3		235	9.51	3.22	0.02	(Faleye et al., 2017)
Anti-malaria								
Chloroquine	$C_{18}H_{26}ClN_3$	319.8		343	10.1	4.63	0.0175	(Drugbank, 2021)
NSAIDs								
Acetyl salicylic acid	$C_8H_8O_4$	180.2		256	-	1.19	3000	(Madikizela et al., 2018b)
Stimulant								
Caffeine	$C_9H_{10}N_4O_2$	194.2		275	10.4	-0.07	1.13	(Bayen et al., 2013b)
Theophylline	$C_7H_8N_4O_2$	180.2		272	8.81	-0.9	22.9	(Drugbank, 2021)

Anti-diabetic II								
Metformin	$C_4H_{12}ClN_5$	129.2		231	12.33	-0.92	1.38	(Drugbank, 2021)

3.2. Development and validation of HPLC method for simultaneous separation of 13 pharmaceuticals

3.2.1. Chromatographic conditions

Chromatographic separations of the thirteen selected pharmaceutical compounds were done through optimization of different factors affecting the character of their peak and the acceptability of the result. Basic factors affecting chromatographic separation such as mobile phase, a separation column, flowrate and absorption wavelength were considered during method development. Different mobile phase compositions and different types of additives were used for the optimization of the mobile phase for good chromatographic separation. The mobile phase of three solvent compositions (A) water with 0.1% formic acid, (B) acetonitrile and (C) methanol in gradient elution was used and the proportion is presented in Table 3.2.1. The mobile phase was prepared daily and degassed by sonication for 20 min prior to use. The analysis was carried out on an Agilent 1260 series HPLC-UV system using Kromasil C18 (150 cm x 4.6 mm) 5 µm analytical column with a detection wavelength of 250 nm. The HPLC-UV system was allowed to warm up for nearly 30 min and the baseline was monitored until it becomes stable before the samples or standards were injected. The injection volume was 10 µL, and the flow rate was maintained at 1 mL/min with a run time of 22 min and the analysis was performed at ambient temperature.

Table 3.2. 1 Mobile phase gradient elution program, A – water with 0.1% formic acid, B – acetonitrile, C – methanol

Time min	**Mobile phase solvent**		
	A [%]	**B [%]**	**C [%]**
0.0	90	1	9
8.0	50	20	30
15	50	20	30
17	30	50	30
20	5	90	5
22	90	1	9

3.2.2. Preparation of standard solutions

Mixed stock solutions of twelve pharmaceutical standards (200 µg/mL) were prepared by dissolving 10 mg with ultra-pure water/methanol (50:50, v/v) in a 50 mL volumetric flask. A stock solution of ciprofloxacin which is not soluble in methanol was dissolved in ultra-pure water (200 µg/mL) and stored in the refrigerator. Series of working standard solutions for method development were daily prepared with a mixture of ultra-pure water/methanol (90:10, v/v).

3.2.3. Preparation of sample solution

For the preparation of sample solution used for method validation, ten tablets of each commercial dosage pharmaceutical were weighed, triturated in a porcelain mortar, mixed and the average weight of the tablet was calculated. Accurately weighed amount of powder equivalent to 10 mg of each tablet was transferred completely to in 50 mL volumetric flask and dissolved with ultra-pure water/methanol (50:50, v/v). Ciprofloxacin powder tablet equivalent to 10 mg was transferred into 50 mL volumetric flask and dissolved with ultra-pure water. All the tablet powders were sonicated for about 30 min in the solvent and then filtered with Whatman filter paper number 1(110 mm) and volume was adjusted and filtered again with a 0.22 µm membrane filter. Mixtures of series of sample solutions were prepared by dissolving with ultra-pure water and methanol (90:10, v/v).

3.2.4. Parameter optimization

Separation in liquid chromatography is highly affected by different factors including mobile phase (both solvent type and composition), the addition of additives, absorption wavelength and mobile phase flow rate. Different solvents and compositions of (water, acetonitrile and methanol) and additives (formic acid and acetic acid) were evaluated for the separation of thirteen pharmaceutical compounds. The mixed standard solution was scanned in the wavelength region of 190–400 nm for wavelength selection for proper separation. The effect of mobile phase flow rate was also investigated using a different flow rate of 0.6, 1 and 1.3 mL/min. The chromatographic parameters were evaluated by taking both the resolution and symmetry of the peak into account.

3.2.5. Validation of the analytical method

The developed method for the determination of selected pharmaceuticals was evaluated as per the ICH Q2 (ICH, 2005) guidelines protocol for, linearity, sensitivity, precision, accuracy, specificity, robustness, and system suitability.

3.3. Optimization and use of solid-phase extraction for the determination of pharmaceutical from water samples

3.3.1. Chromatographic conditions

Chromatographic separations of the thirteen selected pharmaceutical compounds were done using the optimized condition stated in section 3.2.1 and SPE conditions were optimized.

3.3.2. Validation of the analytical method

Mixed stock solutions of selected pharmaceutical standards (200 µg/mL) were prepared by dissolving with ultra-pure water/methanol (50:50, v/v) in a 50 mL volumetric flask. Series of working standard solutions for method development were daily prepared with a mixture of ultra-pure water/methanol (90:10, v/v). The optimized method was evaluated by considering the linearity of standard curves, method detection and quantification limit, relative recovery with spiked river and spiked deionized water and precision of the analytical method (Intra and inter-day) were determined using spiked target compounds.

3.3.3. Water sample collection

The water samples for the application of the method were obtained from Addis Ababa, Ethiopia. Composite 24 h influent and effluent samples were collected from the Akaki sewage treatment plant (ASTP) (8.919839°N 38.75560°E). In addition, grab water samples were collected from Akaki River (8.877650°N, 38.78547°E) which receives the treated effluent, Gefersa dam (9.074092°N, 38.63567°E) and from Saint Paul's Millennium Hospital wastewater (9.047683°N, 38.72810°E) in September 2019 (Figure 3.3.1) Samples for analysis were collected using pre-cleaned amber glass bottles. After collecting the samples were filtered using a 0.45 µm Whatman filter paper and kept in the refrigerator at 4 ^{o}C until analysis with the developed method.

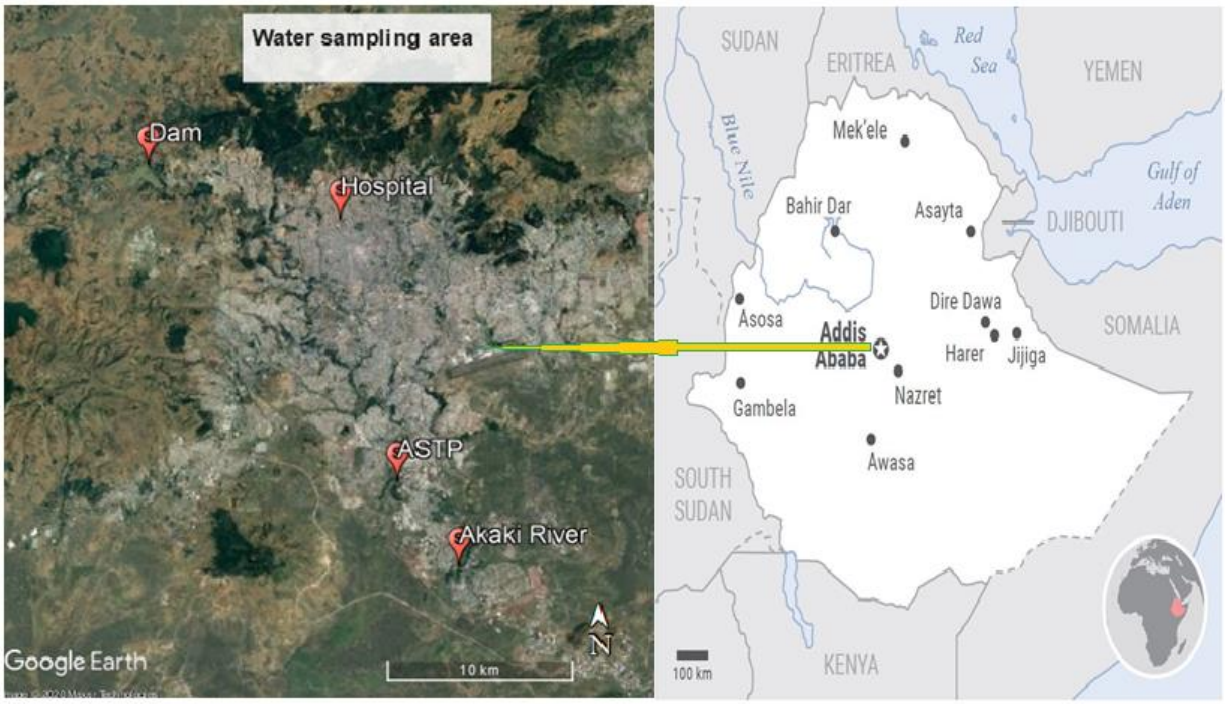


Figure 3.3. 1. Map showing water sampling points

3.3.4. Solid-phase extraction procedure

The solid-phase extraction method was optimized using deionized water spiked with a mixture of a solution of thirteen standard compounds at a concentration of 0.05 µg/mL. Extraction of the water samples was carried out using the optimized conditions, HLB cartridges as a selected sorbent in solid-phase extraction. The cartridge was preconditioned with 6 mL methanol followed by a 6 mL ultrapure water solution, then 150 mL of water sample at pH of 5 containing 0.5% w/v EDTA was loaded using an SPE vacuum manifold. The pH of the spiked sample solution was adjusted using either a solution of 0.01M NaOH or 0.01M HCl. The cartridges after loading were washed with 5 mL ultrapure water and dried for 15 min, and then the analytes were eluted with 6 mL 2% formic acid in water/methanol (20:80, v/v). All eluted extracts were evaporated in a stream of nitrogen near to dryness and reconstituted with 2 mL of water/methanol (90:10, v/v) followed by filtration through a 0.22 µm (PVDF) syringe filter and transferred into a 2 mL amber glass vial for HPLC analysis. A schematic diagram of the proposed SPE procedure is presented in Figure 3.3.2.

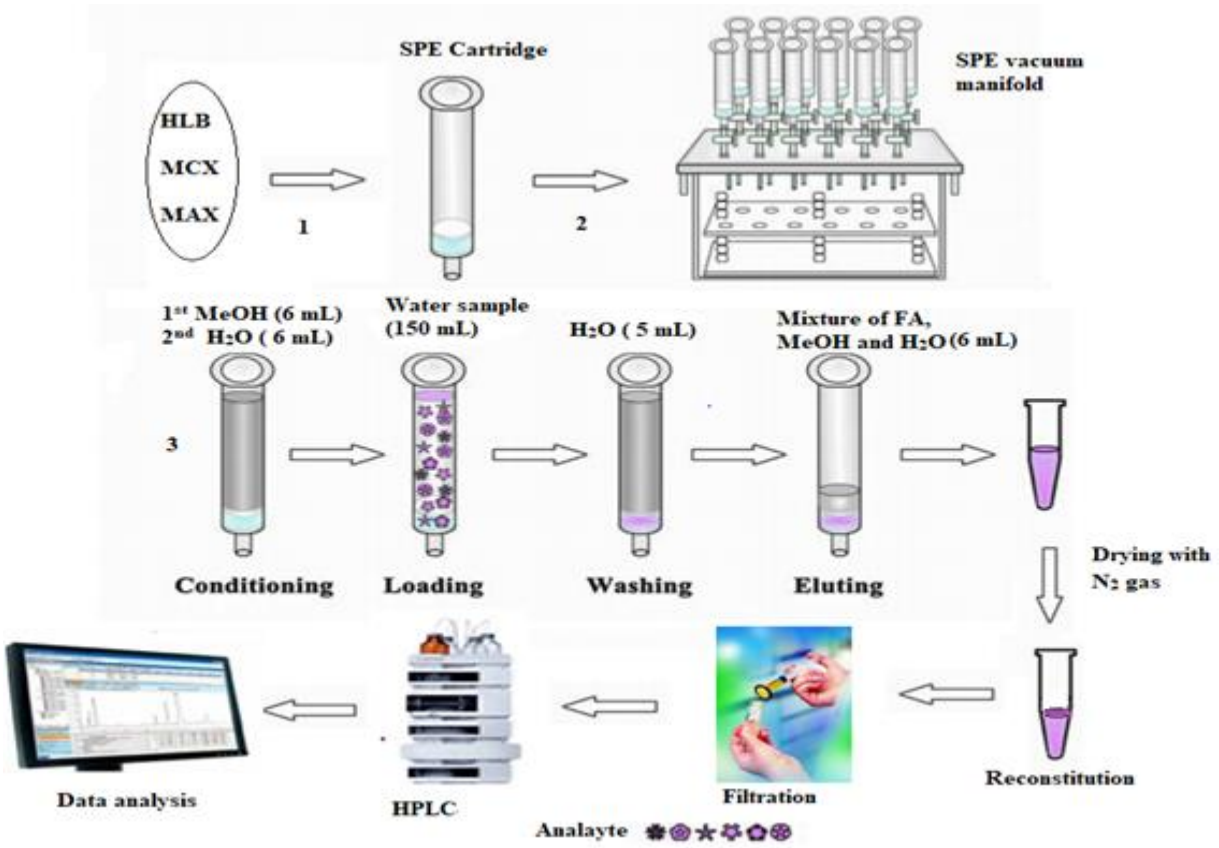


Figure 3.3. 2 Schematic diagram of the proposed solid-phase extraction (SPE) procedure.

3.4. Determination of selected pharmaceutical compounds using UHPLC-MS from different water samples

3.4.1. Chromatographic condition

Chromatographic separations of the ten selected pharmaceutical compounds were done through optimization of different factors affecting the character of their peak and the acceptability of the results. The compound separation was achieved on a UHPLC system equipped with a Kromasil C18, column (4.6 mm x 150 mm, 5 µm). Mobile phase (A) water with 0.1% formic acid and (B) methanol with 0.1% formic acid flow rate 0.8 mL/min. The analysis was carried out using Kromasil C18 (150 cm x 4.6 mm) 5 µm analytical column with a mass spectrometry detector. Different mobile phase compositions and different types of additives were used for the optimization of the mobile phase for good chromatographic separation. Mobile phase of two solvent compositions (A) water with 0.1% formic acid and (B) methanol with 0.1% formic acid in gradient elution (Table 3.4.1) was used. The mobile phase was prepared daily and degassed by sonication for 20 min prior to use. The UHPLC system was allowed to warm up for nearly 30 min and the baseline was monitored until it becomes stable before the samples or standards were injected. The injection volume was 20 µL, and the flow rate was maintained at 0.8 mL/min with a run time of 16 min and the analysis was performed at ambient column temperature.

Table 3.4. 1 Gradient mobile phase program for separation, A - (pure water with 0.1% formic acid, v/v) and B - (methanol with 0.1% formic acid, v/v)

Time (min)	Mobile phase solvent	
	A [%]	B [%]
0.0	95	5
3	70	30
8	50	50
10	50	50
12	20	80
16	95	5

3.4.2. Mass spectrometry conditions

The mass spectrometric analysis was performed in the positive electrospray ionization mode (ESI+) and the spectrometer was operated in the multiple reaction monitoring (MRM) modes. Precursor and product ions and collision energies were optimized for each analyte. Ions were acquired in multiple reactions monitoring (MRM) mode. Precursor ions for each compound were determined by direct infusion of individual compound standards in the standard mode of the spectrometry (at m/z 50–1000). Product scanning was then performed to obtain the product ion, and collision energy (CE) was optimized for each individual analyte. The highest intensity characteristic precursor to product ion MRM transition was used for quantification (quantifier).

3.4.3. Water sampling and treatment

The water samples for analysis were collected from different places in Addis Ababa city, Ethiopia with an expected potentially contaminated source of pharmaceuticals on February 2020 G.C. Composite of 24 h influent and effluent samples were collected from the Akaki sewage treatment plant (ASTP). In addition, grab water samples were collected from rivers, Hospitals and drinking water treatment plants influent and effluent water. Amber reagent bottles (2 L), which had been washed, rinsed with tap water and distilled water were employed for water sample collection. The bottles were rinsed twice with the water sample before the actual water sample was collected. A grab sampling technique was used to collect water samples. Samples were stored at low temperature under ice during transportation to the laboratory and thereafter stored at 4 °C prior to extraction to prevent break down and biodegradation of the compounds of interest. The sampling area information and the geographical map are given in Table 3.4.2 and Figure 3.4.1 respectively. The samples were filtered using 0.45 mm Whatman filter paper and kept refrigerated until analysis with the developed solid-phase extraction and quantification with UHPLC-MS.

Table 3.4. 2 Water sampling point and description for pharmaceuticals analysis

Sites	pH	Elevation (m)	Location		Description of the sample site
S1	7.08	2581	9.076142°N	38.63595°E	Gefersa river
S2	6.87	2579	9.074092°N	38.63567°E	Gefersa water treatment plant influent
S3	9.91	2583	9.064050°N	38.64270°E	Gefersa water treatment effluent
S4	7.78	2407	9.039742°N	38.71769°E	Little Akaki River at Kolfea sub-city
S5	7.86	2500	9.047683°N	38.72810°E	Saint Paulo's Millennium Hospital waste
S6	7.36	2393	9.040397°N	38.77810°E	Keben river before Menilik Hospital waste discharge
S7	7.90	2403	9.039781°N	38.77786°E	Menilik Hospital waste
S8	7.78	2393	9.035047°N	38.77859°E	Kebena River after mixing with Menilik Hospital waste
S9	7.53	2254	8.984500°N	38.70281°E	Little Akaki River at Zenebwork sub-city
S10	9.84	2214	8.964169°N	38.74586°E	Lafto River Lafto sub-city
S11	7.92	2126	8.930022°N	38.74960°E	Little Akaki river after Akaki sewerage treatment plant effluent
S12	8.06	2145	8.920025°N	38.75441°E	Akaki sewerage treatment plant effluent
S13	7.99	2146	8.920261°N	38.75501°E	Akaki sewerage treatment plant after sedimentation
S14	7.94	2555	8.919961°N	38.75532°E	Akaki sewerage treatment plant after filtration
S15	7.93	2565	8.919839°N	38.75560°E	Akaki sewerage treatment plant influent
S16	9.70	2054	8.877650°N	38.78547°E	Great Akaki River at the bridge with human and animal activity

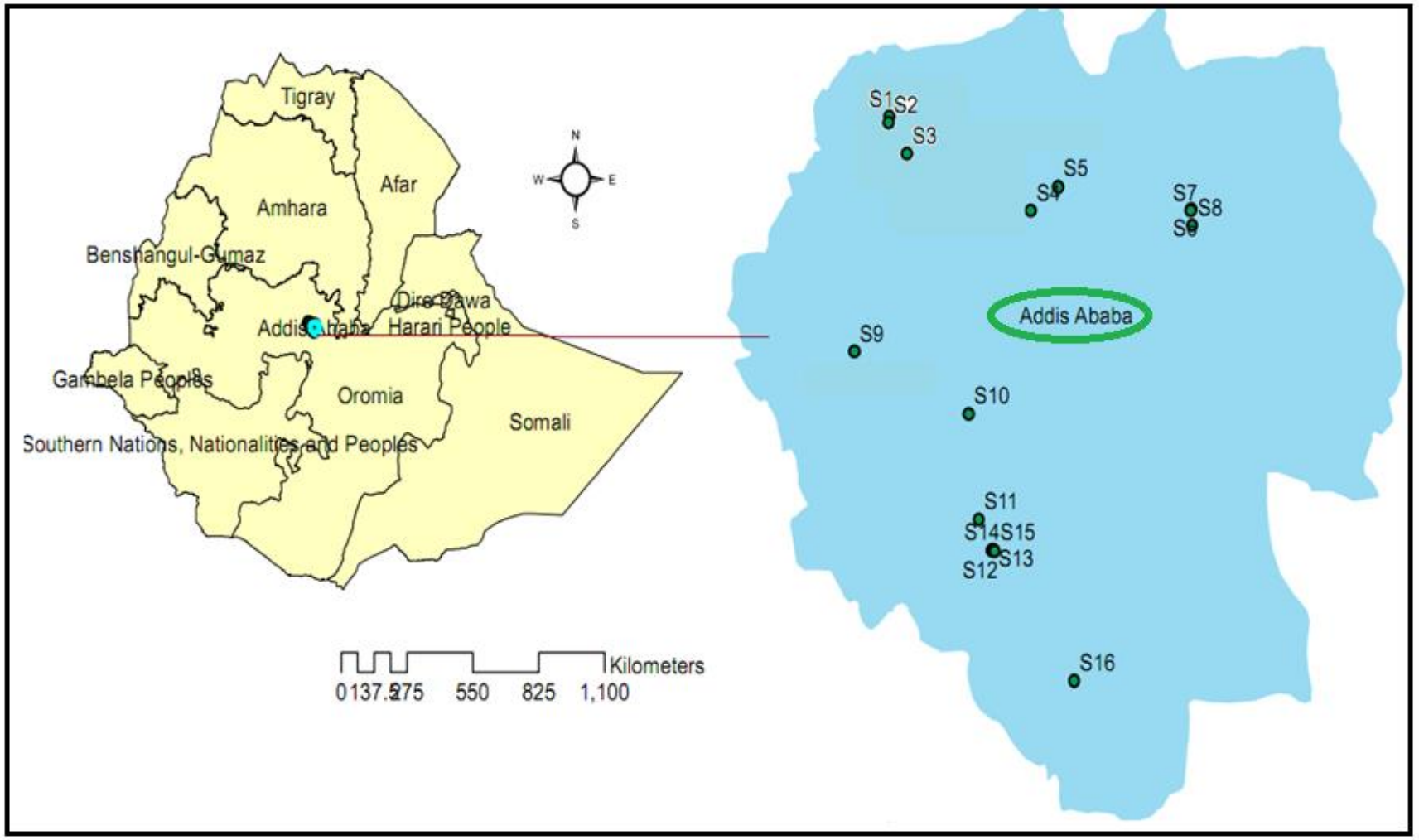


Figure 3.4. 1 Sampling sites of the studied water samples in Addis Ababa.

3.4.4. Water sample extraction procedure

Extraction of water sample was carried out using optimized conditions (section 3.3.5) using HLB cartridge as the selected sorbent in solid-phase extraction. The cartridges were preconditioned with 6 mL methanol followed by 6 mL ultrapure water and then 150 mL of water sample of pH 5 were loaded using an SPE vacuum manifold. The cartridges were washed with 5 mL ultrapure water and dried for 15 min and the analyte was eluted with 6 mL of 2% formic acid in water/methanol (20:80, v/v). All eluted extracts were evaporated in a stream of nitrogen near to dryness and reconstituted with 2 mL of water/methanol (90:10, v/v) followed by filtration through a 0.22 µm syringe filter and transferred into a 2 mL amber glass vial for HPLC analysis.

3.4.5. Method validation

Identification of pharmaceuticals was performed with UHPLC-MS with multiple reaction monitoring (MRM), using the highest characteristic product ion transition. The method validation includes an experimental procedure that tested the calibration, limit of detection (LOD), and quantification (LOQ), recovery and instrumental repeatability. The standard calibration curve was constructed by spiking the water with pharmaceutical standard solutions in the range from 0.5–1000 µg/L (10 points) and the linearity of calibration curves was estimated by fitting a linear mode ($y = a + bx$). The limit of detection (LOD) and limit of quantification (LOQ) were estimated through the calibration curve as $|3.3S|/b$ and $|10S|/b$, respectively, where b is the slope and S the residual standard deviation of the linear function. Recovery experiments were performed on river water spiked with the 10 µg/L target analytes and precision evaluated through the relative standard deviation (RSD). Compounds were identified using the UHPLC retention time a standard and MRM product ion used for quantification.

3.5. QuEChERS method development for the determination of pharmaceuticals from vegetable samples

3.5.1. Vegetable samples and preparation

Vegetable samples (cabbage, carrot and lettuce) for this experiment were collected from Addis Ababa, vegetable market were the vegetable coming from the farm have been sold. Vegetable samples were also collected from irrigated farm using river water from Johannesburg, South Africa. Vegetable samples collected were washed with ultrapure water to remove any suspended wastes on the part and freeze-dried at a temperature of -50 ^{o}C in a freeze drier (LABCONCO). The dried samples then grounded using mortar and pestle to obtain powder and stored in a dry container for extraction and analysis (Figure 3.5.1). Selected pharmaceuticals for analysis of vegetable samples were presented in (Figure 3.5.2).

Figure 3.5. 1 Vegetable sample and dried powder ready for analysis.

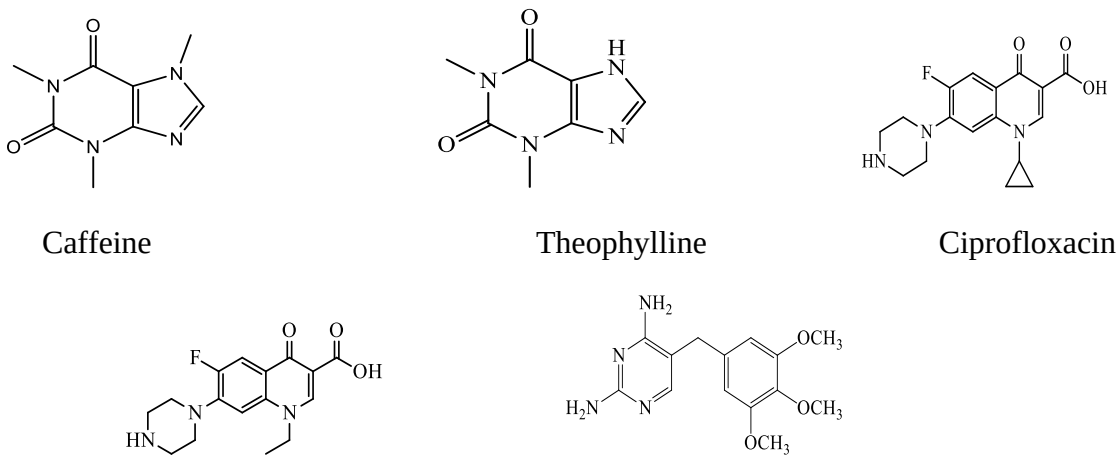


Figure 3.5. 2 Selected pharmaceuticals for QuEChERS method optimization

3.5.2. Chromatographic condition

Chromatographic condition optimization for separations of five selected pharmaceutical compounds used for QuEChERS extraction method development was the first task that was conducted. The mobile phase composition of binary solvent (water with 1% formic acid, v/v and acetonitrile) was obtained as a good solvent for separation of the target compound (Table 3.5.1). The mobile phase was prepared daily and degassed by sonication for 20 min prior to use. The analysis was carried out on an Agilent 1260 series HPLC-DAD system using Kromasil C_{18} (150 cm x 4.6 mm) 5 µm analytical column with a detection wavelength of 270 nm. The HPLC-DAD system was allowed to warm up for nearly 30 min and the baseline was monitored until it becomes stable before the samples or standards were injected. The injection volume was 10 µL, and the flow rate was maintained at 1 mL/min with a run time of 13 min and the analysis was performed at ambient temperature.

Table 3.5. 1 Mobile phase gradient elution program, A – water with 0.1% formic acid, B – acetonitrile

Time in (min)	Mobile phase solvent	
	A [%]	**B [%]**
0.0	95	5
10	80	20
11	50	20
12	20	80
13	95	5

3.5.3. QuEChERS vegetable extraction procedure

A vegetable sample of mass 0.5 g was weighed and transferred to a 50 mL centrifuge tube and 25 µL of the solution of all target compounds in methanol (100 µg/mL) was spiked. Vegetable sample containing the spiked mixture was vortexed for 1 min and placed in a fume hood for methanol to evaporate and then extraction solvent and salt was added (5 mL methanol and 1 mL of (0.135 w/v Na_2 EDTA) (1.5 g $MgSO_4$ and 0.5 g NaCl). The solution mixtures were vortexed for 1 min and kept for 10 min for extraction and centrifuged for 5 min at 4000 rpm. The supernatant solution was transferred to another centrifuge tube containing clean-up sorbent (20 mg C_{18} and 20 mg PSA, 250 mg $MgSO_4$, 50 mg DE) and vortexed for a minute and centrifuged for 5 min and the supernatant was filtered with a syringe filter of 0.22 µm and transferred to HPLC vial for analysis. The schematic diagram of QuEChERS sample extraction is given below in (Figure 3.5.3).

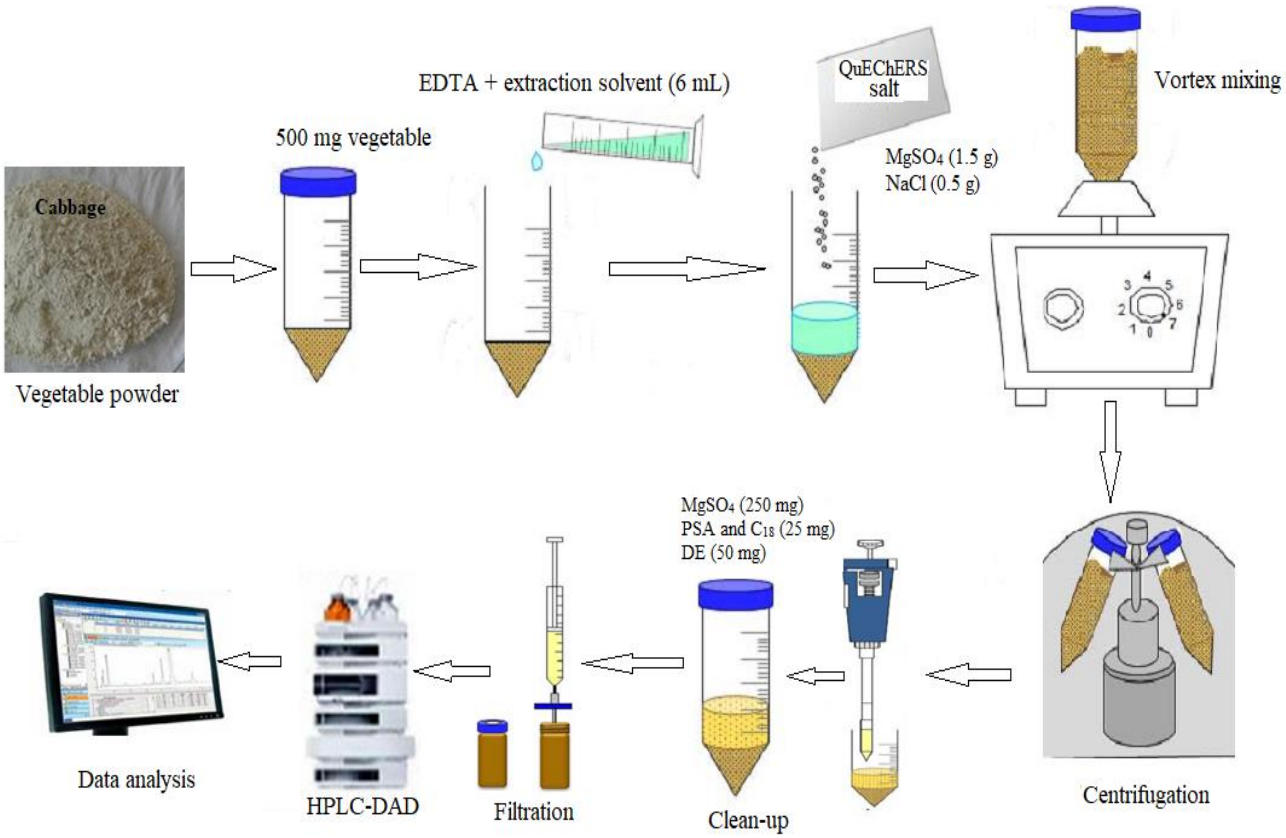


Figure 3.5. 3 Schematic diagram of the proposed QuEChERS extraction procedure.

3.6. Synthesis and characterization of molecularly imprinted polymer for adsorption of selected pharmaceuticals

3.6.1. Chromatographic conditions

Chromatographic condition optimization for separations of four selected pharmaceutical compounds used for adsorption study using molecularly imprinted polymer was the first task that was conducted. The mobile phase composition of binary solvent (water with 1% formic acid, v/v and acetonitrile) was obtained as a good solvent for separation of the target compounds and the (Table 3.6.1). The mobile phase was prepared daily and degassed by sonication for 20 min before use. The analysis was carried out on an Agilent 1260 series HPLC-DAD system using Kromasil C_{18} (150 cm x 4.6 mm) 5 µm analytical column with a detection wavelength of 230 nm for venlafaxine and albendazole and 270 nm norfloxacin and ciprofloxacin (Table 3.6.1). The HPLC-DAD system was allowed to warm up for nearly 30 min and the baseline was monitored until it becomes stable before the samples or standards were injected. The injection volume was 10 µL, and the flow rate was maintained at 1 mL/min with a run time of 18 min and the analysis was performed at ambient temperature.

Table 3.6. 1 Mobile phase gradient elution program, A – water with 0.1% formic acid, B – acetonitrile

Time (min)	**Mobile phase solvent**	
	A [%]	**B [%]**
0.0	95	5
10	80	20
15	50	20
17	80	20
18	95	5

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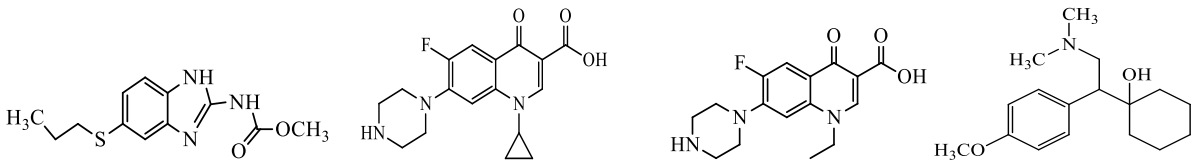


Albendazole Ciprofloxacin Norfloxacin Venlafaxine

Figure 3.6. 1 Selected pharmaceutical for the study of adsorption using molecularly imprinted polymer

3.6.2. Synthesis of molecularly imprinted and non-imprinted polymer

Synthesis and characterization of the polymer for this study were carried out by using the reported protocol (Cormack and Elorza, 2004; Spivak, 2005) and the bulk polymerization method. The synthesis was carried out by dissolving 30 mg of the template venlafaxine (water solubility (1422.2 mg/L) pK_a (9.4), and Log P (0.9)) (Bayen et al., 2013a) in 6 mL of dimethyl sulfoxide (DMSO) at 40 ^oC for 5 min while stirring in a round bottom flask. Function monomer 4-vinyl benzoic acid 60 mg was then added into the mixture while heating for a further 10 min at 40 ^oC. The mixture was then heated to 60 ^oC and the cross-linker ethylene glycol dimethyl acrylate (EGDMA) 300 µL was added. After heating for 10 min, the system was purged with nitrogen gas for 5 min to remove oxygen from the reaction. Initiator 1,1-azobis-(cyclohexanecaranitrile) (98%) 20 mg was then added and the temperature slowly raised to 100 ^oC with a closed reaction system and then polymerization was achieved within 1 h. The molecularly imprinted polymer (MIP) was then transferred to a petri dish and dried in an oven at 70 ^oC for few minutes and transferred into a 50 mL centrifuge vial and 50 mL methanol was added to remove the template to create the cavities. The vial was placed in the Wise Cube shaking incubator, with shaking being done at 150 rpm and temperature maintained at 35 ^oC for 4 h and repeated six times. Then centrifuged for 5 min and the supernatant was analyzed for imprint molecule using HPLC-DAD. This process was repeated until the imprinted molecules were no longer detected. The non-imprinted polymer (NIP) was synthesized and treated likewise but in the absence of a template in the reaction mixture. The chemical structure of the chemical compound used and the schematic diagram for synthesis are given in Figures 3.6.2 and 3.6.3.

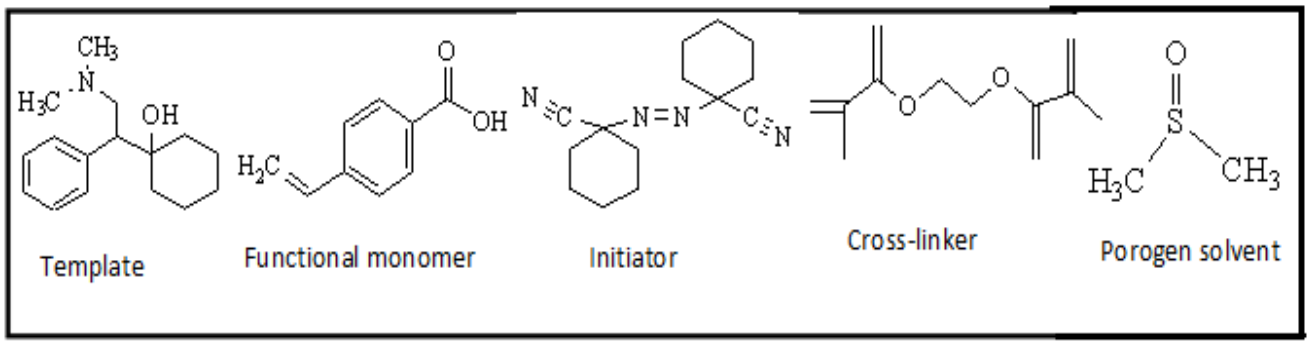


Figure 3.6. 2 Chemical structure of compounds used in molecularly imprinted and non-imprinted polymer synthesis.

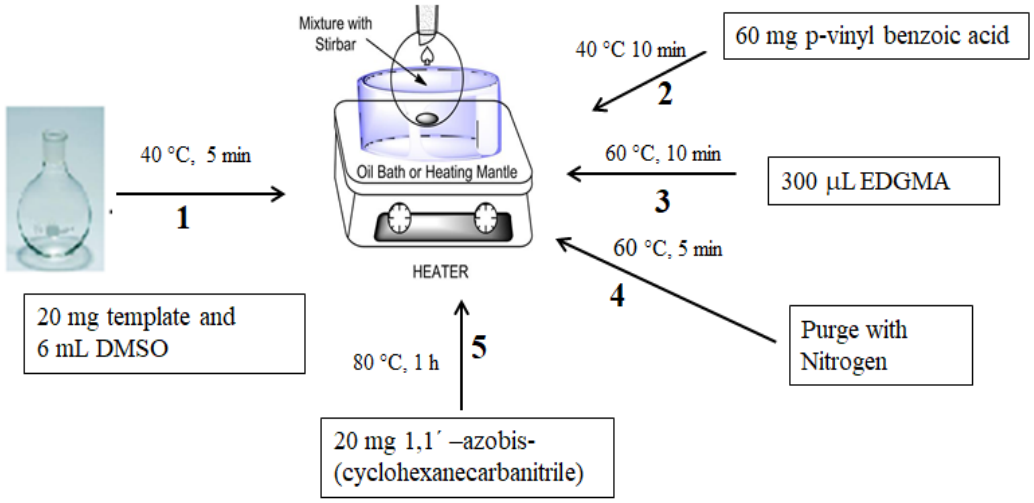


Figure 3.6. 3 The schematic diagram for syntheses of MIP and NIP

3.6.3. Characterization of the polymers

The polymers synthesized were characterized by different techniques to assure the possibility of selective adsorption and extraction of the compounds and stability of the polymer. Therefore, the MIP and NIP characterized by Fourier transform-infrared spectroscopy (FT-IR) equipped with attenuated total reflection (Perkin Elmer, Llantrisant, United Kingdom), thermogravimetric analysis (TGA) were by a ZRY-2P thermal analyzer (Shanghai Balance Instrument Company, China), X-ray diffractions (XRD) from Bruker AXS (Karlsruhe, Germany) were equipped with XRD commander for data collection and Eva software for processing, scanning electron

microscopy (SEM) JOEL model JSM 6700F (Tokyo, Japan) and Brunauer, Emmett and Teller using the Flow Prep 060 instrument from Micromeritics (Aachen, Germany) was used.

3.6.4. Batch adsorption experiments

A batch adsorption experiment on the effect of parameters for adsorption was carried out and the adsorption isotherm and kinetics were evaluated. It was carried out using ultra-pure water that was previously spiked with the selected four pharmaceuticals at room temperature. Parameters affecting the adsorption of the MIP such as polymer amount (10-60 mg), sample pH (3-11), contact time (5-50 min) and initial concentration (2.5-60 mg/L) of target compounds were optimized by varying one parameter at a time while keeping the other constant. To optimize the elution volume, 10 mL of spiked deionized water at1 mg/L concentration at pH of 9 was mixed with 50 mg polymer and stirred for 20 min and the mixture was transferred into l mL empty SPE cartridge with the filter at the bottom. The polymer remained in the cartridge was rinsed with 1 mL water and different solvent (methanol (MeOH), acetonitrile (ACN), water: methanol (H_2O: MeOH) (1:1, v/v), 2% acetic acid in water: methanol (2% HAC: MeOH) (1:1, v/v) and 2% formic acid in water: methanol (2% FA: MeOH) (1:1, v/v) of which 1 mL for elution of the target pharmaceutical was evaluated. After elution, the eluate was filtered with a 0.22 µm syringe filter and injected into HPLC-DAD was used to evaluate the eluting ability of the solvent from the MIP. All experimental parameters affecting the adsorption conducted in triplicate and adsorption efficiency (%) using Eq. (8) and adsorption capacity (mg/g) using Eq. (9) was determined (Hashemzadeh et al., 2018; Madikizela et al., 2018d).

$$\text{Adsorption efficiency (\%)} = \frac{(C_0 - C_e)}{C_o} \text{ x } 100 \quad (8)$$

$$\text{Adsorption capacity (mg/g)} = \frac{(C_0 - C_e)V}{W} \quad (9)$$

Where C_o represents the initial concentration (mg/L) before the adsorption and C_e the final concentration (mg/L) of the target compound remaining in solution after adsorption, V is the volume of the solution in liters and W represents the mass of the polymer in grams.

3.6.5. Cross-selectivity study

The cross-selectivity experiments of the polymer adsorbent were conducted using selected four pharmaceutical compounds targeted for this study. Deionized water spiked with 1 mg/L of the

mixture at a pH of 9 in 10 mL was mixed with 50 mg polymer and stirred for 20 min at room temperature. The selectivity of the MIP towards the selected target pharmaceuticals was evaluated using the imprinting factor (α) and calculated from Eq. (10) and partition coefficient (K_D) using Eq. (11) (Barros et al., 2016; Madikizela and Chimuka, 2016)

$$\alpha = \frac{K_{D(MIP)}}{K_{D}(NIP)} \quad (10)$$

$$K_D = \frac{C_P}{C_S} \quad (11)$$

where: C_P and C_s are the concentration of the pharmaceutical (mg/L) in polymer and solution, respectively, at adsorption equilibrium.

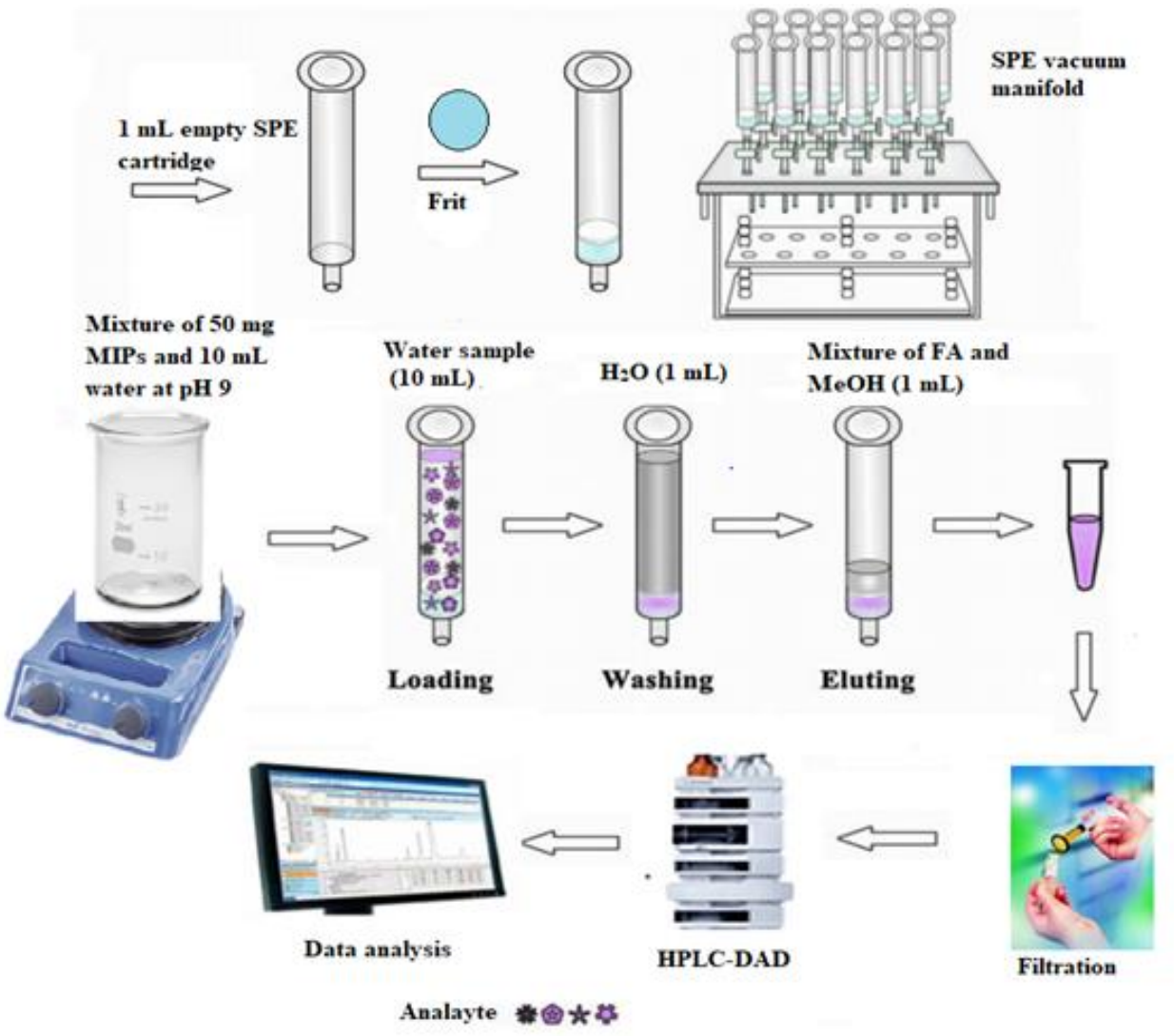


Figure 3.6. 4 The schematic diagram for adsorption and extraction of pharmaceuticals using MIP and NIP as sorbent.

3.6.6. Regeneration and re-use of the MIP

This study was conducted using an amount of 50 mg MIP was added into 10 mL deionized water spiked with 10 mg/L mixture of all selected pharmaceuticals and after 20 min contact time the mixture was centrifuged to remove the MIP. The supernatant was removed and analyzed using HPLC-DAD and the recovered MIP was cleaned with desorption solvent several times followed by methanol, air-dried and used in the next cycle for adsorption study.

3.6.7. Adsorption of the target analyte from contaminated water

River water for application of extraction of selected pharmaceuticals in the polymer using the optimized method was collected from Akaki River (8.877522°N and 38.78547°E) from Addis Ababa, Ethiopia. The water sample was filtered through a 0.45 mm filter paper before using it for application. The water was spiked with 1 mg/L of the target pharmaceuticals to a final volume of 10 mL and a pH of 9 was adjusted. Spiked 10 mL water sample mixed with 50 mg of MIP and stirred for 20 min. After 20 min the mixture was centrifuged for 5 min at 4000 rpm and 2 mL of the supernatant was taken and analyzed in HPLC-DAD. Using the peak area of the analyte, the adsorption efficiency and the recovery were calculated using imprinted polymer.

3.7. Multi-class pharmaceutical removal from water using *Moringa oleifera* seed cake, husk and extracted water-soluble protein

3.7.1. Preparation of seed husk, seed cake and extraction of water-soluble protein from *Moringa oleifera* seeds

The *Moringa oleifera* seeds were collected from Limpopo South Africa washed with distilled water to get rid of dust and soluble contaminants suspended on it, air-dried and divided into two groups one for seed cake and the other for husk and water-soluble protein. Preparation of seed cake was done by squeezing the seed (Figure 3.7.1 (a)) as it is to remove the oil and everything in the seed forms seed cake (Figure 3.7.2 (b)). Further, the seed cake was washed with hexane to remove the oil remaining and dried. The seed cake was prepared in powder form and allowed to pass through a 300 µm size sieve and stored in dry contained for adsorption study.

The other group of the seeds was deshelled and the husk and the seed were separated (Figure 3.7.1 (b) and (c)). After separating, the seed husk was then ground to powder using a domestic blender and then passed through the sieve to obtain a particle size of 300 µm. This was done to obtain a smaller particle size that offers higher sorption rates as the surface area increases. On the other hand, for extraction of water-soluble protein, husk from the seeds were removed and washed with pure water, followed by drying for 24 h in an oven at 80 °C temperature. The dried seeds were then ground to powder using a domestic blender and sieved to get uniform-sized particles. Extraction of water-soluble proteins from the *Moringa oleifera* seeds was adopted from (Ndabigengesere and Narasiah, 1998; Ndabigengesere et al., 1995) with some modification. The powder was mixed with petroleum ether and stirred for 30 min to extract dissolved fats, waxes and oil and then filtered through a Whatman paper no. 3 to separate. The residue was dissolved three times with the extraction solvent to make sure that the yellowish extract is no longer in the seed powder. The residue remains in the filter paper dissolved in ultra-high purity water and stirred for 30 min to extract the water-soluble protein. The solution was then filtered to separate water-soluble protein from any water-insoluble substances. The filtrate was then mixed with ammonium sulfate ($(NH_4)_2SO_4$) to induce the precipitation of proteins. This process was followed by centrifugation at 4000 rpm for 10 min, re-dissolved the supernatant with $(NH_4)_2SO_4$ until no precipitate was observed. The precipitate was dissolved in pure water and filtered to

remove any insoluble substances. The filtrate was dialyzed through a cellulose membrane to purify the protein and then freeze-dried and the powder protein was stored in a dry container at room temperature.

Moringa oleifira (a) seed (b) deshelld seed (c) husk

Figure 3.7. 1 *Moringa oleifera* seed and seed part for preparation of the adsorbent for removal.

(a) Water-soluble protein (b) Seed cake (c) Husk powder

Figure 3.7. 2 *Moringa oleifera* seed adsorbent prepared for removal of pharmaceuticals.

3.7.2. Characterization of the adsorbents

Adsorbents from *Moringa oleifera* seed were characterized by different techniques to assure the possibility of selective adsorption of the pharmaceutical compounds. Therefore, the adsorbent

characterized by Fourier transform-infrared spectroscopy (FT-IR) equipped with attenuated total reflection (Perkin Elmer, Llantrisant, United Kingdom) and scanning electron microscopy (SEM) JOEL model JSM 6700F (Tokyo, Japan).

3.7.3. Determination of pH of point zero charge (pH_{pzc})

The point of zero charge (pH_{PZC}) of the adsorbents was determined by adopting the solid addition method described by (Mall et al., 2006). To a series of 100 mL conical flasks, 30 mL of KNO_3 solution of known strength (0.1 and 0.01 M) was transferred. The pH initial (pH_i) values of the solution were roughly adjusted from 2 to 12 by adding either 0.01 M HNO_3 or NaOH. The pH_i of the solutions was then accurately noted, and 0.04 g of adsorbent was added to each flask, which was securely capped immediately. Equilibration was carried out by shaking for 48 hours using a shaker at the speed of 150 rpm at room temperature. Ultimately the dispersions were filtered and the final pH (pH_f) of the solution was recorded. The difference between the initial and final pH values ($\Delta pH = pH_i - pH_f$) was plotted against the pHi. The point of intersection of the resulting curve at which ΔpH is 0 gave the pH_{PZC}.

3.7.4. Bach adsorption studies

Batch adsorption experiments on pharmaceutical removal efficiency of *Moringa oleifera* seeds prepared adsorbents were carried out using a series of 100 mL Erlenmeyer flasks. The experiment was done using ultra-pure water that was previously spiked with the pharmaceuticals. Adsorbent mass of 40 mg was mixed with 30 mL pharmaceutical solution and then it was agitated at a constant speed of 250 rpm for 80 min in a shaker (Figure 3.7.3). Parameters affecting the removal of pharmaceuticals on the proposed adsorbent such as sample pH (3-11), contact time (10-200 min) and initial concentration (0.5-10 mg/L) of target compounds were optimized by varying one parameter at a time while keeping the other constant. After adsorption, the solution was filtered with a 0.22 µm syringe filter and injected into the UHPLC-DAD was to evaluate the removal efficiency of the adsorbent.

$$\text{Removal (\%)} = \frac{(C_0 - C_e)}{C_o} \times 100 \quad (12)$$

$$\text{Adsorption capacity (mg/g)} = \frac{(C_0 - C_e)V}{W} \quad (13)$$

where C_o represents the initial concentration (mg/L) for pharmaceuticals before adsorption and C_e the final concentration (mg/L) remaining in solution after adsorption, V is the volume of the solution in liters and W represents the mass of the adsorbent (*Moringa oleifera*) in grams.

Figure 3.7. 3 Bach adsorption study set up for the removal of pharmaceuticals

Chapter 4 - RESULTS AND DISCUSSION

4. Results and Discussion

In this part of the thesis, detailed method development results achieved and recorded in each experimental part is discussed. The results in each experimental setup are interpreted with the possible reasons extracted based on nature and how they are behaving. Moreover, the detected compounds among the studied pharmaceuticals, the concentration level at their sampling point have been discussed. The possible removal efficiency of adsorbents was assessed and investigated for selected pharmaceuticals.

4.1. Development and validation of HPLC method for simultaneous separation of 13 pharmaceutical compounds

4.1.1. Optimization of chromatographic conditions

Chromatographic separations for thirteen selected pharmaceutical compounds were carried out through the optimization of different factors affecting the character of their peak and the acceptability of the result. Parameters optimized include, a different type of column, mobile phase (solvent type and composition), different additives and amount in the mobile phase, also different wavelength and mobile phase flow rates were evaluated. The evaluation of different chromatographic conditions was depending on the capability of getting acceptable peak characteristics such as peak shape, symmetry and resolution.

4.1.1.1. Column selection for separation

A column in chromatographic separation is the key to the result intended. For the separation and optimization of the peak shape and separation of selected thirteen pharmaceutical compounds, different types of analytical columns were evaluated for their performance (Figure 4.1.1). The columns considered in this study, were the same but differed with some modification and their length. Luna C_{18}, (100 cm x 4.6 mm) 5 µm, Water Spherisorb phenyl (250 cm x 4.6 mm) 5 µm and Kromasil C_{18} (150 cm x 4.6 mm) 5µm. Luna C18 is modified with (TMS) trimethylsilane, Water Spherisorb phenyl contains a non-end capped silica-based *Phenyl* phase. Chromatographic separation is based on the interaction of the analyte of interest to the stationary and mobile phase, the property of the stationary phase affects the separation and the peak shape. From the three

columns used for separation of the mixture of pharmaceutical compounds, Kromasil C_{18} 150 x 4.6 mm, 5 µm was capable of separating all with good resolution and peak shape than the other which could not separate the entire mixture with analytes of differing polarities and properties (Table 4.1.1 and Figure 4.1.2).

Figure 4.1. 1. Type of chromatographic column used in this study for separation (1- Water Spherisorb phenyl; 2- Kromasil C18; 3- Luna C_{18}).

Table 4.1. 1. Pharmaceutical compound separated with different column

Column	**Pharmaceutical compounds separated**												
Luna C18	MET	AMOX	CHQ	TRM,	THP	CAF	ASA	MET	ALB	CLA			
Water spherisorb phenyl column	ASA	AMOX	THP	CAF	CLA	TRM	MET						
Kromasil C18	MET	AMOX	CHQ	THP	TMP	CAF	NOR	CIP	ASA	DOXH	MTZ	ABZ	CLA

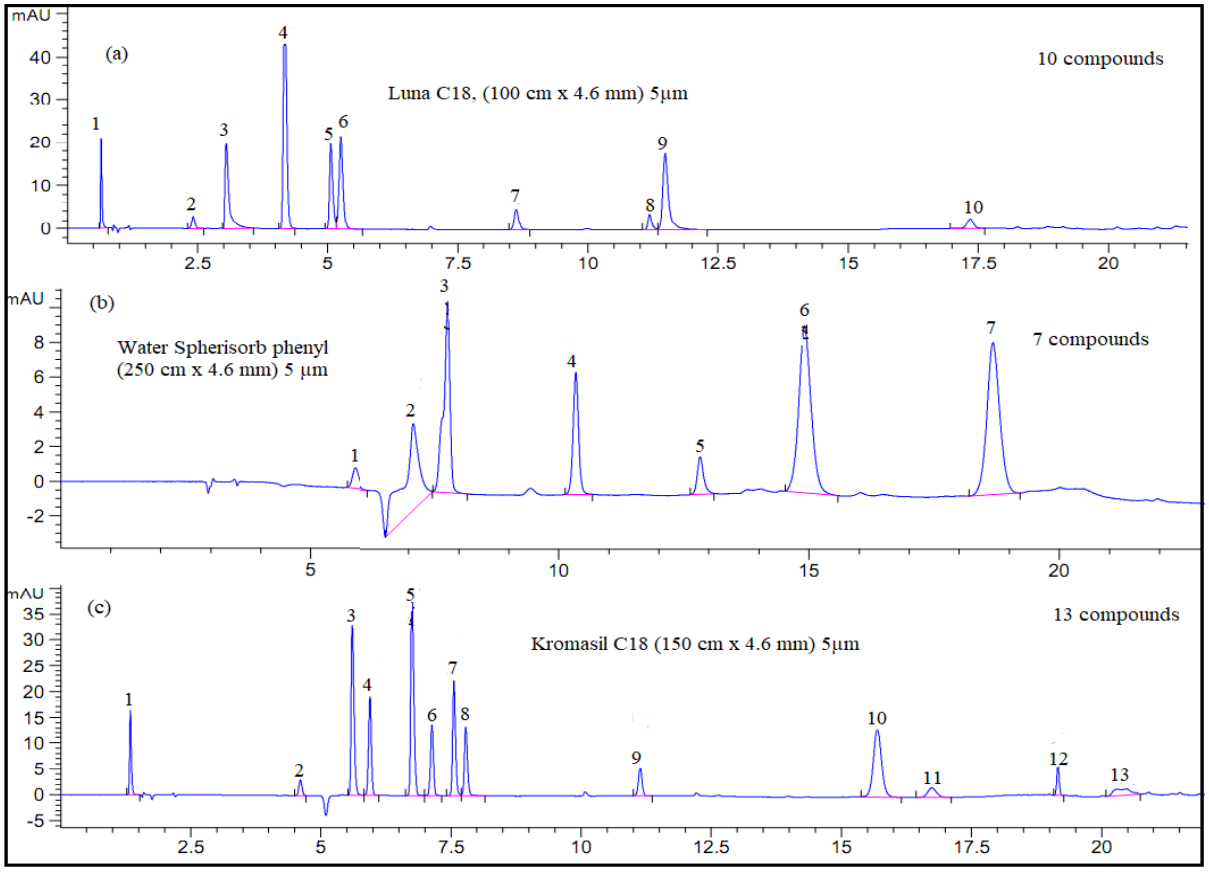


Figure 4.1. 2. Chromatogram of the compound using different analytical column (1-MET; 2-AMOX; 3-CHQ; 4-THP; 5-TRM; 6-CAF; 7-NOR; 8-CIP; 9-ASA; 10-DOXH; 11-MTZ; 12-ALB and 13-CLA).

4.1.1.2. Mobile phase solvent and additive selection

Separation in chromatographic techniques is highly affected by the mobile phase both solvent type and composition within the run time. In the complete separation of selected thirteen pharmaceutical compounds, different types of solvents (water, acetonitrile and methanol), composition (water with methanol, water with acetonitrile and all three) and additives (formic acid and acetic acid) were evaluated. The binary mobile phase composition of water with acetonitrile and methanol was evaluated and found not to be satisfactory separation. The combination of three solvents as a mobile phase for separation was better than the binary combination. An additive in the mobile phase enhances the resolution of the peaks and avoids peak tailing and fronting. Additives in this study added both in aqueous and organic components

of the mobile phase and their separation potential were evaluated however additive addition only in water is selective in its resolution. The addition of acetic acid resulted in a slight improvement in the resolution and separation of analyte peaks than the one without additives. Moreover, the addition of formic acid relatively shows better resolution of the pharmaceuticals in the mixture than acetic acid as reported in the chromatogram (Figure 4.1.3). The addition of a small amount of formic acid led to sharper peaks, better selectivity and sensitivity (Tan et al., 2015). Therefore, from the examined, mobile phase composition and additives, formic acid in water together with, acetonitrile and methanol gives better resolution and short run times. The amount in percent of formic acid added into the water was also examined and 0.1% formic acid shows better performance in the separation with good resolution relatively. The composition of the solvent is monitored through the resolution and symmetry of the peak. Reasonable separation with sharp peaks was obtained with a mobile phase composed of (0.1% formic acid with water, acetonitrile and methanol) programmed for 22 min. Gradient elution in which a technique of altering the composition of the mobile phase during the chromatographic run was presented in the table below (Table 4.1.2).

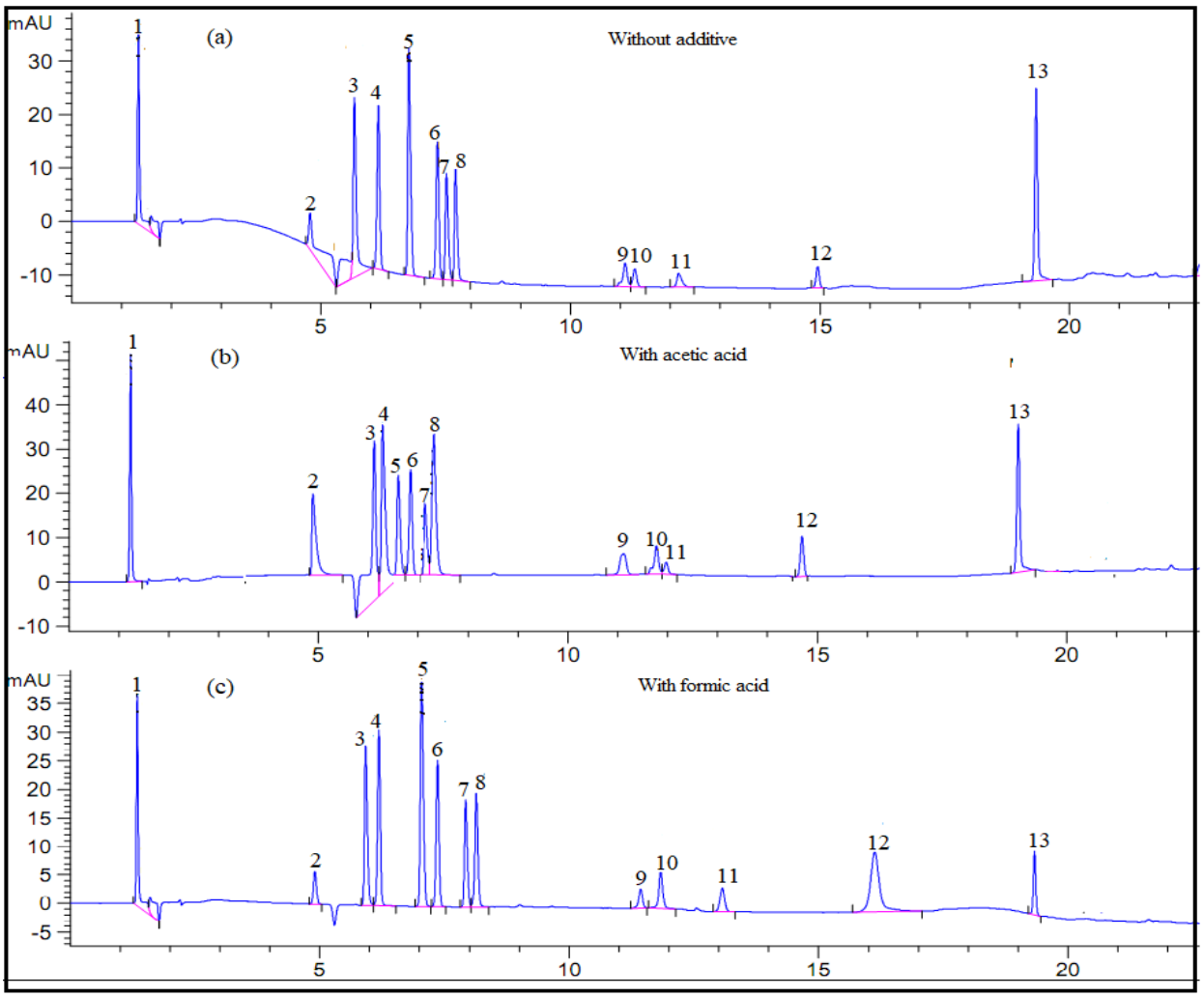


Figure 4.1. 3. Chromatograms obtained using (a) without additive, (b) acetic acid as the additive and (c) formic acid as the additive. (1-MET; 2-AMOX; 3-CHQ; 4-THP,5-TRM; 6-CAF; 7-NOR; 8-CIP; 9-ASA; 10-DOXH; 11-MTZ; 12-ALB and 13-CLA).

Table 4.1. 2. Mobile phase gradient elution program, A – water with 0.1% formic acid, B – acetonitrile, C – Methanol

Time minutes	Mobile phase solvent		
	A [%]	B [%]	C [%]
0.0	90	1	9
8.0	50	20	30
15	50	20	30
17	30	50	30
20	5	90	5
22	90	1	9

4.1.1.3. Wavelength selection

The optimum wavelength should be the one that shows good absorbance for all the selected compounds. The mixed standard solution was scanned in the wavelength region of 190–400 nm. It is evident from Figure 1 that 250 nm yielded the largest overall relative peak height for all the analytes compared to those obtained at 240 nm, 270 or 340 nm. It should be noted that (Table 3.1.1) shows that three compounds (amoxicillin, metronidazole and chloroquine) have a maximum UV absorption at 340 nm or close to 340 nm. However, Figure 4.1.4 shows that only chloroquine had a good absorbance at 340 nm while amoxicillin and metronidazole do not had good absorbance at 340 nm. This is because the absorbance of a compound depends on the type of solvent, concentration and molar absorptivity. This indicates that amoxicillin and metronidazole have smaller molar absorptivity in the solvent used. No absorption of amoxicillin and metronidazole at 340 nm may also be due to the effect of the other compounds present in the mixture. Therefore, 250 nm was selected as an optimum wavelength at which all the thirteen compounds showed good absorbance. It should also be noted that using a wavelength of 250 nm (the selected wavelength) would certainly diminish the sensitivity of the detector towards analytes with UV max above 300 nm. Although this would not matter for pharmaceutical products containing high concentrations of the active ingredient, since the analytes are easily

detected, small differences in concentrations would not be picked up for these compounds.

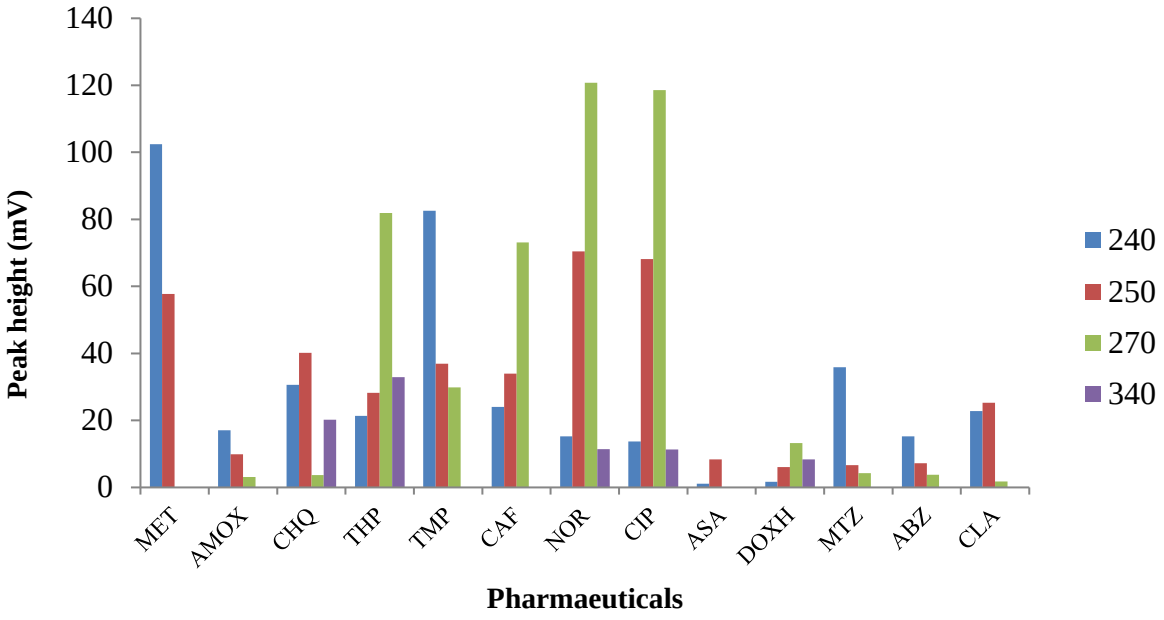


Figure 4.1. 4. Selection of common wavelength on the absorption of selected pharmaceutical compounds

4.1.1.4. Mobile phase flow rate optimization

The interaction of the compound with the retention and the mobile phase affects widely the resolution of the peaks in addition to the effect of the flow rate of the mobile phase. It was observed that the increase in flow rate (1.3 mL/min) decreases the retention time of all analyte compounds but it adversely affected the resolution of some compounds. It is because there is insufficient time for the analyte to interact with the stationary phase. While decreasing the flow rate (0.6 mL/min) increases the retention time, the run time and leads to the broadening of peaks and ends with poor resolution compared to 1.0 mL/min which was selected as an optimum flow rate (Figure 4.1.5). Having the above result flow rate of 1.0 mL/min was selected and used for validation of the method. In this study the first peak was controlled to make sure that the pharmaceutical elute in the retention time by avoiding contamination with frequent injection. The complete chromatogram of selected pharmaceutical compounds is presented in (Figure 4.1.6) and parameters obtained as optimum conditions are in (Table 4.1.3) respectively.

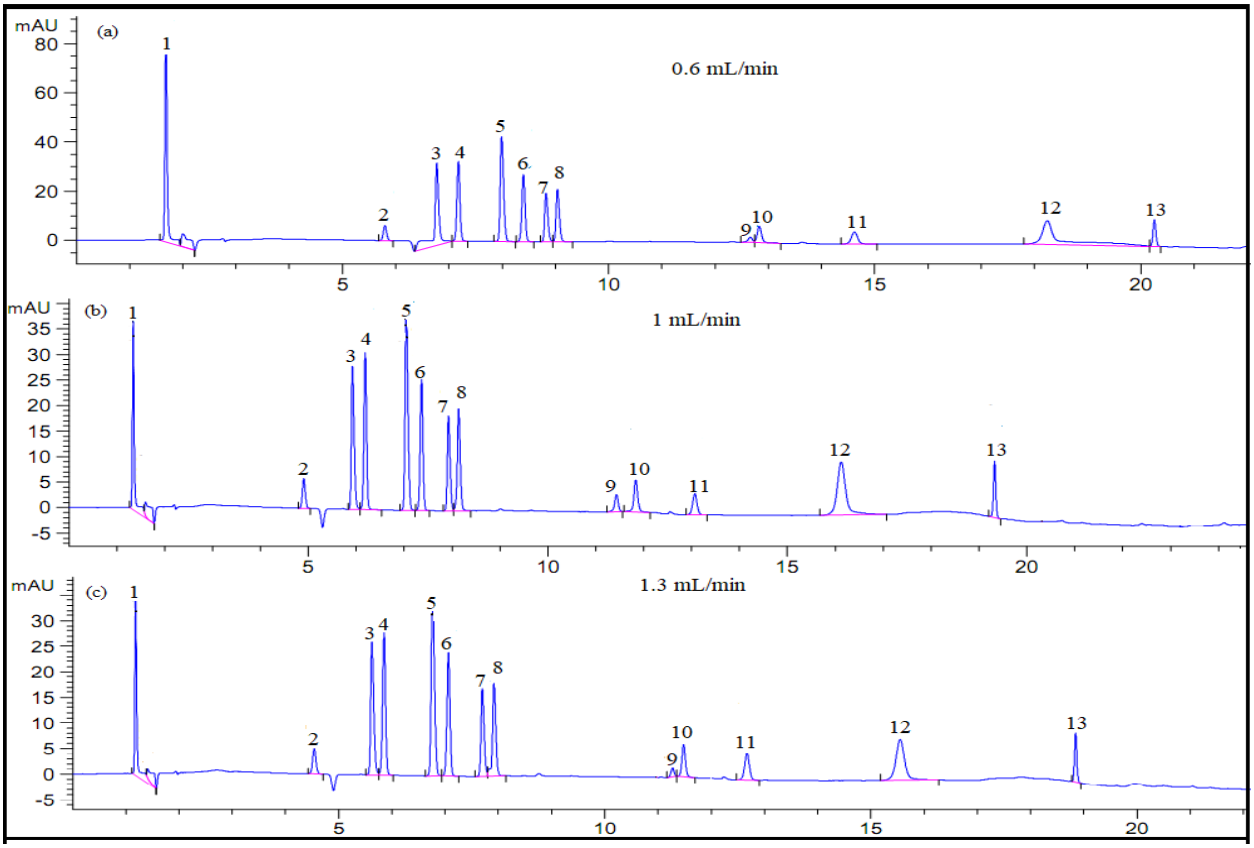


Figure 4.1. 5. Chromatograms obtained using different flow rate (a) 0.6 mL/min, (b) 1 mL/min (c) 1.3 mL/min. (1-MET; 2-AMOX; 3-CHQ; 4-THP,5-TRM, 6-CAF; 7-NOR; 8-CIP; 9-ASA; 10-DOXH; 11-MTZ; 12-ALB and 13-CLA).

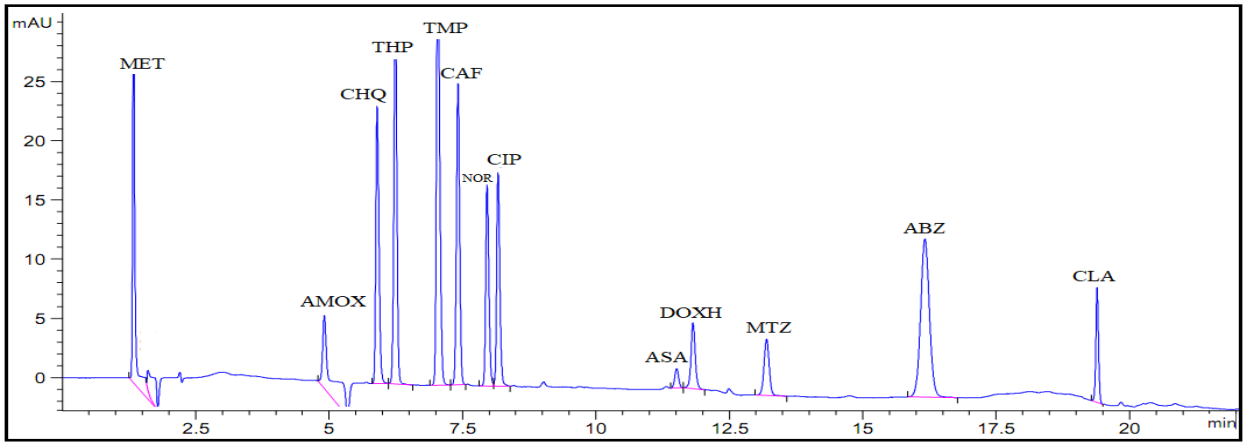


Figure 4.1. 6. Complete chromatogram showing the peaks of 13 selected pharmaceuticals.

Table 4.1. 3. Optimized chromatographic conditions

Separation variables	Optimized conditions
Column	Kromasil C_{18}, 5 µm (4.6 mm x 150 mm, 5µm)
Mobile phase	Ultra-pure water + 0.1% formic acid (A), acetonitrile (B) and methanol (C)
Flow rate	1 mL/min
Column temperature	Ambient
Detection wavelength	250 nm
Run time	22 min

4.1.2. Validation of the analytical method

Validation is required for any new or amended method to ensure that it is capable of giving reproducible and reliable results when used by different operators employing the same equipment in the same or different laboratories. The methods were validated according to the International Conference on Harmonization (ICH) guidelines for validation of analytical procedu res (Toomula et al., 2011). The developed method for simultaneous detection of selected pharmaceuticals in this study was validated as per the ICH guidelines for, linearity, sensitivity, precision, accuracy, specificity, robustness, suitability and stability (ICH, 2005).

4.1.2.1. Linear range

The ability of the assay to give data that is directly proportional to the amount of analyte that the sample contains referees to linearity. Similarly, the range refers to the highest and lowest amount of the analyte that the detector detects with an appropriate amount of accuracy, precision, and linearity (Sutariya et al., 2012). The linearity of the method measured as peak areas versus the corresponding concentration of the pharmaceutical compounds were estimated by the correlation coefficient (R^2) were calculated and evaluated. The linearity range as it was reported in Table 4.1.4 compounds like amoxicillin ranged from 2.5 - 100 µg/mL, trimethoprim ranged from 0.25 - 100 µg/mL, cloxacillin and norfloxacin 0.7 - 100 µg/mL, ciprofloxacin, doxycycline hyclates and metronidazole 1 - 100 µg/mL and the rest pharmaceutical compounds ranged from 0.5 - 100 µg/mL. On the other hand, correlation coefficients (R^2) of the selected compounds were between

0.9985 - 0.9998 which was good and promising to use the method for further analysis. Calibration curves for thirteen pharmaceutical compounds (Y-axis peak area, X-axis concentration) were reported in (Appendix A1-13).

Table 4.1. 4. Analytical parameters for simultaneous determination of selected pharmaceuticals

Pharmaceutical compound	**Linear range (µg/mL)**	**Regression equation**	**R^2**
Acetyl salicylic acid	1 – 100	y = 3.6181x + 1.3002	0.9998
Albendazole	0.5 – 100	y = 8.8529x + 13.169	0.9988
Amoxicillin	2.5 – 100	y = 2.4260x + 0.2300	0.9998
Caffeine	0.5 – 100	y = 10.167x + 6.0284	0.9996
Chloroquine	0.5 – 100	y = 11.150x + 7.5555	0.9997
Ciprofloxacin	1 – 100	y = 9.8126x +5.7892	0.9987
Cloxacillin	0.7 – 100	y = 3.422x + 2.7597	0.9991
Doxycycline hyclates	1 – 100	y = 0.9325x + 5.5146	0.9987
Metformin	0.5 – 100	y = 9.0233x + 7.2918	0.9993
Metronidazole	1 – 100	y = 4.4886x + 9.6939	0.9985
Norfloxacin	0.7 – 100	y = 7.5441x + 1.4492	0.9998
Theophylline	0.5 – 100	y = 11.644x + 8.8687	0.9994
Trimethoprim	0.25 – 100	y = 16.536x + 9.2685	0.9997

4.1.2.2. Sensitivity

Limit of detection (LOD) the lowest concentration of analyte that can be detected and limit of quantification (LOQ) the lowest concentration that can be quantified is means of measuring the sensitivity of the method (Carmona and Pico, 2018). LOD and LOQ of the developed method were calculated from the standard deviation of the response and slope of the calibration curve of pharmaceutical compounds using the formula as per ICH guideline, $3\,\sigma/s$ and $10\,\sigma/s$, respectively. Where σ is the standard deviation of y-intercepts of the regression line and s is the slope of the calibration curve. The results are reported in Table 4.1.5, which shows that LOD ranged from 0.056 - 0.669 µg/L and LOQ from 0.131 - 2.232 µg/L.

4.1.2.3. Precision

The precision of the developed method was evaluated by performing intra-day and inter-day precision studies using concentration of 1 µg/mL solution. The measured peak areas were used to calculate the percent relative standard deviations (% RSD). Intra-day precision was carried out by analysing three replicates of a mixture of the standard pharmaceuticals on the same day. The inter-day precision study was performed on three consecutive days using all the thirteen pharmaceutical drugs in triplicate and the % RSDs were calculated and presented in (Table 4.1.5). The result obtained for precision study is acceptable for analysis due to its lower % RSD that ranged from 1.1 - 5.3% which are lower than the stipulated values of 15% (UNODC, 2009).

Table 4.1. 5. Analytical parameters for simultaneous determination of selected pharmaceuticals

Pharmaceutical compound	**LOD (µg/L)**	**LOQ (µg/L)**	**Precision % RSD**	
			Repeatability (% RSD, n = 3)	**Reproducibility (% RSD, n = 3)**
Acetyl salicylic acid	0.07	0.23	2.8	5.1
Albendazole	0.04	0.15	1.7	4.3
Amoxicillin	0.27	0.91	1.2	4.9
Caffeine	0.02	0.08	1.1	2.0
Chloroquine	0.03	0.12	1.4	3.3
Ciprofloxacin	0.03	0.09	1.2	5.1
Cloxacillin	0.16	0.53	1.3	2.6
Doxycycline hyclates	0.16	0.54	3.1	3.9
Metformin	0.15	0.33	3.0	5.2
Metronidazole	0.10	0.58	1.3	3.9
Norfloxacin	0.08	0.27	1.7	4.1
Theophylline	0.05	0.16	1.4	3.1
Trimethoprim	0.02	0.10	1.3	5.3

4.1.2.4. Accuracy

To study the accuracy of the proposed method, recovery studies were carried out by standard addition method at three different levels. The known amount of the mixture of pharmaceutical compounds (50%, 100% and 150%) was added to the pre-analyzed standard and tablet sample solution, and percentage recoveries were calculated as reported in (Table 4.1.6). Recoveries ranged from 86.3 - 102% in pure compounds and 90.9 - 106 % in tablet dosage forms which is in the acceptable range (UNODC, 2009). The preparation of the solution and the ingredients other than the active compounds in the marketed tablet might be the reason for the difference in the recovery.

Table 4.1. 6. Results of the accuracy studies of standard and sample solution

Pharmaceutical compound	Pre-analyzed standard			Pre-analyzed tablet	
	Spiked amount	Recovery (%)	Mean recovery ± SD	Recovery (%)	Mean recovery ± SD
Acetyl salicylic acid	50 %	86.78	86.3±1.0	89.40	90.9±10
	100 %	85.18		81.38	
	150 %	86.97		101.9	
Albendazole	50%	89.72	93.8±7.7	89.20	95.1±5.2
	100%	102.6		97.78	
	150%	88.96		98.46	
Amoxicillin	50 %	88.89	86.8±1.8	99.55	97.0±3.5
	100%	86.03		93.04	
	150%	85.51		98.48	
Caffeine	50 %	92.50	93.5±1.7	83.12	91.9±8.2
	100%	92.55		99.40	
	150%	95.55		93.08	
Chloroquine	50 %	88.69	87.9±2.4	93.74	94.9±11
	100%	85.23		107.0	
	150%	89.87		83.90	
Ciprofloxacin	50 %	89.86	102±10	89.40	97.4±7.0
	100%	104.8		102.6	
	150%	110.7		100.2	
Cloxacillin	50 %	95.95	92.2±3.5	110.3	106±4.0
	100%	89.03		104.1	
	150%	91.58		102.8	
Metformin	50 %	89.37	87.5±1.7	104.9	105±1.7
	100%	87.24		103.9	
	150%	85.9		107.2	
Doxycycline hyclates	50 %	108.4	100.1±7.6	116.5	101±9.6
	100%	93.73		95.17	

	150%	97.92		94.65	
Metronidazole	50 %	94.81	100.9±10	115.2	104±12
	100%	112.5		90.12	
	150%	95.48		105.9	
Norfloxacin	50 %	85.62	95.3±9.2	97.99	99.6±9.2
	100%	96.34		109.4	
	150%	103.9		91.28	
Theophylline	50 %	85.23	87.9±2.8	103.4	101±6.1
	100%	87.57		105.1	
	150%	90.89		93.80	
Trimethoprim	50 %	86.54	86.3±0.8	107.8	98.5±11
	100%	85.44		101.9	
	150%	86.90		85.78	

4.1.2.5. Specificity

The specificity of the developed HPLC method was established by injecting the blank (solvent without the pharmaceuticals) and placebo solution (without active pharmaceutical ingredient containing the remaining components in the formulation) into the HPLC system using the optimized conditions. The representative chromatogram of blank and placebo is shown in (Figure 4.1.7 (a) and (b)), respectively. No peaks were detected at the retention times of any of the target analyte compounds considered in this study. This study confirms the specificity of the developed method for the selected compounds.

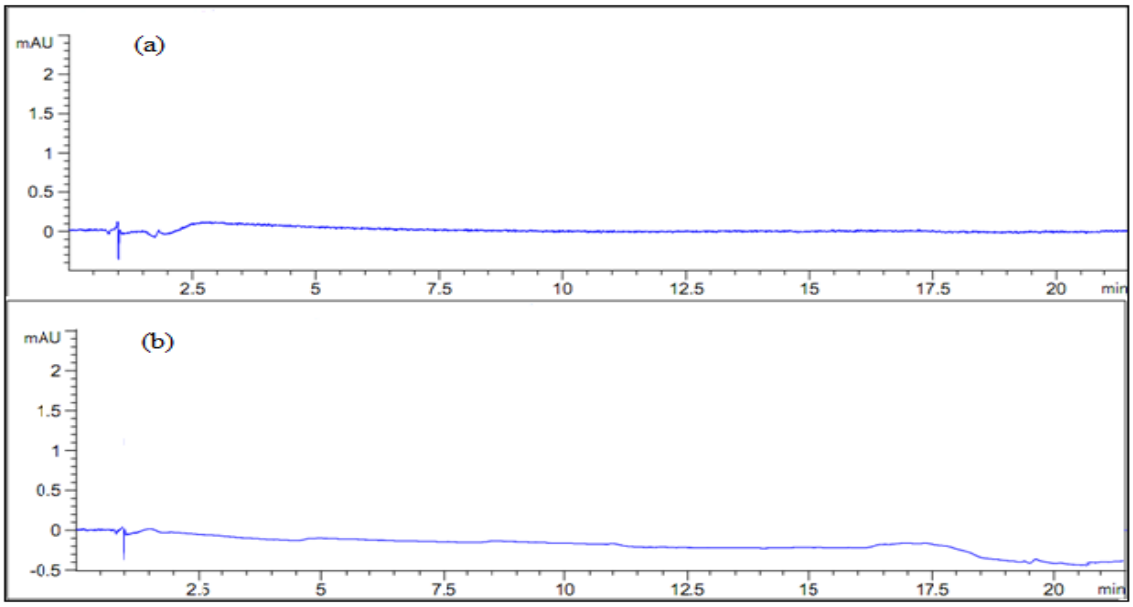


Figure 4.1. 7. Chromatogram of the blank solvent (a) and placebo solution (b) using the developed method

4.1.2.6. Robustness

The robustness of an analytical procedure refers to its capability to remain unaffected by small but deliberate variations in method parameters (Shah et al., 2018) and indicates its reliability during application. The robustness of this method was investigated under minor modifications of conditions including changes of the flow rate of the mobile phase, percent of additive (formic acid) in the mobile phase and wavelength for absorption. The mixture of all selected pharmaceutical compounds was injected in triplicate for every condition and percent relative standard deviations (%RSD) of the peak area, retention time and peak symmetry of small deliberate variation in the method were recorded. The results of the robustness studies (Table 4.1.7) demonstrate that small but deliberate changes in the chromatographic condition considered in this study did not show a significant shift in retention time and the value of peak symmetry and % RSD were still in the accepted range ($p < 0.05$). The results reveal that the method is robust, and the peaks are well separated and elute with acceptable symmetry and resolution within the studied boundaries of the parameters.

Table 4.1. 7. Results of the method robustness tests

Compounds		Parameters changed for robustness study								
		Flow rate (mL/min)			Wavelength (nm)			% Formic acid		
		0.8	1.0	1.2	248	250	252	0.05	0.1	0.15
Albendazole	Peak symmetry	1.116	0.913	0.879	0.379	0.913	0.927	1.077	0.913	0.919
	% RSD	0.362	1.731	3.267	1.971	1.731	2.962	0.166	1.731	2.146
	Rt	16.03	16.17	15.55	16.07	16.17	16.08	16.18	16.17	16.62
Amoxicillin	Peak symmetry	0.883	0.218	0.911	0.585	0.218	0.47	0.903	0.218	0.837
	% RSD	0.557	1.205	3.566	0.838	1.205	3.077	2.722	1.205	2.939
	Rt	5.344	4.910	4.541	4.823	4.910	4.812	4.584	4.910	3.758
Acetyl	Peak symmetry	1.038	0.995	1.091	1.26	0.995	0.782	0.912	0.995	1.100
salicylic acid	% RSD	4.641	2.778	2.158	1.794	2.778	2.963	3.608	2.778	0.307
	Rt	11.53	11.51	11.11	11.42	11.51	11.50	11.16	11.51	11.12
Caffeine	Peak symmetry	0.960	0.995	0.988	0.988	0.995	0.995	0.611	0.995	1.005
	% RSD	3.057	1.142	4.339	2.050	1.142	0.544	3.629	1.142	2.204
	Rt	7.731	7.417	7.064	7.032	7.417	7.293	7.162	7.417	7.167
Chloroquine	Peak symmetry	1.735	0.676	0.630	1.46	0.676	1.592	0.650	0.676	0.393
	% RSD	3.125	1.430	3.110	2.478	1.430	0.878	0.660	1.430	1.257
	Rt	6.241	5.900	5.623	5.634	5.900	5.476	5.626	5.900	4.940
Ciprofloxacin	Peak symmetry	0.770	0.717	0.866	0.913	0.717	0.908	0.897	0.717	0.630
	% RSD	2.564	1.198	2.894	3.398	1.198	0.676	3.994	1.198	1.684
	Rt	8.308	8.166	7.918	8.008	8.166	8.014	7.700	8.166	7.518
Cloxacillin	Peak symmetry	0.911	0.925	0.912	0.270	0.925	0.968	0.948	0.925	0.856
	% RSD	3.832	1.323	3.909	2.008	1.323	0.785	3.578	1.323	2.096
	Rt	19.278	19.39	18.85	19.03	19.39	18.99	19.23	19.393	19.16
Metformin	Peak symmetry	0.672	0.503	0.515	1.002	0.503	0.999	0.406	0.503	0.721
	% RSD	2.818	3.020	1.961	1.961	3.020	3.030	1.171	3.020	0.969
	Rt	1.635	1.334	1.185	1.333	1.334	1.332	1.348	1.334	1.333
Doxycycline	Peak symmetry	0.844	0.859	0.851	0.659	0.859	0.740	0.247	0.859	0.846
hyclates	% RSD	4.545	3.104	3.040	4.140	3.104	2.433	3.101	3.104	2.674
	Rt	11.76	11.82	11.56	11.52	11.82	11.45	11.78	11.82	11.35
Metronidazole	Peak symmetry	1.019	0.953	1.009	0.771	0.953	1.245	0.948	0.953	1.154
	% RSD	1.742	1.294	0.754	2.286	1.294	3.116	3.838	1.294	0.881
	Rt	13.11	13.19	12.67	13.04	13.19	13.01	12.24	13.19	12.33
Norfloxacin	Peak symmetry	0.780	0.714	0.712	1.037	0.714	1.026	0.671	0.714	0.547

	% RSD	1.890	1.713	4.006	1.389	1.713	1.949	2.163	1.713	0.081
	Rt	8.114	7.960	7.699	7.654	7.960	7.556	7.494	7.960	7.269
Theophylline	Peak symmetry	0.963	0.942	1.003	0.792	0.942	0.783	0.637	0.942	0.952
	% RSD	1.141	1.414	2.710	1.916	1.414	1.068	2.850	1.414	0.816
	Rt	6.616	6.242	5.853	5.934	6.242	6.103	5.950	6.242	5.972
Trimethoprim	Peak symmetry	0.772	0.691	0.701	1.021	0.691	1.047	0.963	0.691	0.499
	% RSD	2.578	1.269	2.277	3.044	1.269	0.544	1.292	1.269	1.263
	Rt	7.348	7.038	6.764	7.010	7.038	7.021	6.751	7.038	6.371

Rt = retention time in (min)

4.1.2.7. System suitability

System suitability test of the chromatographic method used to verify that the chromatographic systems are adequate for the analysis to be done. Parameters for this test include retention time, resolution (to the adjacent peak); peak symmetry and number of the theoretical plate were investigated using the optimized chromatographic conditions. The results can be seen in (Table 4.1.8) which shows good performance and acceptable levels for all analytes.

Table 4.1. 8. System suitability result of the developed chromatographic method

Pharmaceutical compounds	**Retention time (min)**	**Resolution**	**Peak symmetry**	**Theoretical plates (N)**
Acetyl salicylic acid	11.5	3.7	0.995	341762
Albendazole	16.2	13	0.953	28382
Amoxicillin	4.8	9	0.218	18916
Caffeine	7.4	8.5	0.955	212283
Chloroquine	5.9	5	0.676	139880
Ciprofloxacin	8.2	47	0.717	262543
Cloxacillin	19.4	-	0.925	2071273
Metformin	1.3	30	0.503	13950
Doxycycline hyclates	11.8	14.5	0.859	311071
Metronidazole	13.2	21	0.953	251295
Norfloxacin	7.6	3.2	0.714	250709
Theophylline	6.2	12.5	0.942	170141

Trimethoprim	7.0	5.8	0.691	174425

4.1.2.8. Solution stability

The stability of analytical (standard and tablet sample) solutions was determined by analyzing at 0 hours, after 24 hours in the refrigerator and after 24 hours at room temperature. Triplicate injections were executed at each time and the stability of the solution was evaluated by calculating percent recovery and relative standard deviation (% RSD) of peak area. The recovery (%) was > 85% and % RSD not more than 5% as reported in (Table 4.1.9). Standard and tablet sample solutions prepared as described in (sections 2.3 and 2.4), respectively, were stable even after 24 hours at room temperature.

Table 4.1. 9. Result of stability studies on a mixture of standard and tablet sample solution of 13 pharmaceutical compounds

Pharmaceutical compounds	**Stability study**				
	Standard solution			**Tablet sample solution**	
	Conditions	**Recovery (%)**	**RSD (%) of peak area**	**Recovery (%)**	**RSD (%) of peak area**
Albendazole	0 h	-	0.844	-	1.889
	24 h in refrigerator	86.18	2.077	98.8	1.493
	24 h at room temp	88.34	3.194	100.1	1.784
Amoxicillin	0 h	-	4.699	-	1.812
	24 h in refrigerator	86.06	2.510	98.01	1.278
	24 h at room temp	90.34	0.504	102.9	2.004
Acetyl salicylic acid	0 h	-	4.183	-	0.985
	24 h in refrigerator	97.80	1.095	86.09	0.831
	24 h at room temp	88.04	0.961	98.05	0.682
Caffeine	0 h	-	1.370	-	1.723
	24 h in refrigerator	105.3	0.135	96.10	1.088
	24 h at room temp	105.2	0.314	97.44	2.823
Chloroquine	0 h	-	0.107	-	1.300
	24 h in refrigerator	102.9	0.277	97.99	1.278

	24 h at room temp	93.79	3.937	100.4	2.004
Ciprofloxacin	0 h	-	4.324	-	1.869
	24 h in refrigerator	85.60	0.041	103.3	0.851
	24 h at room temp	86.40	0.485	98.95	1.343
Cloxacillin	0 h	-	3.881	-	2.505
	24 h in refrigerator	97.07	3.765	103.3	2.481
	24 h at room temp	108.1	4.193	96.30	4.292
Metformin	0 h	-	0.249	-	0.128
	24 h in refrigerator	103.4	0.438	90.85	1.552
	24 h at room temp	105.3	1.528	96.98	1.809
Doxycycline hyclates	0 h	-	3.835	-	2.783
	24 h in refrigerator	100.7	1.300	86.32	1.859
	24 h at room temp	103.5	1.344	97.38	2.132
Metronidazole	0 h	-	2.387	-	1.259
	24 h in refrigerator	90.61	3.801	94.16	2.464
	24 h at room temp	91.10	1.445	106.9	1.987
Norfloxacin	0 h	-	4.629	-	2.963
	24 h in refrigerator	95.48	0.257	98.89	1.859
	24 h at room temp	96.70	0.660	98.25	2.214
Theophylline	0 h	-	3.504	-	1.458
	24 h in refrigerator	88.49	2.348	90.79	1.275
	24 h at room temp	87.86	2.836	101.3	2.270
Trimethoprim	0 h	-	3.022	-	0.784
	24 h in refrigerator	86.93	1.550	100.6	0.373
	24 h at room temp	86.20	1.205	99.17	1.840

4.2. Optimization and use of solid-phase extraction for the determination of pharmaceutical from water samples

4.2.1. Optimization of solid-phase extraction conditions

The optimization of the sample extraction method was performed to secure the best extraction conditions for the compounds of interest. The conditions optimized were the type of sorbent, pH of the sample solution, best elution solvent, sample volume and effect of the addition of EDTA in the sample. Each parameter is optimized at a time by keeping the other condition constant.

4.2.1.1. Sorbent type

The result of the extraction recovery using different sorbent types is shown in (Figure 4.2.1). Three commercially available sorbents tested here are HLB (hydrophilic-lipophilic balance), MAX (mixed-mode anion exchanger) and MCX (mixed-mode cation exchanger) with HLB giving superior results. The performance of HLB as SPE sorbent was known but its capability in comparison with other sorbents for those selected compounds for their extraction recovery was not reported and known. The extraction recovery is higher with HLB compared with the other sorbent type for almost all compounds. The average extraction recovery on the selected sorbent considered in this study is in the order of HLB > MCX > MAX. Solid-phase extraction using HLB sorbent was also reported by other studies concerned with the analysis of pharmaceuticals in comparison with other sorbents (Abafe et al., 2018; Ngubane et al., 2019; Ngumba et al., 2016b) as it can adsorb compounds in acid, basic and neutral forms better than the other adsorbents. Evaluating the capability of different sorbents for their simultaneous extraction recovery of different pharmaceuticals is important because of their diverse physicochemical properties (Ngumba et al., 2016b).

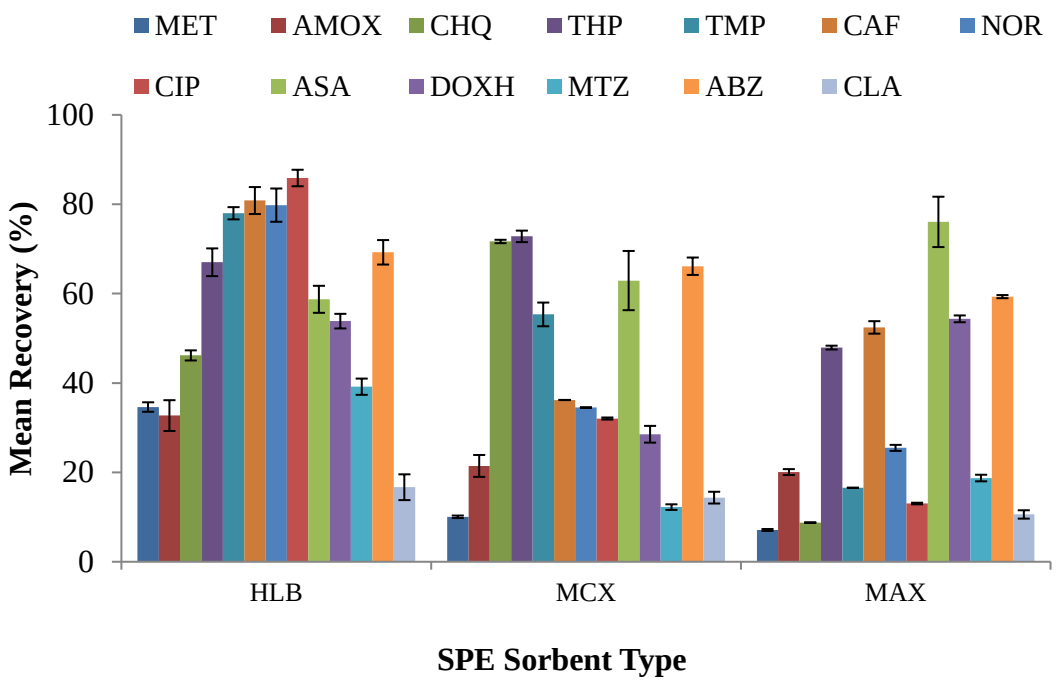


Figure 4.2. 1. Effect of sorbent type on the extraction of the pharmaceutical compounds using solid-phase extraction

4.2.1.2. pH of sample solution

The result of the optimization of the pH of the water sample is shown in (Figure 4.2.2). Solution pH range from 3-11 including acid, base and neutral level of the sample solution was considered in the optimization. From the pH range under consideration, pH 5 showed good average recovery for the majority of the pharmaceutical compounds in solid-phase extraction. The basic solution was not favorable for all compounds to retain in the sorbent. A pH of 5 was therefore considered as an optimal pH and used in subsequent experiments, and this result fell within the manufacturer's pH specified range. A pH of 5 was therefore considered as an optimal pH and used in subsequent experiments. The optimum pH here is comparable with the result obtained in another study by Ngumba et al., (2016b) that examined ten different antibiotics and anti-retroviral pharmaceuticals using HLB cartridge for solid-phase extraction. The pH of the sample solution is important as it affects the ionization status of the compounds which in turn influences their binding capabilities to the sorbent (Gezahegn et al., 2019).

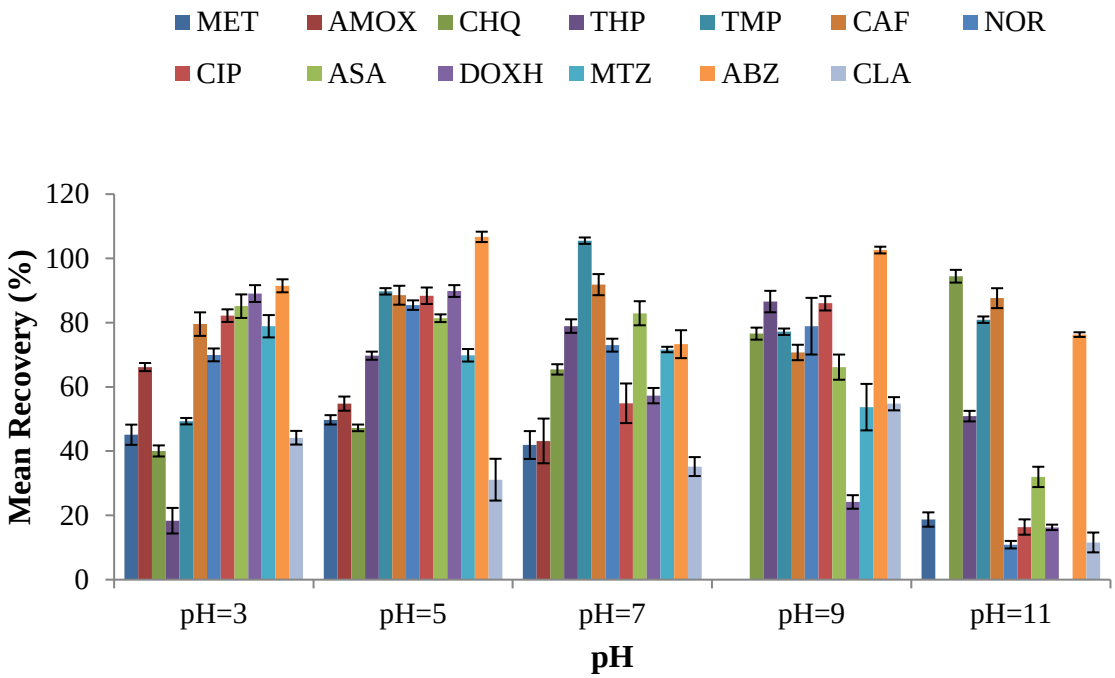


Figure 4.2. 2. Effect of sample pH on the extraction of the pharmaceutical compounds using solid-phase extraction

4.2.1.3. Elution solvent

The result of extraction recovery involving different elution solvent on the extraction efficiency is shown in (Figure 4.2.3). The elution solvents investigated in detail are a mixture of acetonitrile (ACN) and methanol (MeOH) (20:80%, v/v), 2% formic acid (FA) in ultrapure water and methanol (20:80%, v/v) and 2% acetic acid (HAC) in water and methanol (20:80%, v/v). These solvents were selected based on the fact that its suitability to desorb all the analytes from sorbent at minimal volume. The result in (Figure 4.2.3) showed that each mixture of elution solvent was able to elute a significant amount of analyte from the sorbent HLB solid-phase extraction cartridge. The use of a mixture of acetonitrile and methanol as an elution solvent gives minimum recovery for all compounds compared to the other mixture because pharmaceuticals have different polarities. Improvement was observed in the addition of additives in methanol in the place of acetonitrile. From the additives used together with methanol, formic acid resulted in a better recovery than acetic acid. Therefore, the best elution solvent was found to be 2% formic acid in water and methanol (20:80, v/v) in 6 mL volume. Other researchers have also reported

that they examined different solvents for elution of selected compounds from the solid phase extraction sorbent (de Oliveira et al., 2019).

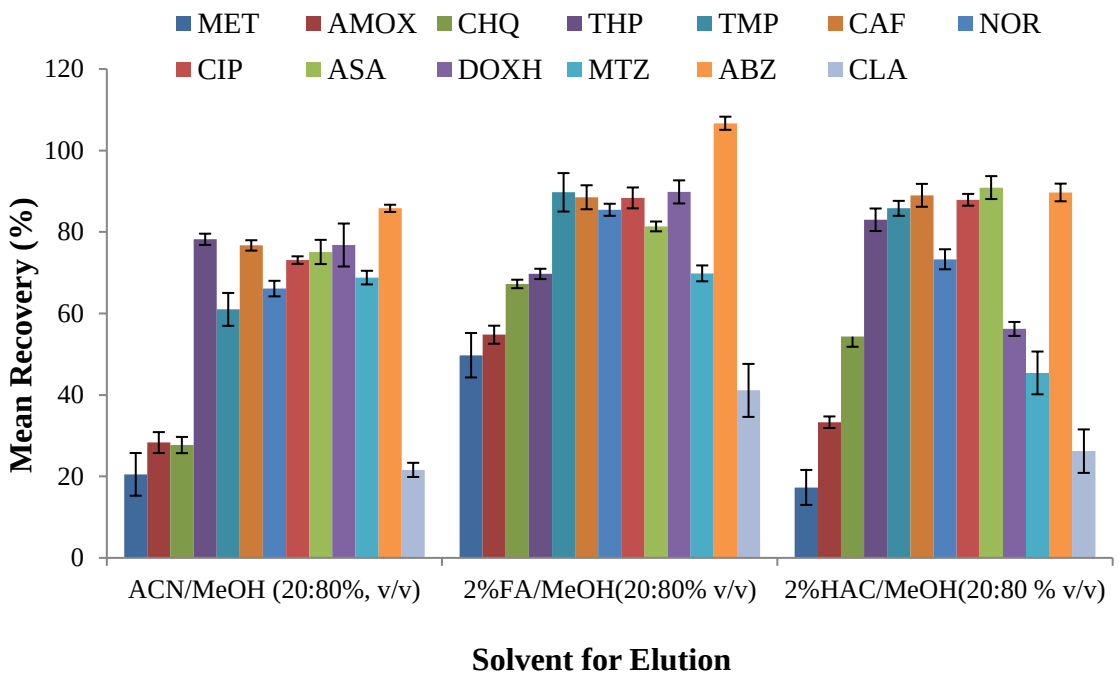


Figure 4.2. 3 Effect of elution solvent on the extraction of the pharmaceutical compounds using solid-phase extraction.

4.2.1.4. Effect of load volume of sample solution

Figure 4.2.4 shows the result of the influence of the sample load volume on the recovery of pharmaceutical compounds. The sample volume of (50, 100, 150 and 200 mL) was considered for their extraction recovery of selected pharmaceutical compounds. The sample volume of 150 mL was comparably selected as the optimal volume of the sample solution that gives the relatively highest extraction recovery. Beyond this volume, the recovery decreases as the sorbent breakthrough volume is exceeded. Furthermore, sample solution volume beyond 200 mL was not tested due to that the average recovery fell beyond the optimum. The sample volume for solid-phase extraction is a condition that has to be optimized to avoid over-loading the SPE sorbent to avoid saturation of adsorption sites that affects the results (Madikizela and Chimuka, 2017;

Madikizela et al., 2014; Tan et al., 2015). The final volume obtained depends basically on the analyte-sorbent interaction.

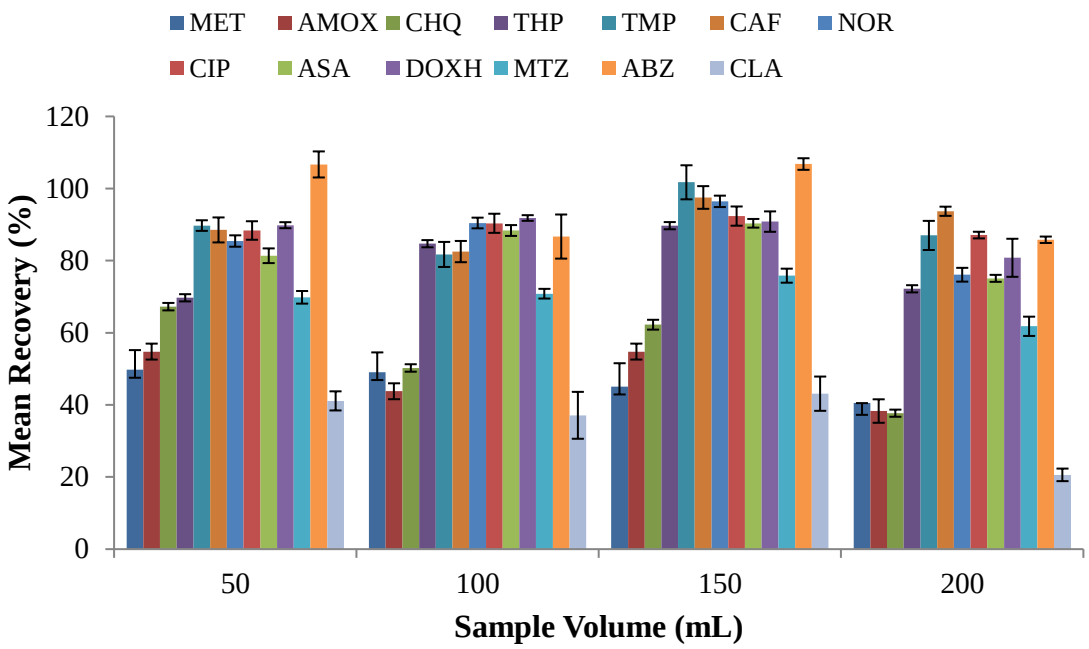


Figure 4.2. 4. Effect of sample volume on the recovery of pharmaceutical compounds

4.2.1.5. Effect of ethylene diamine tetraacetic acid (EDTA)

The result of the effect of the addition of EDTA on extraction recovery is shown in (Figure 4.2.5). The statistical t-test with and without the addition of EDTA in the sample solution gives the p-value higher than 0.05 for all pharmaceuticals except DOXH and MTZ which indicates that there is no statistically significant difference in the recovery. However for antibiotic pharmaceuticals DOXH and MTZ the result shows there is the significant difference in the recovery result on the use of EDTA. The addition of EDTA in the sample solution increases the recovery of these compounds which is good for the application of the method. The result agrees with the reported result that addition of EDTA affects the recovery of some antibiotics by de Oliveira et al., (2019) and addition of EDTA in the sample solution was used throughout the analysis.

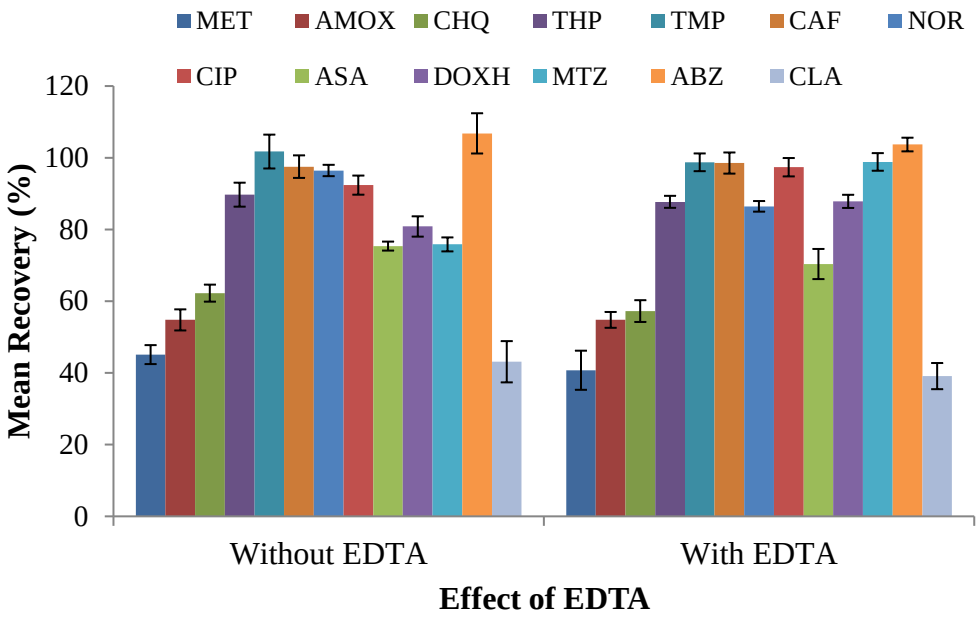


Figure 4.2. 5. Effect of EDTA in the extraction recovery of selected pharmaceuticals from the water sample solution

4.2.2. Validation of the proposed method

Analytical method validation was carried out encompassing different performance parameters using the optimized conditions following the protocols and standards stated (UNODC, 2009; USEPA, 2008). The correlation coefficients (R^2) for the calibration curves of the targeted compound were within the range of 0.9985-0.9998. The limits of detection (LOD) and limits of quantification (LOQ) of the developed HPLC method were calculated from the standard deviation of the response and slope of the calibration curve of pharmaceutical compounds. The limit of detection ranged from 0.1-0.8 µg/L and the limit of quantification ranging from 0.2-2.6 µg/L. Precision studies, intra-day precision (repeatability) carried out by running three replicates of spiked and extracted water samples on the same day, and measured peak areas used to calculate percent relative standard deviation. The inter-day precision (reproducibility) study performed on three different analysis days and percent relative standard deviation (% RSD) was calculated and reported as shown in Table 4.2.1.

Table 4.2. 1. Analytical parameters for simultaneous determination of selected pharmaceutical compounds using optimized solid-phase extraction.

Pharmaceutical compound	R^2	LOD (µg/L)	LOQ (µg/L)	Precision % RSD	
				Repeatability (% RSD, n = 3)	Reproducibility (% RSD, n = 3)
Acetyl salicylic acid	0.9998	0.361	1.204	1.9	2.8
Albendazole	0.9988	0.101	0.339	1.1	1.9
Amoxicillin	0.9998	0.772	2.574	6.6	7.6
Caffeine	0.9996	0.081	0.305	2.2	3.3
Chloroquine	0.9997	0.371	1.237	5.5	4.6
Ciprofloxacin	0.9987	0.052	0.173	1.7	2.0
Cloxacillin	0.9991	0.281	0.934	1.7	1.3
Doxycycline hyclates	0.9987	0.557	1.857	2.3	2.3
Metformin	0.9993	0.300	0.999	3.2	2.0
Metronidazole	0.9985	0.278	0.928	3.6	4.7
Norfloxacin	0.9998	0.204	0.680	2.7	1.8
Theophylline	0.9994	0.232	0.773	1.3	3.4
Trimethoprim	0.9997	0.096	0.320	1.5	2.1

The applicability of the proposed method was evaluated by extracting the analytes from a real water sample following the optimized SPE-HPLC-DAD analytical technique. Consequently, the applicability of the technique was further investigated by extracting the analytes from water samples spiked at the concentration level 1 of 0.25 µg/mL and level 2 of 0.05 µg/mL, the peak areas of which were compared with those obtained from the spiked reagent water at the same concentration levels, a similar study reported by (Ebele et al., 2020) also uses this to assess the matrix effects. The relative recovery (%RR), defined as the ratios of the peak areas of spiked real water extracts to the peak areas of spiked reagent water extracts gives good analytical views about the matrix effect to the target analytes (Gure et al., 2014). The advantage of calculating the relative recovery was to understand the matrix effect on the optimized extraction method and to know how the complex matrix where the analytes were affects the availability. The complex matrix will have a positive and negative impact meaning they could magnify its availability or

suppress the availability. In this study, four different water samples were employed and the matrix effect was evaluated in terms of relative recovery for each pharmaceutical compound. As it is reported in (Table 4.2.2), it was in the range of 70–117% except in the case of amoxicillin and acetylsalicylic acid where it goes down to 62% and 65% in Akaki sewage treatment plant (ASTP) influent, respectively. Therefore, the result gives a clue that the selectivity of the SPE sorbent affected by other organic compounds potentially present in the water sample. In addition to this the availability of some compounds like amoxicillin and acetylsalicylic acid in water samples potentially that have several different pollutants present, could have the extraction negatively affected. However, the method was still acceptable for analysis due to its % RSD which ranged from 0.3-11% being lower than the stipulated values of 15% (UNODC, 2009).

Table 4.2. 2. Percentage relative recovery (% RR) for spiked tap water and river water of selected pharmaceuticals

Pharmaceutical Compound	Relative recovery (%) ± % RSD							
	River water		ASTP influent		ASTP effluent		Hospital wastewater	
	Level 1	Level 2	Level 1	Level 2	Level 1	Level 2	Level 1	Level 2
Acetylsalicylic acid	94.5±8.1	88.1±5.5	70.2±5.1	74.5±4.6	75.1±10.1	83.9±4.8	76.1±1.4	87.8±1.8
Albendazole	84.9±5.5	78.7±4.3	75.7±9.4	70.2±9.9	69.8±5.4	71.4±2.8	71.8±5.9	76.4±8.4
Amoxicillin	86.2±7.5	105±8.9	78.8±1.1	84.1±6.7	81.6±5.2	96.5±10.3	71.7±1.7	84.4±9.2
Caffeine	95.1±2.3	117±1.2	94.6±4.1	86.3±3.4	71.4±2.9	75.2±4.4	99.6±5.8	105.2±7.1
Chloroquine	76.4±1.2	97.8±9.9	96.5±2.1	86.1±9.9	102.4±1.7	96.1±10.3	102±5.4	90.6±3.4
Ciprofloxacin	78.5±0.3	115±3.8	76.6±6.6	87.2±8.6	88.3±2.3	83.3±7.2	79.3±5.7	86.3±5.7
Cloxacillin	83.9±6.1	84.8±8.1	74.2±8.0	85.3±4.4	76.3±11	77.4±0.5	70.3±3.6	76.3±2.3
Doxycycline hyclates	85.9±4.4	82.3±9.3	93.7±2.9	84.6±8.9	84.5±9.4	86.8±5.4	92.1±7.7	75.6±9.7
Metformin	91.3±6.2	90.9±8.9	102.5±8.5	97.8±6.8	101.5±5.2	89.7±8.8	78.9±3.6	75.1±2.9
Metronidazole	89.6±7.8	82.7±9.3	76.6±1.1	86.7±8.7	78.4±6.3	79.7±9.5	71.8±11.3	79.7±9.5
Norfloxacin	83.5±2.6	72.7±7.4	80.8±3.5	90.9±4.8	69.1±2.2	90.6±4.3	106.8±0.4	89.6±5.3
Theophylline	102±5.3	81.2±2.2	102.7±1.3	80.5±3.9	75.3±1.7	88.4±9.1	102.7±9.8	98.1±10.9
Trimethoprim	89.9±9.1	102±6.7	102.1±1.1	102.1±3.1	70.9±6.3	104.8±1.3	106.5±2.2	93.4±2.5

Level 1 (250 µg/L) and level 2 (50 µg/L)

4.2.3. Application of the method for environmental water sample

Water samples from different sources were collected for the application of the proposed method to assess the level of selected pharmaceuticals. Physicochemical properties including (pH, conductivity, total dissolved solids (TDS) salinity and turbidity) of the water samples were measured and reported in (Table 4.2.3). The conductivity and total dissolved solids are higher in sewerage treatment plant influent which was expected as it contained wastes from different sources.

Table 4.2. 3. Physicochemical properties of water sample used for analysis (n = 3, %RSD).

Sample	pH	Conductivity (µs/cm)	Total dissolved solids (mg/L)	Salinity (psu)	Turbidity (FTU)
River water	7.6(0.1)	328(5.3)	157(4.0)	0.2(0.0)	12(2.5)
ASTP effluent	7.4(0.3)	717(2.5)	344(4.2)	0.3(0.0)	3(0.5)
ASTP influent	7.8(0.1)	863(6.1)	416(3.5)	0.3(0.1)	190(4.0)
Hospital wastewater	6.6(0.1)	299(6.6)	144(5.1)	0.1(0.0)	30(3.2)
Dam water	7.9(0.1)	77(2.1)	36(2.9)	0.0(0.0)	22(2.9)

Identification of target pharmaceuticals was done based on matching the retention times obtained in sample extract to the standard solutions and confirmation with photodiode array (PDA) spectra obtained with the standard solution as the detector allows (Ngubane et al., 2019). The chromatogram in Figure 4.2.6 (a), and (b) shows for standard mixture solution and the extract hospital wastewater sample respectively. In a real water sample, many compounds might be present and the retention time will not be the only means of identification rather the PDA spectra should be used to confirm, spectra for the detected compound in this study is given in (Figure 4.2.7-12). The detected compound and concentration level are summarized in (Table 4.2.4). Compounds including antibiotics, trimethoprim, ciprofloxacin and norfloxacin, stimulant caffeine and anthelmintic albendazole pharmaceuticals were quantified in the water samples except norfloxacin which was below the limit of quantification of the method. The concentration detected in this study will be related to the consumption of those pharmaceutical compounds in

arround the sampling place. Others pharmaceuticals might be below the detection limit of the method or they are not present in the water during the sampling time.

Table 4.2. 4. Concentrations of the evaluated pharmaceuticals found in real water samples from different sources in Addis Ababa

Pharmaceuticals	**Water sample (in µg/L, n = 3, %RSD)**				
	River water	**Dam water**	**Hospital wastewater**	**ASTP influent**	**ASTP effluent**
Trimethoprim	n.d.	n.d.	7.8(1.1)	0.5(0.02)	n.d.
Caffeine	n.d.	n.d.	3.2(0.4)	n.d.	n.d.
Norfloxacin	n.d.	n.d.	<LOQ	n.d.	n.d.
Albendazole	n.d.	n.d.	2.1(0.1)	n.d.	n.d.
Ciprofloxacin	n.d.	n.d.	n.d.	0.3(0.01)	n.d.

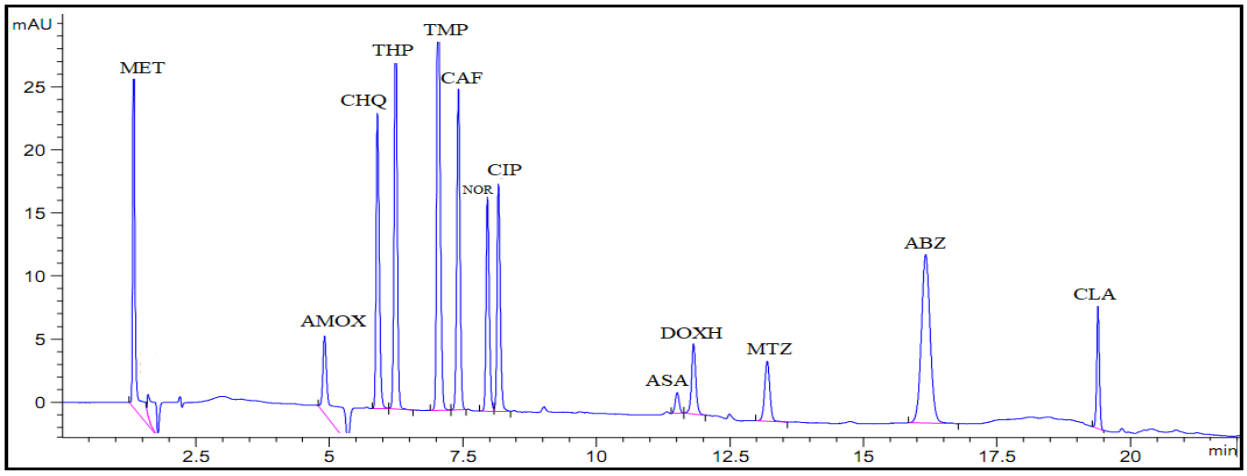
n.d. = not detected

Previous studies have shown that hospital wastewater is a source of different pharmaceutical compounds (Bertrand-Krajewski, 2018; Souza et al., 2018). In this study, norfloxacin, trimethoprim, and caffeine were detected. These have been reported in other studies of extracted hospital wastewater (Kanama et al., 2018; Kosma et al., 2010; Verlicchi et al., 2012). The concentration of antibiotic trimethoprim obtained from hospital wastewater in this study is higher than that reported in Portugal by Santos et al., (2013) and in Turkey by (Aydin et al., 2018; Santos et al., 2013). However, caffeine was found to be lower than reported by other studies in Western Greece (Kosma et al., 2010; Oliveira et al., 2015).

Trimethoprim and ciprofloxacin antibiotics were also detected and quantified from the sewage treatment plant influent sample. However, wastewater treatment plants mostly were unable to remove pharmaceutical compounds they rather decrease the level in effluents than influent (Al et al., 2019; Pereira et al., 2017) and the level in the effluent was found below the limit of detection of the proposed method for this study. These antibiotics have also been detected in a South Africa wastewater treatment plant (Faleye et al., 2019). The concentration reported by Faleye et al., (2019) is much higher than detected in this study. The concentration of antibiotics ciprofloxacin result reported in South Africa by Kanama et al., (2018) in the wastewater

treatment plant was congruent but other studies reported in the same country by (Agunbiade and Moodley, 2016) higher than the concentration in this study. In this study, the reported concentration of trimethoprim in wastewater treatment plant influent is significantly higher than that reported in Turkey by (Aydin et al., 2018). These differences are due to the population density within a given area and their healthy life style.

(a)



(b)

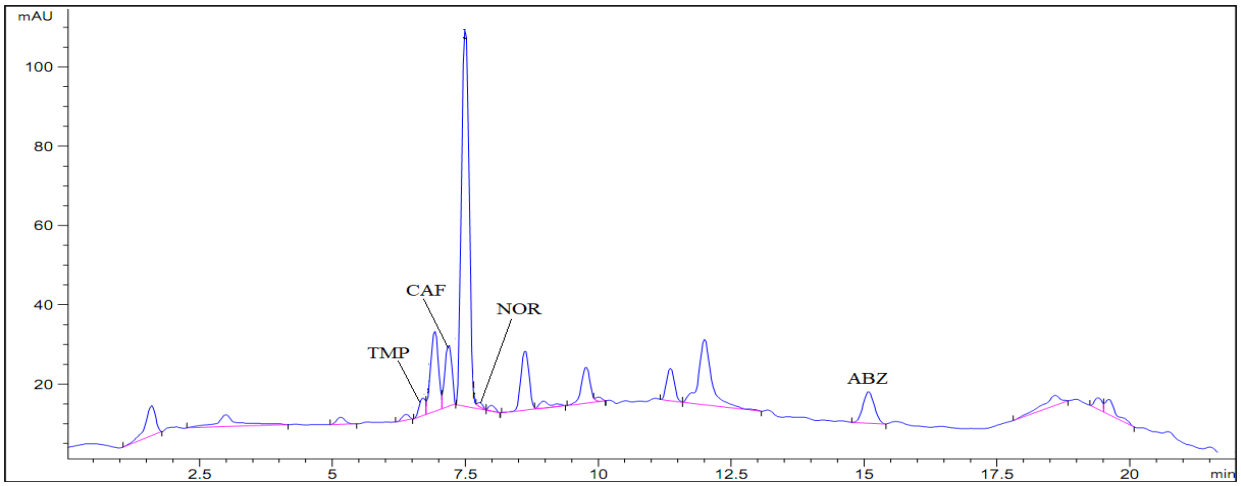


Figure 4.2. 6. Chromatogram of standard mixture solution (a) and extracted hospital wastewater using optimized SPE (b).

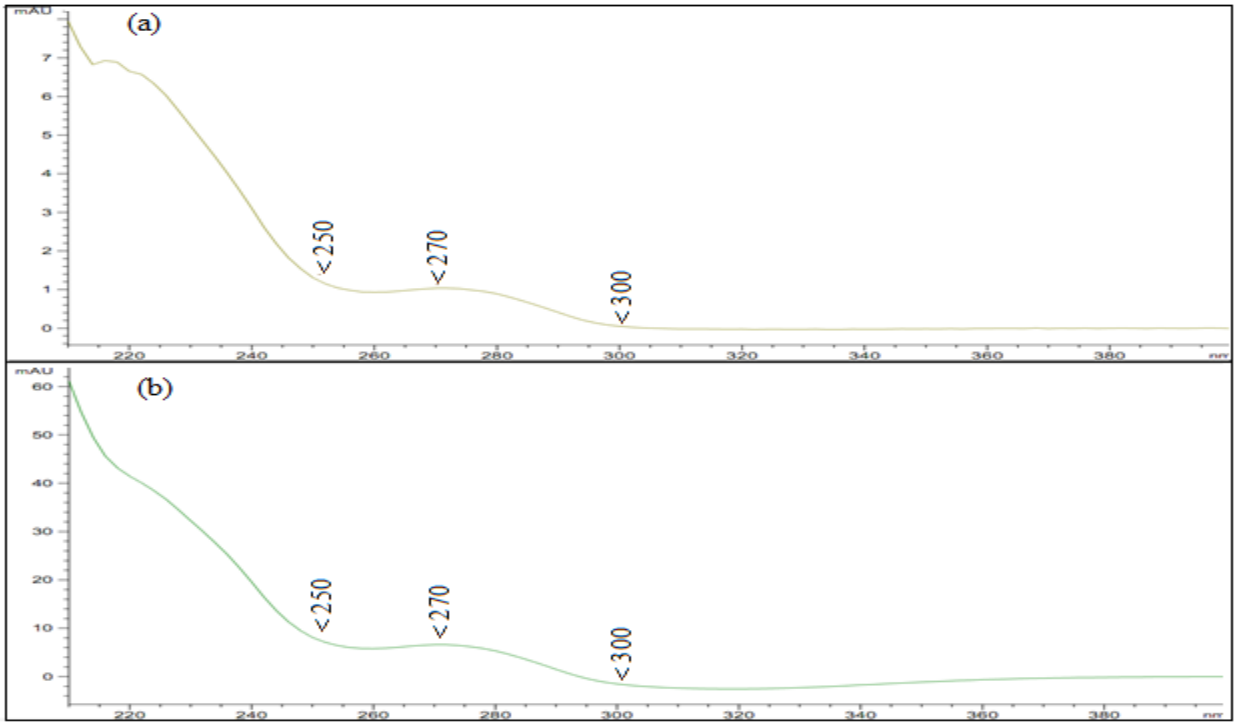


Figure 4.2. 7. PDA spectra of trimethoprim (TRM) standard (a) and trimethoprim (TRM) in hospital wastewater (b).

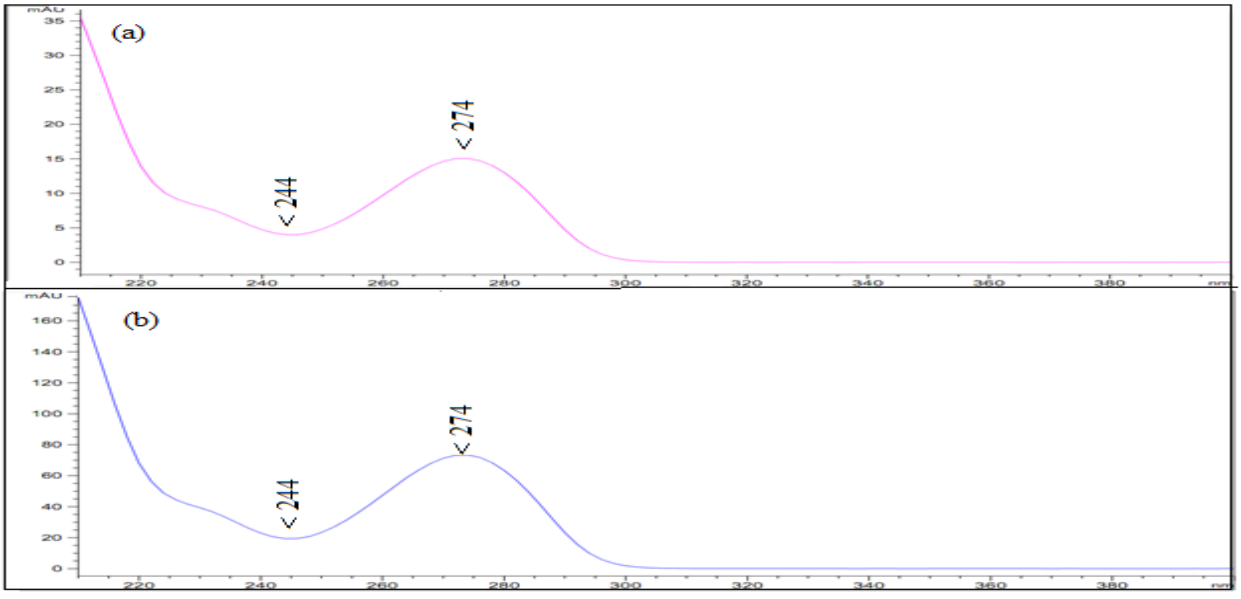


Figure 4.2. 8. PDA spectra of caffeine(Appendino et al.) standard (a) and caffeine(Appendino et al.) in hospital wastewater (b)

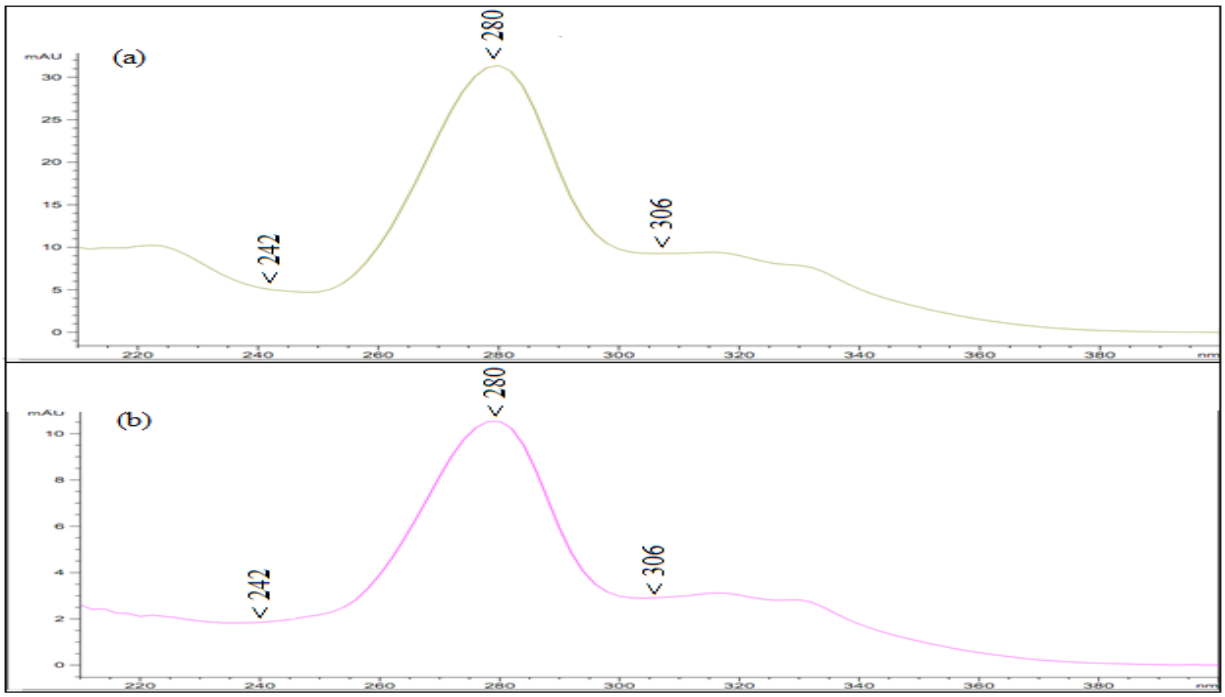


Figure 4.2. 9. PDA spectra of norfloxacin (NOR) standard (a) and norfloxacin (NOR) in hospital wastewater (b).

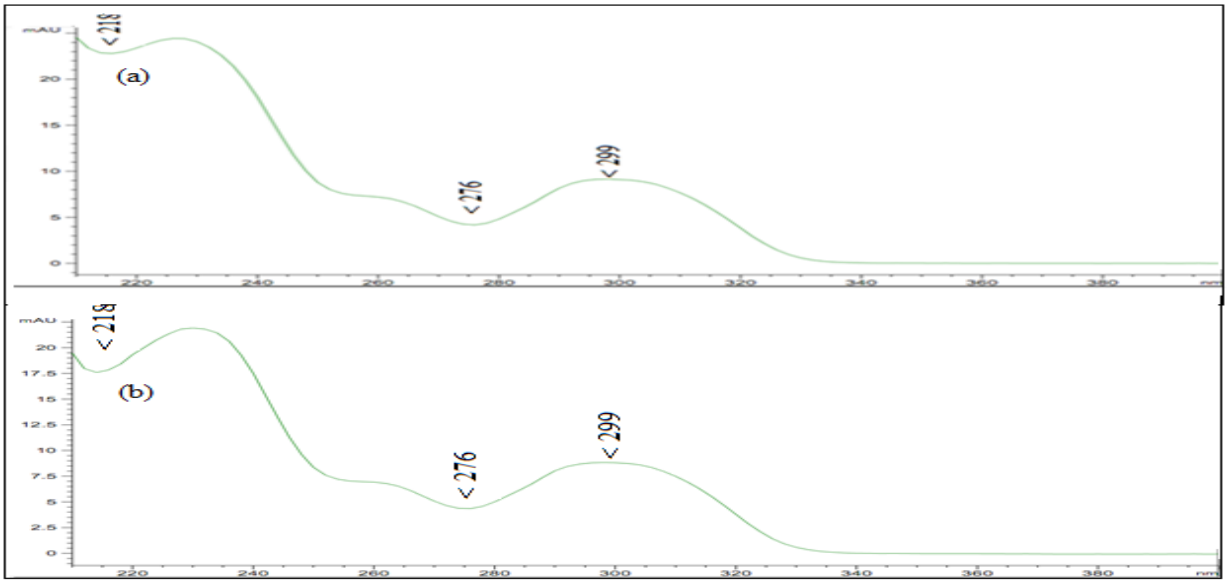


Figure 4.2. 10. PDA spectra of albendazole (ALB) standard (a) and albendazole (ALB) in hospital wastewater (b)

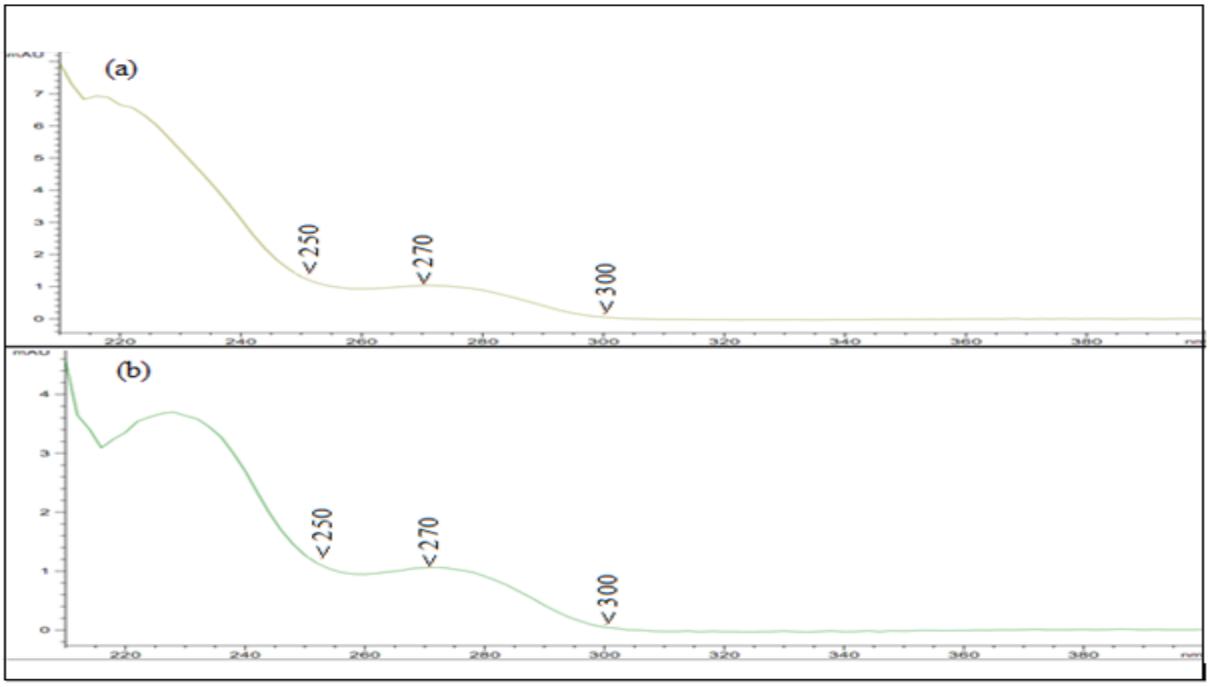


Figure 4.2. 11. PDA spectra of trimethoprim (TMP) standard (a) and trimethoprim (TMP) in sewerage treatment plant influent (b).

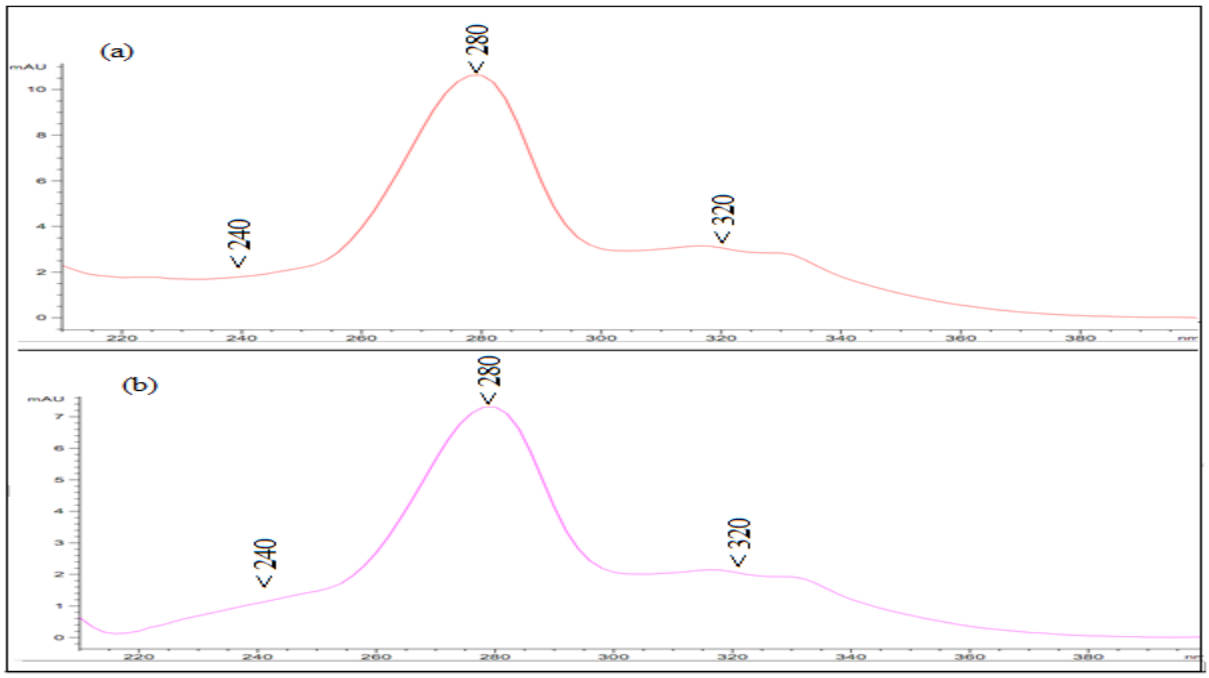


Figure 4.2. 12. PDA spectra of ciprofloxacin (CIP) standard (a) and ciprofloxacin (CIP) in sewerage treatment plant influent (b).

4.2.4. Comparison of the proposed method with similar literature reports

To evaluate the performances of the developed method, it was compared with previously reported studies in the literature both with LC with MS detector and LC with UV detector. As there is no previous work done in pharmaceutical determination in Ethiopian environment the result obtained was compared with reported values in other countries. Details of the comparison are shown in (Table 4.2.5). The results show that this method is comparably better than some other reported methods using UV detector but the difference is visible in mass detector. Such technique for analysis is not accessible and very expensive for the developing country like Ethiopia. The comparison focused on parameters such as type of SPE sorbent used, simplicity of elution solvent used and its step, recovery and limit of detection. In other developed solid phase extraction methods where as many pharmaceuticals were extracted, complex elution solvents combinations have been used.

Table 4.2. 5. Comparison between the proposed method and some reported methods for pharmaceuticals determination from water samples.

Methods	Number of pharmaceuticals	Type of SPE sorbent	Elution solvent	LOD (µg/L)	References
LC-MS/MS	10(4)	HLB	Acetonitrile/methanol (1/1, (v/v))	0.002-0.018	(Ngumba et al., 2016b)
UHLC-MS/MS	20(3)	Strata™-X	1% (v/v) acetic acid in acetonitrile and methanol (50:50 % , v/v)	0.003-0.03	(de Oliveira et al., 2019)
LC-MS	7(2)	HLB	Methanol	0.0001-0.0028	(Afonso-Olivares et al., 2013)
HPLC-DAD	15(1)	HLB	Methanol	0.004-0.183	(Baranowska and Kowalski, 2011)
LC-MS/MS	6(2)	MIP	Acetonitrile/methanol/acetic acid (50:50:2 v/v)	0.004-0.037	(Ngumba et al., 2016a)
HPLC-DAD	7(1)	HLB	Methanol/Dichloromethane (70:30)	0.04-0.233	(Madureira et al., 2010)
HPLC-DAD	13	HLB	2% formic acid in water and methanol (20:80%, v/v)	0.05 - 0.56	This study

'a(b)', 'a' is the number of pharmaceuticals in the report and the 'b'in the bracket is the common pharmaceutical with this study

4.3. Determination of pharmaceutical compounds using UHPLC-MS from different water samples collected from Addis Ababa, Ethiopia

Selected ten pharmaceutical compound residues were determined in water samples collected from potentially contaminated sampling points in the capital city Addis Ababa, Ethiopia. The determination was done using a UHPLC-MS analytical sensitive instrument in which method parameters were optimized and validated using selected compounds. The results obtained from these sampling points are presented and discussed in different ways and compared with the sampling points and potential contaminants source as shown below.

4.3.1. Optimization of instrumental conditions

To obtain the selective separation and quantification results from the chromatography and best detection condition from the detector, conditions affecting their performance should be optimized. In this study, UHPLC-DAD for separation of thirteen selected pharmaceuticals was optimized by considering conditions like mobile phase solvent, injection volume and flow rate. Similarly, in the detector, ESI-TOF-MS for the target compounds conditions affecting the detection capability were optimized and reported as follows.

4.3.1.1. Chromatographic conditions

A number of experiments to achieve optimum chromatographic separation for all the target analytes were conducted. Efficient analyte resolution in the chromatographic separation was the preliminary experimental practice usually considered. This can be achieved by performing a series of experiments while varying the parameters that have a potential effect. Consequently, mobile phase compositions with different types and proportions were evaluated. The composition of the mobile phase, water with acetonitrile and 50:50 methanol and acetonitrile were evaluated at the beginning and it results in poor resolution of the compound. On the use of formic acid additives, all the compounds eluted before 10 min but the resolution and the appearance of the peak were not good. For the other trial, the mobile phase composition of water and methanol gives a good separation with good resolution except for chloroquine (CHQ), acetylsalicylic acid (ASA) and cloxacillin. These compounds are appearing everywhere in the chromatogram and their intensity was found to be low in simultaneous separation. Therefore,

three pharmaceuticals were not considered out of thirteen and ten compounds were used for further optimization and analysis in the real water samples.

In the liquid chromatography separation method development, further addition of formic acid (0.1%), the resolution and the peak appearance become relatively good as they took 16 min to separate 10 compounds. An injection volume of the standard mixture was also evaluated and 20 µL was found to be optimum for good separation of the compounds. The other factor during the method development was the flow rate. As it is obvious in chromatography flow rate affects the peak separation and resolution of the compounds. The effects of the mobile phase flow rate evaluated in the range of 0.3–0.8 mL/ min. It was observed that both the retention times and peak widths of the target pharmaceuticals were increased with an increase of the flow rate, in addition to which the resolution between norfloxacin and ciprofloxacin (CIP) was improved. Thus a flow rate of 0.8 mL/min was chosen as the optimum as it is the maximum value in the instrument used throughout the analysis. The column temperature was at room temperature and the DAD monitoring wavelength of 250 nm was adjusted and used throughout the analysis. Total ion chromatogram for ten analytes is shown in Figure 4.3.1.

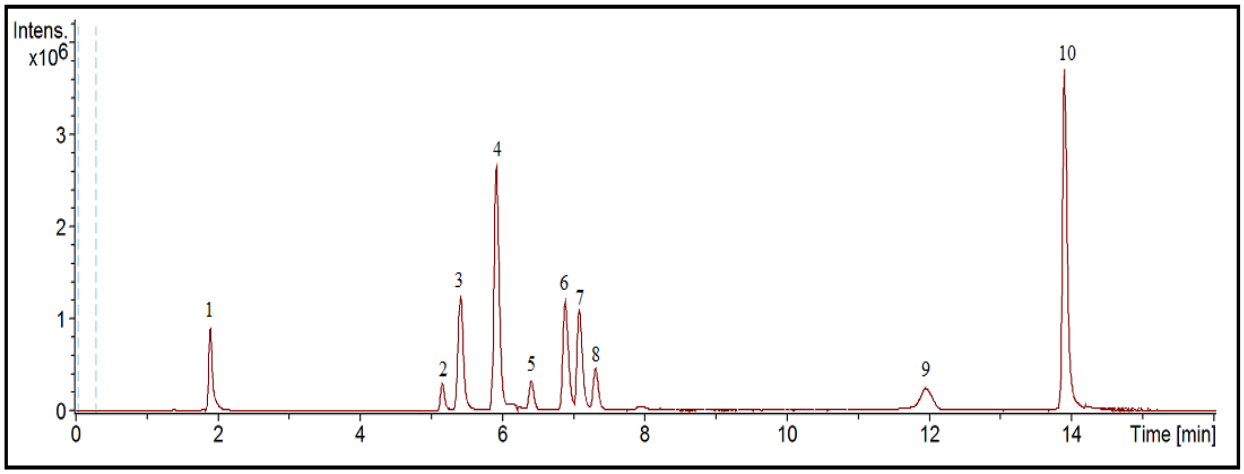


Figure 4.3. 1. UHPLC-MS total ion chromatograms of representative compounds of pharmaceuticals: ((1) (MET), (2) (AMOX), (3) (MTZ), (4) (TRM), (5) (THP), (6) (NOR), (7) (CIP), (8) CAF, (9) (DOXH), (10) (ALB)).

4.3.1.2. Mass spectrometry conditions

Mass spectrometry parameters were optimized by direct infusion of the individual analyte standard solution using a syringe pump. The conditions for the analysis were as follows: drying gas temperature, 220 ^{o}C; capillary voltage, 4.5 kV; drying gas flow (nitrogen), 9 L/min; and nebulizer pressure (nitrogen), 1.8 Bar. For all the compounds, positive ionization was selected and precursor ion was $[M + H]^{+}$. Multiple reactions monitoring (MRM) was carried out using TOF-MS spectrometer equipped with an electrospray ionization (ESI) interface, using the positive-ion mode and parameters was optimized and presented in Table 4.3.1 and the extracted ion chromatogram of each compound are combined and shown in Figure 4.3.2. Total chromatogram of all compounds, the precursor ion (m/z) in the multiple reaction monitoring and product ion are shown in (Appendix D 1,2 and 3).

Table 4.3. 1 Mass spectrometer parameters for the detection of target analytes

Compounds	**Molecular mass (g/mol)**	**Precursor ions $[M + H]^{+}$ (m/z)**	**Product ions (m/z)**	**Collision energy (V)**	**RT (min)**	**Ion (mode)**
Albendazole	265	266	234	22	13.68	ESI +
Amoxicillin	365	366	114	32	4.83	ESI +
Caffeine	194	195	138	43	7.37	ESI +
Ciprofloxacin	331	332	231	58	7.19	ESI +
Doxycycline hyclates	444	445	428	30	12.21	ESI +
Metformin	129	130	71	23	1.90	ESI +
Metronidazole	171	172	128	32	5.37	ESI +
Norfloxacin	319	320	302	68	6.98	ESI +
Theophylline	180	181	124	38	6.47	ESI +
Trimethoprim	290	291	123	46	5.90	ESI +

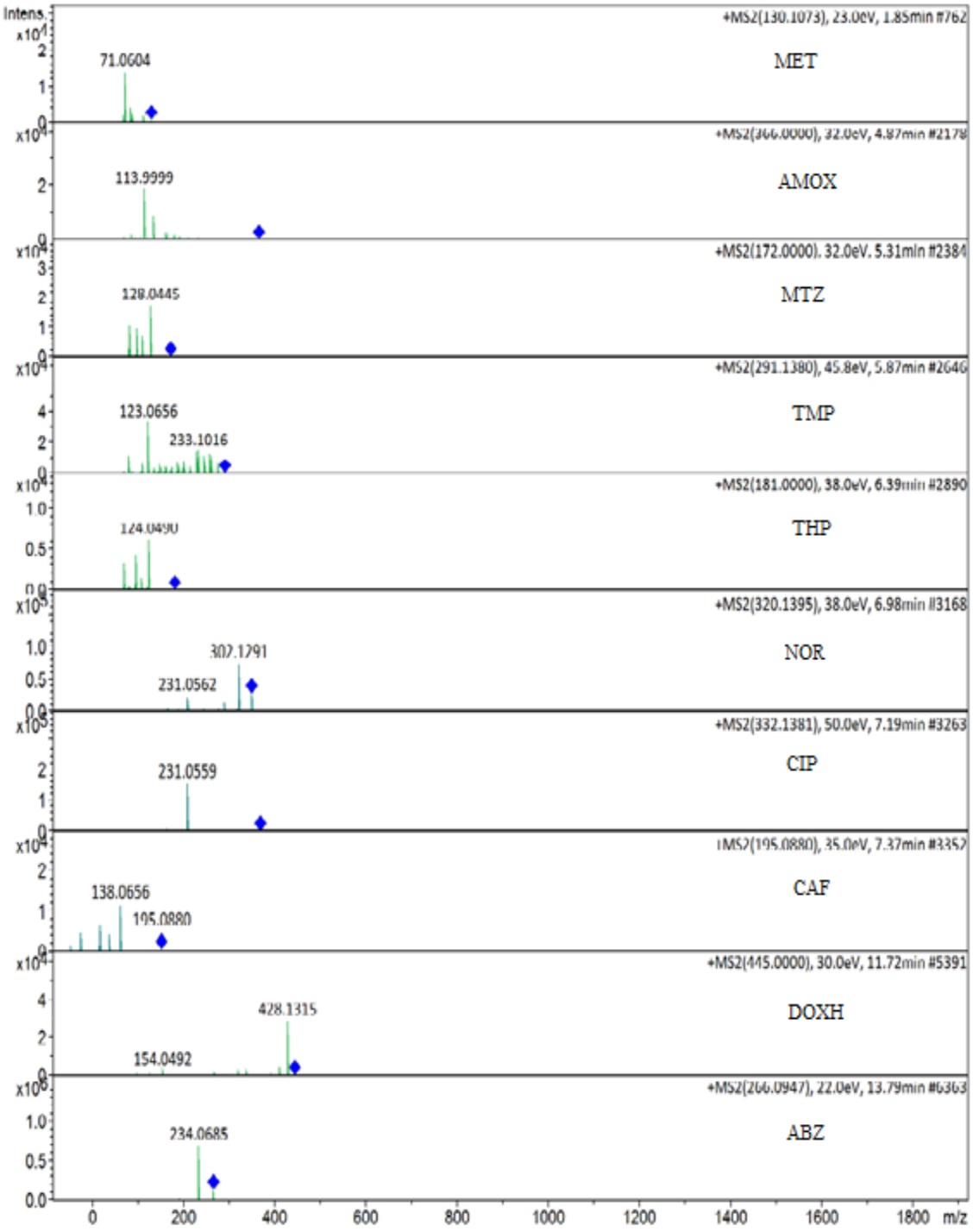


Figure 4.3. 2. Extracted ion chromatogram of the pharmaceutical compounds

4.3.2. Method validation

Identifications of pharmaceuticals in water samples were made by comparing the retention time, identifying the target and quantifier ions. Acceptance criteria for identification consisted of retention times within (± 0.50 min of the expected). Analytical quality control was performed encompassing different performance criteria such as sensitivity (LOD and LOQ), linear range, recovery and precision (Table 4.3.3). Linearity was studied analyzing standard solutions at ten concentration levels corresponding to the range of 0.5 - 1000 µg/ L. Linearity, achieved for every compound in the working standard solutions, was reliable as shown by the fact that the correlation coefficients (R^2) > 0.99. LOD and LOQ values ranged from 0.010 - 0.039 µg/ L and from 0.033 - 0.130 µg/ L. Recovery experiment on spiked river water resulted in > 65% for 70 % of the compound and the rest also recovery > 55% was obtained and which is comparable with the study reported by Kosma et al.,(2010). The developed method still gave lower %RSD values that ranged from 0.4-7.9% which are lower than the stipulated values of 15% (UNODC, 2009).

Table 4.3. 2 Performance characteristics of the proposed analytical method

Compounds	Linear range (µg/L)	Correlation coefficient (R^2)	Regression equation	LOD (µg/L)	LOQ (µg/L)	Recovery (%)	% RSD
Albendazole	0.5-1000	0.9968	Y= 1677.2x + 32654	0.024	0.079	54.9	1.3
Amoxicillin	0.5-1000	0.9979	Y = 85.266x – 1126.2	0.021	0.069	57.8	3.0
Caffeine	1.0-1000	0.9983	Y = 7.4577x + 36.472	0.021	0.071	56.7	2.2
Ciprofloxacin	1.0-1000	0.9957	Y = 349.24x+ 8929.1.7	0.039	0.130	107	6.6
Doxycycline hyclates	1.0-1000	0.9983	Y = 156.1x – 2264.6.4	0.021	0.070	84.2	7.9
Metformin	0.5-1000	0.9988	Y = 33.56x+ 546.22	0.018	0.059	65.9	3.7
Metronidazole	0.5-1000	0.9992	Y = 79.053x + 765.81	0.016	0.052	71.5	4.5
Norfloxacin	0.5-1000	0.9996	Y = 42.367x + 566.6	0.010	0.033	109	5.0
Theophylline	1.0-1000	0.9991	Y = 16.993x + 199.66	0.018	0.060	79.0	6.9
Trimethoprim	0.5-1000	0.9980	Y = 110.59x + 1227	0.019	0.062	73.7	0.4

4.3.3. Occurrence of pharmaceuticals in water samples

The determined concentration levels of pharmaceutical compounds in various samples are summarized in Table 4.3.3 which varied considerably among the sampling areas. Amoxicillin (AMOX) was the pharmaceutical compound not detected in all sampling points and caffeine was the pharmaceutical compound found in high concentration. The concentration and detection frequency (Table 4.3.4) of the nine pharmaceuticals ranged from not detected to 48.8 µg/L and 25 to 100%, respectively. Most of the frequently detected compounds in this study agrees with the study by Lin et al., (2008). Of the detected pharmaceuticals, CAF, CIP, DOXH, NOR and TRM were compounds found in all the studied samples and on average CAF was the highest in concentration followed by NOR. The presence of these compounds in water samples might be related to the commonly used pharmaceuticals for human medication in Addis Ababa, Ethiopia. The high concentration of caffeine in samples could also be due to its commonly found in foods such as coffee, some tea, cocoa, soft drinks, chocolate, dairy desserts and besides it is also a component of hundreds of prescription and over-the-counter pharmaceuticals (Kosma et al., 2010). The mean concentration of the detected pharmaceuticals increased in the order of MET < DOXH < MTZ < ALB < TRM < CIP < THP < NOR < CAF. This order is also supported by the Box and Whisker plot (Figure 4.3.3). The box and whisker plot shows the mean ± %RSD concentration and the point inside each box shows the mean concentration. Lines in each box show the first (lower) and the second (upper) quartile of the concentration values for each pharmaceutical compound. The line inside each box shows the median concentration and outlier values are marked with * symbols. The Box and Whisker plot give the indication which compound was dominantly present in the environments as it related with the consumption rate and the persistent nature of the compounds.

Table 4.3. 3 Concentration of pharmaceuticals in the sampling sites (µg/L, n = 3, %RSD)

Sites	ALB	AMOX	CAF	CIP	DOXH	MET	MTZ	NOR	THP	TRM
S1	0.034±0.013	ND	4.224±1.864	3.911±0.431	6.321±3.219	ND	3.364±1.318	6.13±0.568	2.035±1.611	3.601±0.469
S2	<LOQ	ND	1.341±0.006	1.013±0.202	0.980±0.049	ND	0.303±0.158	1.122±0.132	1.191±0.537	0.876±0.185
S3	<LOQ	ND	2.137±0.152	0.658±0.213	1.067±0.270	ND	ND	0.694±0.03	ND	0.371±0.173
S4	<LOQ	ND	2.041±0.310	1.986±0.234	0.950±0.082	0.193±0.048	0.465±0.145	2.554±0.521	9.354±3.646	4.471±0.367
S5	2.150±0.287	ND	1.291±0.650	2.283±0.158	0.811±0.062	<LOQ	0.263±0.130	2.954±0.776	0.828±0.466	1.851±0.278
S6	<LOQ	ND	2.645±0.777	1.158±0.158	1.083±0.021	ND	0.238±0.084	4.636±0.491	1.045±0.308	2.365±0.354
S7	3.518±0.217	ND	46.164±6.231	5.537±0.023	1.405±0.383	0.088±0.038	9.140±2.000	9.607±0.583	10.987±3.751	3.280±0.721
S8	2.482±0.082	ND	48.801±12.47	1.39±0.205	0.929±0.045	0.473±0.285	1.053±0.141	1.692±0.317	18.647±2.801	1.197±0.363
S9	0.019±0.001	ND	16.526±0.965	3.424±0.187	0.886±0.048	ND	0.333±0.153	5.663±0.518	5.784±0.450	1.581±0.195
S10	0.064±0.006	ND	10.911±2.839	0.920±0.141	1.280±0.094	ND	6.493±0.848	0.605±0.064	1.432±0.474	0.404±0.153
S11	0.155±0.030	ND	6.393±2.014	8.240±0.461	1.018±0.237	ND	0.439±0.119	25.054±0.855	5.793±1.455	0.403±0.076
S12	0.483±0.048	ND	14.770±1.819	14.920±0.512	1.213±0.112	ND	0.444±0.206	6.874±0.904	7.514±0.744	4.670±0.738
S13	0.049±0.034	ND	5.554±2.347	2.320±0.262	1.243±0.473	ND	0.361±0.075	1.654±0.346	2.637±1.138	1.398±0.309
S14	<LOQ	ND	3.687±2.042	2.341±0.235	1.396±0.724	<LOQ	0.702±0.368	1.072±0.163	2.623±1.512	0.393±0.117
S15	18.80±1.491	ND	31.801±1.921	18.503±0.400	1.975±0.136	2.385±0.858	1.117±0.151	13.946±0.331	17.551±2.653	23.013±0.988
S16	<LOQ	ND	1.452±0.296	2.702±0.240	2.488±0.815	ND	2.494±0.761	14.692±1.438	0.838±0.187	2.248±0.502

(ND = Not detected)

Table 4.3. 4 Frequency of detection, mean, median and ranges of pharmaceuticals detected in water samples

	ALB	CAF	CIP	DOXH	MET	MTZ	NOR	THP	TRM
Mean	2.78	12.48	4.46	1.57	0.79	1.81	6.184	5.88	3.26
Median	0.32	4.89	2.33	1.15	0.33	0.47	3.80	2.64	1.72
Ranges	0.02-18.80	1.29-48.80	0.66-18.50	0.81-6.32	0.09-2.39	0.24-9.14	0.61-25.05	0.83-18.65	0.37-23.01
FD (%)	63	100	100	100	25	94	100	94	100

(FD = frequency of detection)

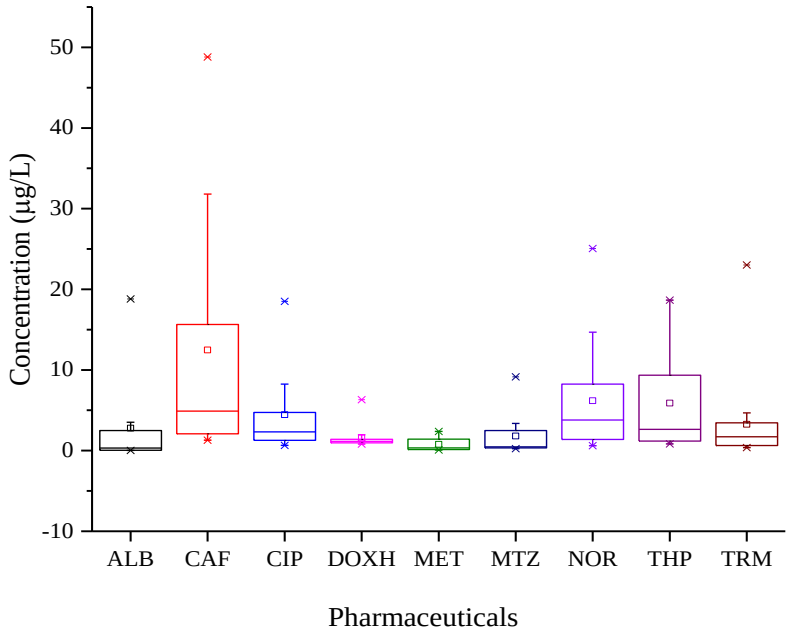


Figure 4.3. 3 Concentration of pharmaceuticals in the water sample in the Box and Whisker plot

4.3.4. Distribution of pharmaceuticals in the water samples

The distribution of the detected pharmaceuticals in the water sample from Addis Ababa, Ethiopia shows wide variations among the sampling points (Figure 4.3.4). Among nine pharmaceutical compounds detected in this study, all nine were detected in the influent of Akaki sewerage treatment plant (S15), in Menilik hospital wastewater (S7) and river water sample after the discharge of hospital wastewater (S8) sampling points. Beside the detection of all targeted pharmaceuticals, the highest cumulative concentration of compound was observed in the sewerage treatment plant influent sample. Many studies reported that hospital wastewaters contribute to the load of the pharmaceuticals in influent of a wastewater treatment plant which leads to a high presence of such compounds (Aydin et al., 2019). Some of the fragment ions in MRM mode for water sample collected for analysis were reported in (Appendix D4-7).

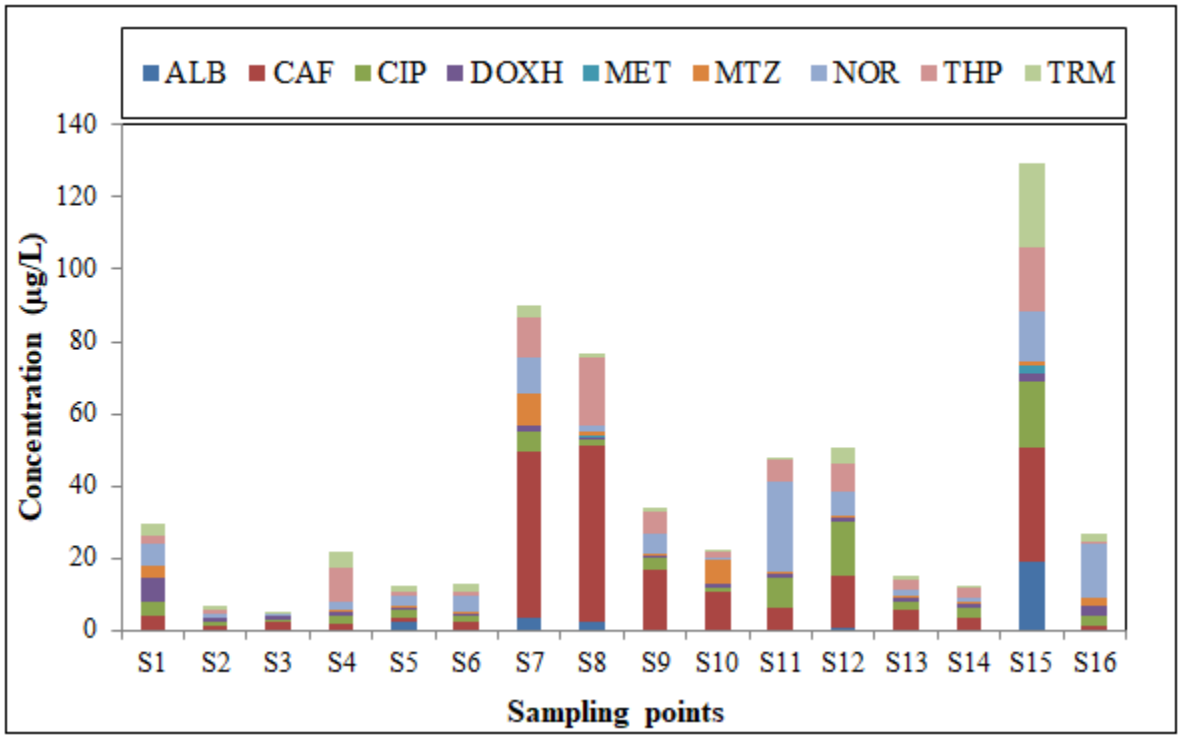


Figure 4.3. 4 Concentration of pharmaceuticals and distribution in the sampling points

4.3.5. Pharmaceuticals in the contamination source and therapeutic classes

In this section pharmaceuticals found at each potential contaminated source and distribution of the target, compounds are discussed. Targeted pharmaceuticals found at each potential contaminated source are shown in (Figure 4.3.5 (a)) and the concentration reported in (Appendix D8). Targeted pharmaceuticals are found mostly in the Akaki sewerage treatment plant (ASTP) sample followed by hospital wastewater sample and then a river water sample and finally drinking water treatment plant. However, the study done in Kenya and reported by Ngumba et al., (2016a), the concentration of pharmaceuticals in the river water sample was found to be higher than in the waste treatment plant. In the same study the concentration of CIP and TRM detected in Nairobi, Kenya river water sample was found to be much lower than reported in this study (Ngumba et al., 2016a). Sewerage treatment plant receives all household wastes, hospitals and also discharges of waste from pharmaceutical manufacturing companies. In river water samples it is also expected that pharmaceuticals can enter the river in a similar manner to that of

improper discharge of treatment plants. However, Grfersa water treatment plant (GWTP) shows less concentration of pharmaceuticals than others. This might be due to that, treatment plant collecting water far from human and animal activities.

Pharmaceuticals considered in this study can be grouped in five therapeutic classes to understand how they are distributed in the contamination source. Figure 4.3.5 (b) presented the pharmaceuticals found at each source and describes the distribution of the various classes in each source and the concentration reported in (Appendix D8). Stimulant caffeine was found in high concentration in all the contamination sources followed by xanthine theophylline. As it is obvious caffeine is found in most of the common foods, the concentration in the environmental water samples was found in high concentration. Caffeine is observed in high concentration in Hospital waste samples which shows additional intake through medication exists (Kosma et al., 2014). Antibiotics are also observed in high concentration in all contamination sources that implies the consumption and disposal of these pharmaceuticals are higher in the city. However, antidiabetic pharmaceutical was the one detected in minimum concentration even not detected in GWTP at all.

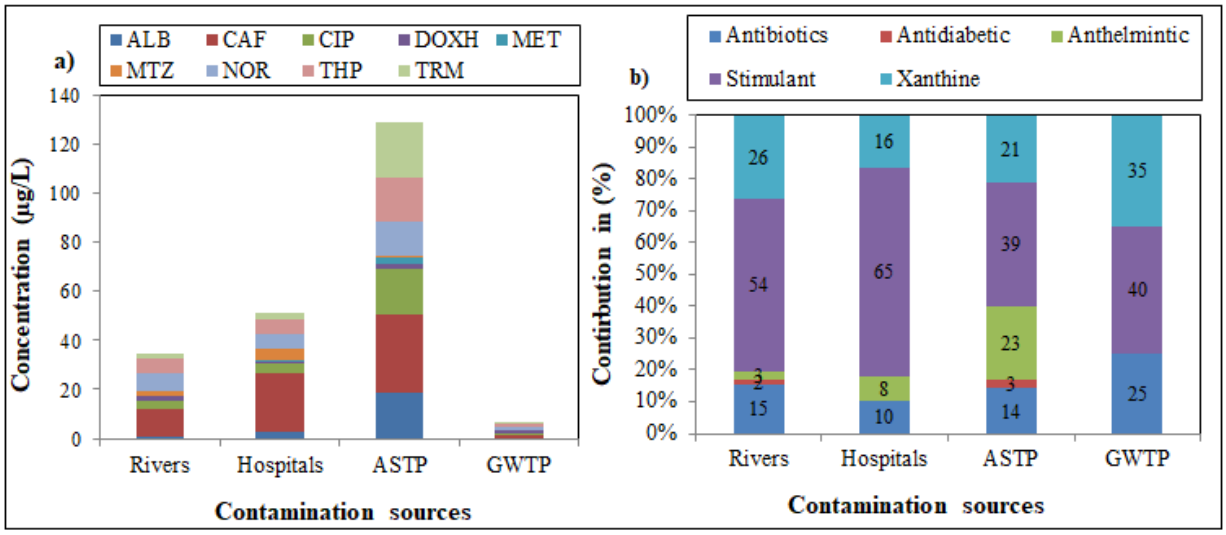


Figure 4.3. 5 Concentration of pharmaceuticals in their potential contamination sources (a) and therapeutic classes (b)

4.3.6. Efficiency of treatment plants for the removal of pharmaceuticals

The removal of organic micro-pollutants in wastewater unit processes will be influenced both by the biodegradability and physicochemical properties such as their water solubility and hydrophobicity of the compound in question and of the unit treatment process employed at the treatment plant itself (Kosma et al., 2010). Removal of pharmaceutical compounds in the wastewater treatment plant was generally the result of adsorption, transformation and biodegradation (Qiang et al., 2013; Sun et al., 2016). The removal (%) of pharmaceutical residues in the treatment plant can be calculated using the following equation considering the influent concentration ($C_{influent}$) and effluent concentration ($C_{effluent}$) (Pereira et al., 2015).

$$\% \text{ Removal} = \frac{(C_{influent} - C_{effluent})}{C_{influent}} \; x \; 100$$

Akaki sewerage treatment plant (ASTP) removal efficiencies of detected compounds was found as ALB (97.4%), CAF (53.6%), CIP (19.4%), DOXH (38.6%), MET (100%), MTZ (60.3%), NOR (50.7%), THP (57.2%) and TRM (79.7%). The removal efficiency ranged between 19.4% and 100% and the higher removal efficiency were observed for MET and ALB followed by TRM. However, lowest removal was observed for CIP which agrees with the properties of this compound having the lowest solid-liquid partitioning coefficient (Aydin et al., 2019). The high removal efficiencies from the wastewater indicated these pharmaceuticals were liable to degrade during the biological treatment or were adsorbed in the sludge (Sun et al., 2016). Removal of pharmaceuticals which were around and above 50%, indicates the partial removal of compounds in the treatment plant. On the other hand, Gefersa water treatment plant (GWTP) removal efficiency shows removal of detected pharmaceuticals as follows: CAF (-59.4%), CIP (35.0%), DOXH (-8.9%), MTZ (100%), NOR 38.1%), THP (100%) and TRM (57.6). The possible reason for the poor removal during the treatment processes might be due to the lack of pharmaceutical degrading bacteria in the microbial community of the treatment system. Besides, the grab sampling uncertainty might add some variations of pharmaceutical levels and accordingly affect the evaluation of the removal (Ort, 2010).

4.3.7. Environmental risk assessment

Environmental risk assessment of the detected pharmaceuticals in this study was evaluated with the risk quotient ((a ratio between measured effluent concentration (MEC) and predicted no-effected concentration (PNEC)). The predicted-none effect concentration (PNEC) of each compound detected was calculated for three trophic levels (daphnia manga, algae and fish) organisms by using LC_{50} and EC_{50} obtained from ECOSAR vs 2.0 and uncertainty factor (UF) of 1000 (Table 4.3.5). The representative organism algae EC_{50} was obtained while for others LC_{50} was obtained using the predictive model ECOSAR and used for the calculations of PNEC of the compounds in water sampling sources.

Table 4.3. 5 Predicted no-effect concentration (PNEC) of the selected pharmaceuticals for algae, daphnia magna and fish for the studied pharmaceuticals

Pharmaceuticals	**CAS no.**	**PNEC µg/L Algae[a,b]**	**PNEC µg/L Fish[c,b]**	**PNEC µg/L Daphnia magna [c,b]**
ALB	54965-21-8	0.3872	1.0831	3.5945
CAF	58-08-2	6.6339	693.89	163.56
CIP	85721-33-1	1621.6	13131	1240.4
DOXH	24390-14-5	1998.9	13861	1117.3
MET	657-24-9	4624.6	27737	1927.7
MTZ	443-48-1	6.9196	878.33	179.75
NOR	70458-96-7	2567.5	20081	1830.8
THP	58-55-9	10.982	2234.5	324.04
TRM	738-70-5	20.745	211.62	6.3804

[a]EC50 was estimated with ECOSAR, [b]UF = 1000 and [c]LC50 was estimated with ECOSAR v.2.0

The risk quotient calculated for all contaminated source used for water sampling. However, Gefersa water treatment plant (GWTP) influent and effluent was not included because more than half of the compounds was not detected in the sample water. RQs were deemed for all the

detected pharmaceuticals in Rivers, Hospitals and Akaki sewerage treatment plant influent and effluent are presented in Table 4.3.6 and the graphic presentation in Figure 4.3.6. In risk quotient calculation, MEC was the average concentration of the detected pharmaceuticals in each contamination sources considered in this study.

In contamination sources river, the risk quotient value presented in the Table below and in Figure 4.3.6 (a) for the three selected organisms (algae, daphnia magna and fish). This contamination source for algae community shows risk quotient values ranged from no risk, RQ < 0.1(CIP, DOXH, MET, NOR and TRM) to low risk, 0.1< RQ < 1 (MTZ and THP) and moderate risk,1< RQ <10 (CAF and ALB). A daphnia magna risk quotient value shows no risk, for all pharmaceuticals except TRM which could cause low risk. Fish risk quotient values shows no risk for all pharmaceuticals except ALB which could cause low risk.

In contamination sources hospital, the risk quotient value presented in Table 4.3.6 and in Figure 4.3.6.(b) for the three selected organisms. This contamination source for algae community shows risk quotient values ranged from no risk, RQ < 0.1 (CIP, DOXH, MET and NOR) to low risk, 0.1< RQ < 1 (THP and TRM) and moderate risk,1< RQ <10 (CAF and ALB) which is somehow similar to the river contamination sources effects in the organisms. The daphnia magna risk quotient shows no risk for all pharmaceuticals except for ALB and CAF which causes low risk to the community level. The fish risk quotient observed from no risk for all pharmaceuticals except ALB which shows moderate risk.

In contamination sources ASTP, the risk quotient was calculated in to a condition that is using concentration in the influent and effluent separately and the risk was characterized accordingly. The risk quotient value presented in Table 4.3.6 and in Figure 4.3.6. (c) for the influent and Figure 4.3.6. (d) for effluent respectively, using the three selected organisms. This contamination source ASTP influent for algae community shows risk quotient values ranging from no risk, RQ < 0.1(CIP, DOXH, MET and NOR), moderate risk 1< RQ <10 (CAF, THP and TRM) and high risk, RQ > 10 (ALB). The daphnia magna risk quotient shows moderate risk for ALB and TRM and CAF which causes low risk to the community level. The rest of the pharmaceuticals pose no risk to the organism in fish.

On the other hand ASTP effluent, for algae community shows risk quotient values ranging from no risk for majority of pharmaceuticals to moderate risk for (ALB and CAF) and low risk for (THP and TRM) in the organism. The daphnia magna risk quotient observed from no risk for all pharmaceuticals except for ALB and TRM which could pose low risk. The fish risk quotient observed from no risk for all pharmaceuticals except for ALB which could pose low risk to the community.

The result indicates that the risk of the selected analytes in water sample collected might be substantially higher than the estimated values. In addition, the risk was considered for individual compound, but it should be noted that pharmaceutically active compounds normally are simultaneously present in the environment, which increases the overall risk via the mixture effect (Ngumba et al., 2016a).

Table 4.3. 6 Risk quotients for the different contamination sources based on the mean detected concentration

Analyte	River			Hospital			ASTP influent			ASTP effluent		
	Algae	Daphnia magna	Fish	Algae	Daphnia magna	Fish	Algae	Daphnia magna	Fish	Algae	Daphnia magna	Fish
ALB	1.42E+00	1.53E-01	5.08E-01	7.31E+00	7.88E-01	2.62E+00	4.85E+01	5.23E+00	1.74E+01	1.25E+00	1.34E-01	4.46E-01
CAF	1.75E+00	7.12E-02	1.68E-02	3.58E+00	1.45E-01	3.42E-02	4.79E+00	1.94E-01	4.58E-02	2.23E+00	9.03E-02	2.14E-02
CIP	1.83E-03	2.39E-03	2.26E-04	2.41E-03	3.15E-03	2.98E-04	1.14E-02	1.49E-02	1.41E-03	9.20E-03	1.20E-02	1.14E-03
DOXH	9.35E-04	1.67E-03	1.35E-04	5.54E-04	9.92E-04	7.99E-05	9.88E-04	1.77E-03	1.42E-04	6.07E-04	1.09E-03	8.75E-05
MET	7.20E-05	1.73E-04	1.20E-05	1.90E-05	4.56E-05	3.17E-06	5.16E-04	1.23E-03	8.59E-05	---	---	---
MTZ	2.69E-01	1.03E-02	2.12E-03	6.79E-01	2.62E-02	5.35E-03	1.61E-01	6.21E-03	1.27E-03	6.42E-02	2.47E-03	5.06E-04
NOR	2.97E-03	4.17E-03	2.97E-03	2.45E-03	3.43E-03	3.13E-04	5.43E-03	7.62E-03	6.94E-04	2.68E-03	3.75E-03	3.42E-04
THP	5.11E-01	1.73E-02	2.51E-03	5.38E-01	1.82E-02	2.64E-03	1.59E+00	5.42E-02	7.85E-03	6.84E-01	2.32E-02	3.36E-03
TRM	9.80E-02	3.19E-01	9.61E-03	1.24E-01	4.02E-01	1.21E-02	1.11E+00	3.61E+00	1.09E-01	2.25E-01	7.32E-01	2.21E-02

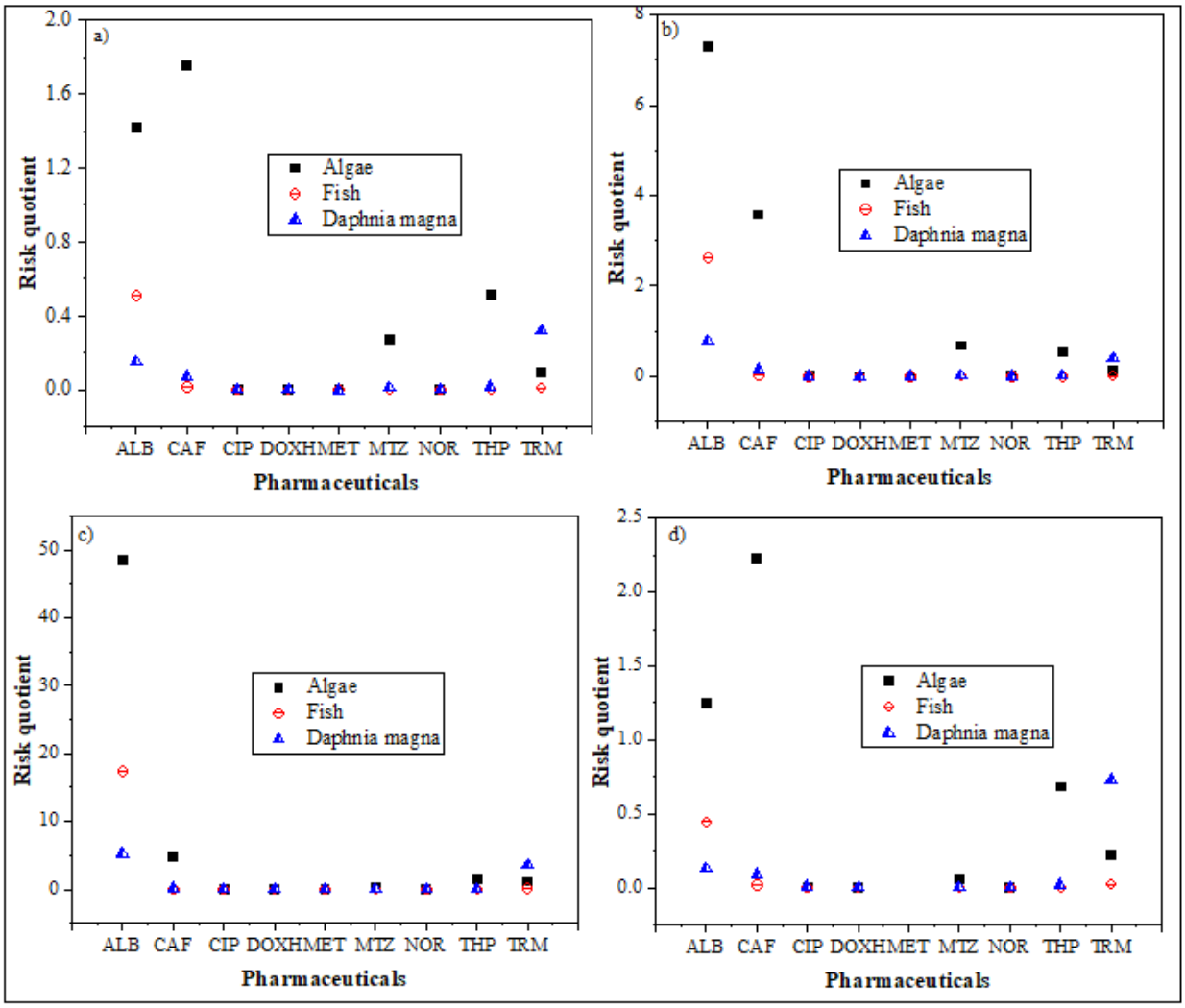


Figure 4.3. 6. Environmental risk for sensitive aquatic organisms calculated based on risk quotients using mean as measured concentrations of detected pharmaceuticals (a), River; b), Hospitals; c), ASTP influent and d), ASTP effluent)

4.4. Development of modified QuEChERS method for the determination of selected pharmaceuticals in vegetable samples

Vegetable samples were used to develop a modified QuEChERS extraction method as sample preparation for pharmaceutical compounds residue analysis. QuEChERS was first used for the analysis of pesticides from vegetables. However, in this study, we are going to modify the original method to make it more suitable for the analysis of pharmaceuticals. Parameters affecting the extraction efficiency of this method were optimized and discussed here. Figure 4.4.1 shows the vegetable powder and extraction with the QuEChERS modified method.

Figure 4.4. 1. Vegetable powder sample for extraction using the QuEChERS method

4.4.1. Modified QuEChERS method

The original QuEChERS method was successfully applied for the extraction of pesticides in cereals, fruits, vegetables, and others (Anastassiades et al., 2003). This method uses acetonitrile as an extraction solvent followed by a partition step, promoted by the addition of magnesium sulfate and sodium chloride. However, due to the effectiveness of the method, it has been applied in other applications for different types of analytes and matrices through several modifications (Barci et al., 2020). Many modifications of the QuEChERS method have been proposed in recent years for different applications. In this study, to get the best extraction result of the QuEChERS method, various parameters affecting the extraction efficiency were optimized. Several factors including clean up sorbent, the effect of diatomaceous earth, amount of diatomaceous earth, the effect of EDTA, amount of EDTA, extraction solvent, the volume of extraction solvent, salt type

and extraction time were optimized. The peak area of the selected analyte was used to evaluate the optimum parameters affecting the extraction efficiency. Results of the various optimized parameters are given below.

4.4.1.1. Type of sorbent for cleanup

The effective cleanup step in the QuEChERS extraction method on the complex matrices reduces co-extracted interferences and many studies considered the use of different sorbents (Kaczyński and Łozowicka, 2017). Particularly from plant extract, large pigment molecules may clog the chromatographic column and interfere with the separation process (Tankiewicz, 2019). Dispersive solid-phase extraction (d-SPE) cleanup steps employ a different type of sorbent including, magnesium sulfate ($MgSO_4$), primary secondary amine (PSA), graphitized carbon black (GCB), and octadecyl (C_{18}). This experiment aims to see the potential of different cleanup sorbent in the QuEChERS extraction of selected pharmaceuticals from vegetable samples. Cleanup steps based on different d-SPE sorbents (PSA, PSA+C_{18} and PSA+C_{18}+GCB with $MgSO_4$) combinations and without cleanup were compared (Figure 4.4.3). As shown in (Figure 4.4.2) and the cleanup d-SPE sorbents combination of PSA+C_{18} with $MgSO_4$ gives a relatively higher peak area for all compounds except for trimethoprim. However, the addition of GCB decreases the peak area of some analytes which could be due to GCB being planer which might allow it to bind to some compounds with a similar structure.

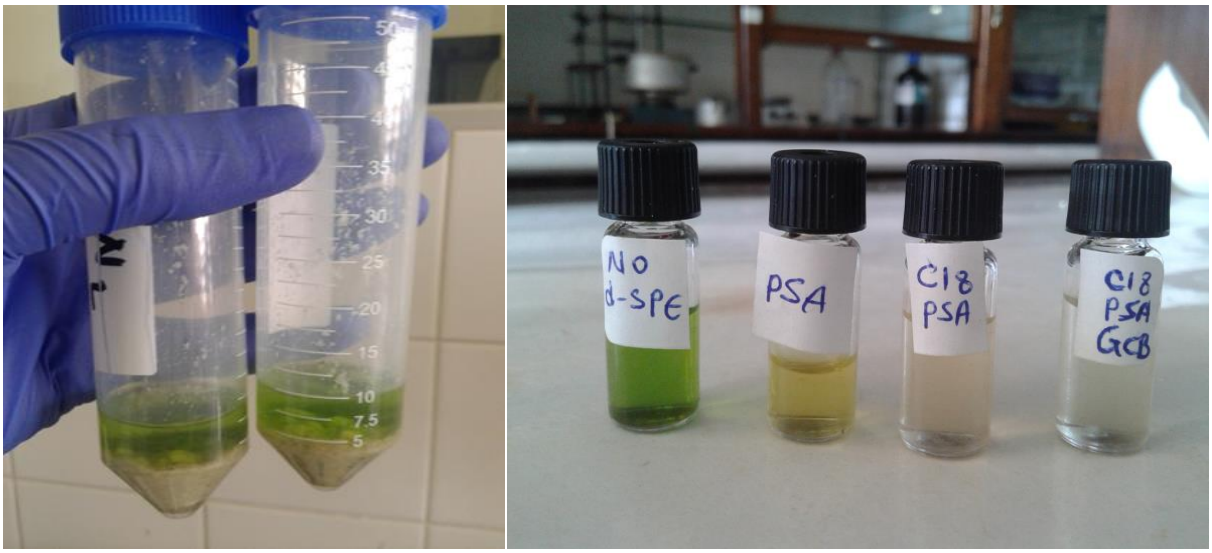


Figure 4.4. 2. Effect of clean-up sorbent on QuEChERS extraction

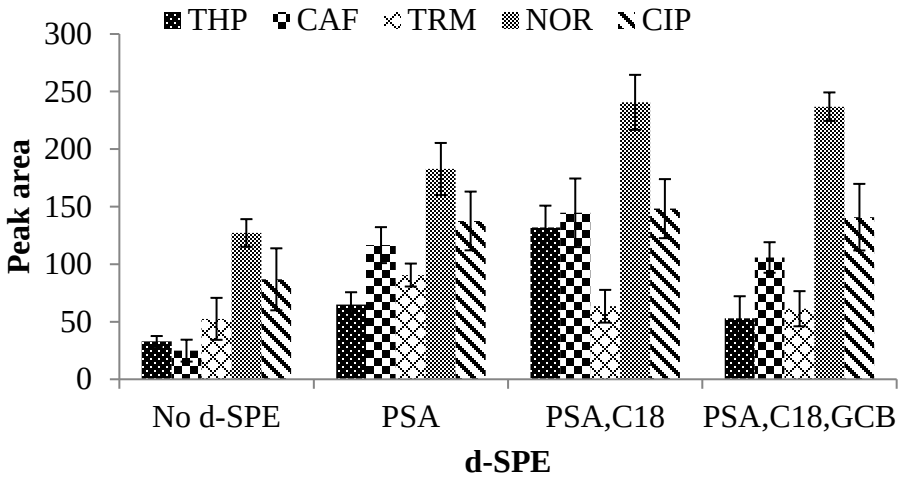


Figure 4.4. 3. Effect of type of d-SPE sorbent for cleanup (THO, theophylline; CAF, caffeine; TRM, trimethoprim; NOR, norfloxacin and CIP, ciprofloxacin). Extraction condition: vegetable sample 0.5 g: extraction solvent methanol: volume of solvent 4 mL: salt $MgSO_4$: NaCl extraction time of 8 min and spiked concentration 5 µg/g for each compound, n = 3

4.4.1.2. Effect of diatomaceous earth (DE) as clean-up and the effect of its dosage in the extraction

Diatomaceous earth is used for cleanup in the QuEChERS extraction method for the vegetable matrix to avoid interferences as it is an environmentally friendly natural sorbent (Kaczyński and Łozowicka, 2017). The effect of the addition of diatomaceous earth was evaluated after the d-SPE sorbent was selected. Two sets of the experiment were conducted to compare the effect of with and without diatomaceous earth as cleanup after extraction to reduce the interference that increases the value of the target compound in the detection. In the same extraction condition, the cleanup of a mixture of diatomaceous earth (50 mg) together with the d-SPE sorbent selected in the section above and the other with only d-SPE sorbent was compared. As it is reported in (Figure 4.4. 4) below the presence of diatomaceous earth in clean up increases the peak is of the target compound compared with the one without it.

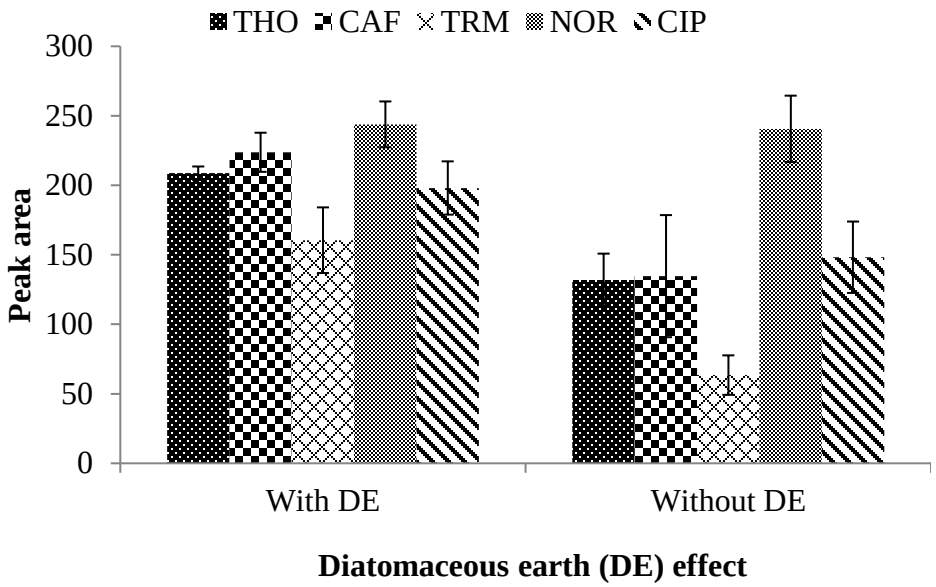


Figure 4.4. 4. Effect of diatomaceous earth (DE) on the extract cleanup. Condition: vegetable sample 0.5 g: extraction solvent methanol: volume of solvent 4 mL: salt $MgSO_4$: NaCl, extraction time of 8 min: cleanup $MgSO_4$ (250 mg), C_{18} (25 mg), PSA (25 mg), and spiked concentration 5 µg/g for each compound, n = 3.

As it is observed that the addition of diatomaceous earth in the cleanup step has a positive effect in extraction from the vegetable matrix, the amount of it also should be optimized to obtain a suitable amount for cleanup. Different amounts of diatomaceous earth in cleanup (5, 10, 50, 100 and 200 mg) were used to evaluate their extraction efficiency by reducing the matrix interference. As it is reported in (Figure 4.4. 5), at the beginning as the amount of diatomaceous earth increases the peak area also increases up to 50 mg. However, the peak area starts to decrease after 50 mg of diatomaceous earth amount as cleanup sorbent. So that amount of 50 mg diatomaceous earth was used as an optimum amount of sorbent for cleanup also with the selected d-SPE cleanup sorbet for selected pharmaceutical compounds.

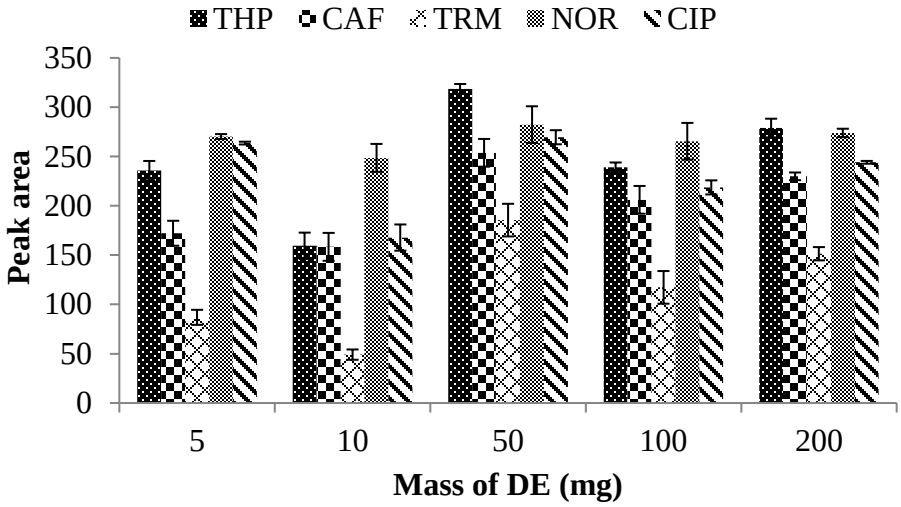


Figure 4.4. 5. Amount of diatomaceous earth (DE) on the extract cleanup. Conditions: vegetable sample 0.5 g: extraction solvent methanol: volume of solvent 4 mL: salt $MgSO_4$: NaCl, extraction time of 8 min: cleanup $MgSO_4$ (250 mg), C_{18}(25 mg), PSA(25 mg), and spiked concentration 5 µg/g for each compound, n = 3

4.4.1.3. Effect of EDTA on the extraction and amount in the extraction

Many studies reported the use of EDTA to increase the extraction recovery of pharmaceuticals from environmental samples to facilitate the extraction of bound compounds by avoiding the complexation of analytes with cations (Aguilera-Luiz et al., 2008; Bourdat-Deschamps et al.,

2014). The effect of EDTA on the extraction of pharmaceuticals from vegetables using the QuEChERS extraction technique was evaluated. As is reported in Figure 4.4.6 the extraction with and without EDTA has a significant difference for extraction as the peak area improved with the presence of EDTA. The result of extraction improvement on the addition of EDTA (highly in ciprofloxacin and norfloxacin) is in agreement with the study reported by Choi et al., (2009). The next step could be obtaining the optimum amount of EDTA which will be sufficient for the extraction of pharmaceuticals from the vegetable sample. Different concentration levels of EDTA were evaluated (0.015, 0.045, 0.135 and 0.405, (% w/v)) (Figure 4.4. 7) for their extraction efficiency using the peak area of each compounds. The concentration level of 0.135 % w/v EDTA was found to be sufficient for extraction in comparison with the other level. As the concentration level of EDTA increases beyond this, the peak area decreases that is because the EDTA starts to interact with the compound that the compound and decreases the solubility to the extraction solvent.

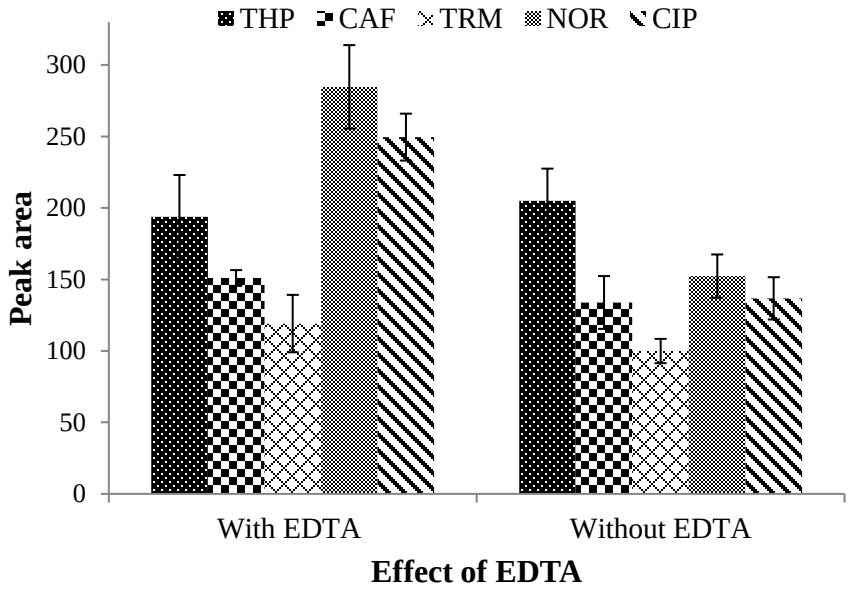


Figure 4.4.6. Effect of EDTA on the extraction. Condition: vegetable sample 0.5 g: extraction solvent methanol: volume of solvent 4 mL: salt $MgSO_4$: NaCl, extraction time of 8 min: cleanup $MgSO_4$ (250 mg), C_{18}(25 mg), PSA(25 mg), and spiked concentration 5 µg/g for each compound, n = 3

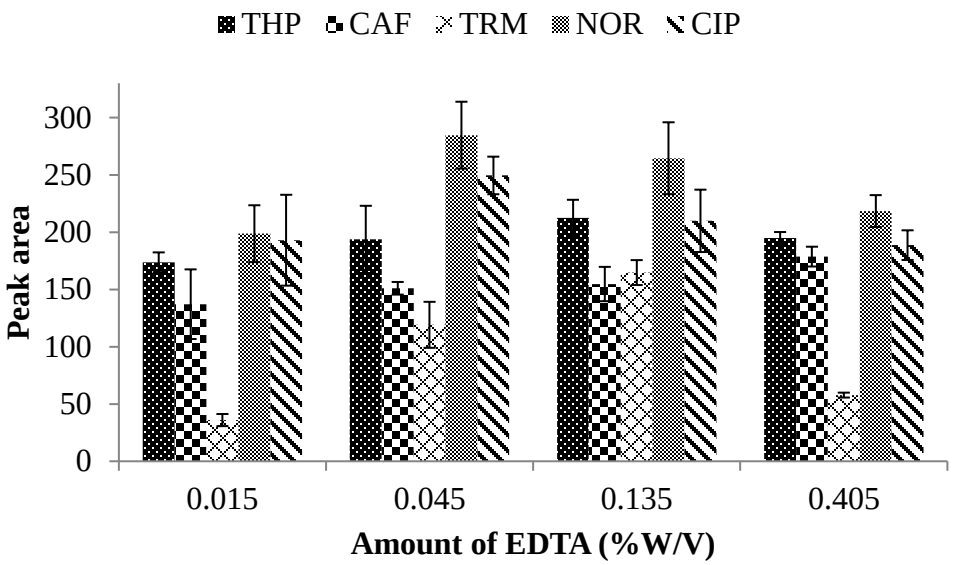


Figure 4.4. 7. Effect of amount of EDTA on the extract. Extraction condition: vegetable sample 0.5 g: extraction solvent methanol: volume of solvent 4 mL: salt $MgSO_4$: NaCl, extraction time of 8 min: cleanup $MgSO_4$ (250 mg), C_{18} (25 mg), PSA (25 mg) and spiked concentration 5 µg/g for each compound, 1 mL EDTA, n = 3

4.4.1.4. Effect of type of extraction solvent and volume in the extraction

The choice of extraction solvent is one of the most important parameters in the QuEChERS sample extraction technique (Vera et al., 2013). For extraction of selected pharmaceuticals in vegetable samples five sets of different solvent was employed for the extraction efficiency. The solvents involved in this study were acetonitrile, methanol, acetonitrile with methanol (50;50, v/v), acetic acid with methanol (20:80, v/v) and formic acid with methanol (20:80, v/v). The extraction efficiency was examined with the spiked vegetable sample considering the peak area of each compound. The highest peak area was obtained when pure methanol was used as an extraction solvent for all pharmaceutical compounds as shown in Figure 4.4.8. As it is obvious pharmaceuticals are polar, so they need to have polar solvent to extract. On the other hand, acetonitrile as it is shown in the result it cannot extract even all compounds from the vegetable sample with only theophylline and caffeine appearing. The addition of additives like acetic acid and formic acid in methanol and acetonitrile does not improve the extraction over the pure methanol. The volume of the selected solvent was evaluated for the optimum amount to be

sufficient for the extraction of selected pharmaceuticals from vegetable samples. The different volume of methanol 4, 5, 6 and 8 mL was employed as the extraction solvent and the extraction efficiency was employed by considering the peak area of the target compounds. As it is reported in (Figure 4.4. 9) a volume of 5 mL methanol was observed to be the optimum volume for maximum extraction of pharmaceuticals.

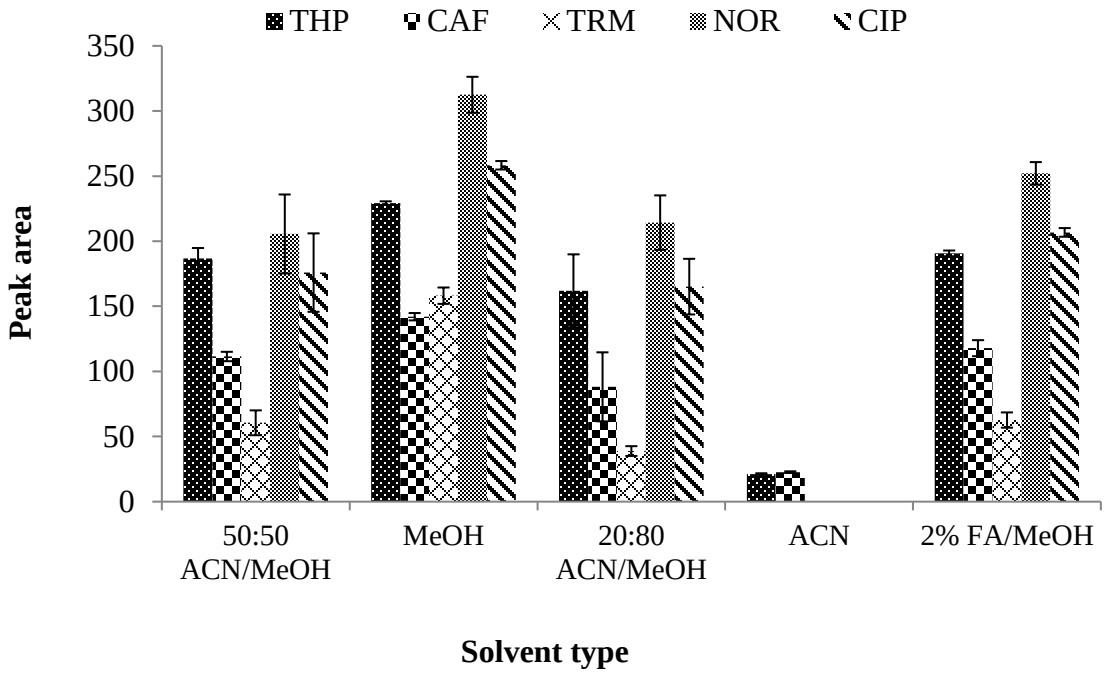


Figure 4.4.8. Effect of type of extraction solvent for extraction. Extraction condition: vegetable sample 0.5 g: volume of solvent 4 mL: salt $MgSO_4$: NaCl, extraction time of 8 min: cleanup $MgSO_4$ (250 mg), C_{18}(25 mg), PSA(25 mg), DE (50 mg), 0.135 (% w/v) 1 mL EDTA and spiked concentration 5 µg/g for each compound, n = 3

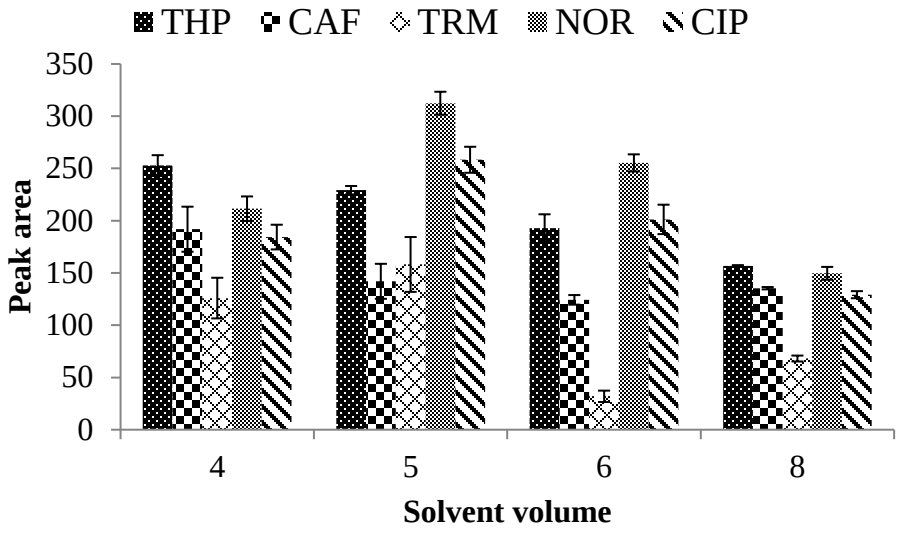


Figure 4.4.9. Effect of volume of extraction solvent for extraction. Extraction condition: vegetable sample 0.5 g: extraction solvent methanol: salt MgSO4: NaCl, extraction time of 8 min: cleanup $MgSO_4$ (250 mg), C_{18} (25 mg), PSA (25 mg), DE (50 mg), 0.135 (% w/v) 1 mL EDTA and spiked concentration 5µg/g for each compound, n = 3.

4.4.1.5. Effect of type of extraction salt

In QuEChERS, the initial single-phase extraction with solvent is followed by the addition of salts to induce phase separation (Vera et al., 2013). Different salts were used to assess their salting-out effect for partitioning. Including QuEChERS common salt magnesium salt and sodium chloride ($MgSO_4$: NaCl) other salts like sodium acetate (NaOAC) alone and with sodium chloride and ammonium acetate with sodium chloride (NH_4OAC: NaCl). From the salts considered for their extraction efficiency, the QuEChERS common salt magnesium sulfate and sodium chloride was the one that gives the highest extraction efficiency in terms of peak area of the targeted compound from the vegetable sample (Figure 4.4.10).

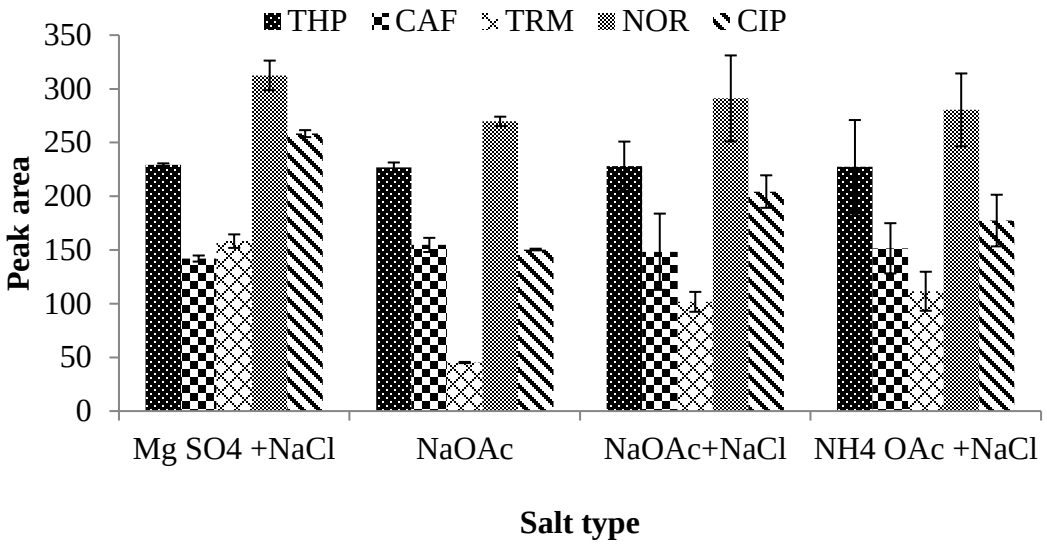


Figure 4.4.10. Type of salt for extraction: Extraction condition: vegetable sample 0.5 g: extraction solvent methanol 5 mL, extraction time of 8 min: cleanup $MgSO_4$ (250 mg), C_{18} (25 mg), PSA (25 mg), DE (50 mg), 0.135 (% w/v) 1 mL EDTA and spiked concentration 5 µg/g for each compound, n = 3

4.4.1.6. Effect of extraction time

Extraction time in the QuEChERS extraction method plays a role as the mass transfer takes time to transfer from one phase to the other phase. This parameter is reported by Vera et al.,(2013) found to be one of the basic to be optimized in the extraction method optimization. The extraction time was the time after mixing the extraction solvent with the extraction salt together with the spiked vegetable sample. Extraction time from 5 min, 10 min, 15 min and 20 min was employed for the extraction time to extract the analyte from the vegetable sample. As it is presented in (Figure 4.4. 11) 10 min extraction time was the maximum time that gave the highest peak area for all compounds.

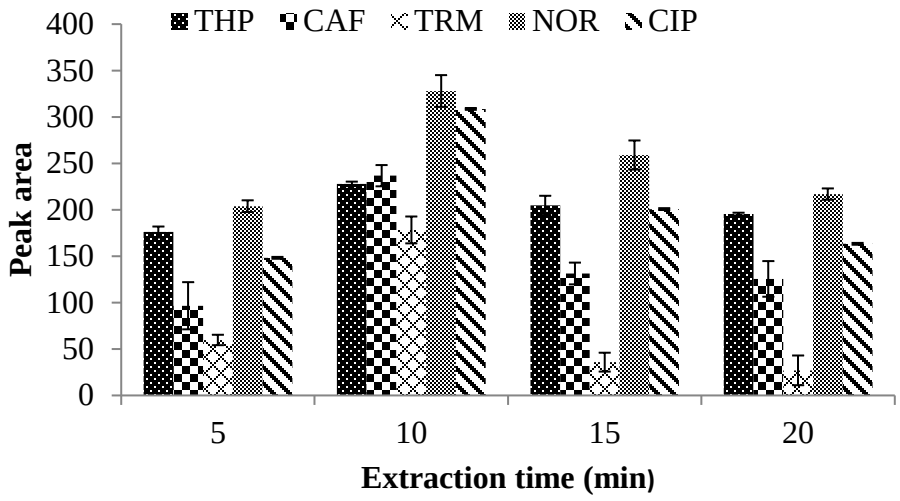


Figure 4.4.11. Effect of extraction time. Extraction condition: vegetable sample 0.5 g: extraction solvent methanol: volume of solvent 5 mL: extraction time of 8 min: cleanup $MgSO_4$ (250 mg), C_{18} (25 mg), PSA (25 mg), DE (50 mg), 0.135 (% w/v) 1 mL EDTA and spiked concentration 5 µg/g for each compound, n = 3

4.4.2. Analytical performance characteristics

The optimized QuEChERS method for analysis of selected pharmaceutical compounds was examined using analytical parameters. The linearity (using the standard solution and matrix-matched calibration curve), the limit of detection (LOD), limit quantification (LOQ), accuracy and precision were parameters considered in this study.

4.4.2.1. Linearity, the limit of detection and quantification

Linearity was evaluated using standard mixture solution and matrices matched using spiked cabbage vegetable at a different level in the range 10-10,000 µg/L and 1-10 µg/g, respectively. The peak areas of each analyte were plotted against the concentrations and linear regression analysis was performed to determine the linearity equation and correlation coefficient. The result obtained in Table 4.4.1 and 4.4.2 confirmed good linearity between analytical signal and analyte concentration for all selected pharmaceutical compounds considered in this study. The sensitivity of the optimized QuEChERS method for the determination of selected pharmaceutical

compounds from the vegetable sample was evaluated using the limit of detection (LOD) and limit of quantification (LOQ) of each analyte.

Table 4.4. 1. Analytical performance of QuEChERS for the determination of selected pharmaceuticals in vegetables using a standard mixture

Compound	Range (µg/L)	R^2	Equation	LOD (µg/L)	LOQ (µg/L)
Caffeine	10-10,000	0.9956	y = 81.417x - 6.906	0.416	1.26
Ciprofloxacin	10-10,000	0.9965	y = 157.59x - 8.0178	0.277	0.839
Norfloxacin	10-10,000	0.9964	y = 179.04x - 10.488	0.282	0.854
Theophylline	10-10,000	0.9956	y = 90.407x - 7.8265	0.416	1.26
Trimethoprim	50-10,000	0.994	y = 25.277x - 0.853	0.58	1.78

Table 4.4. 2. Analytical performance of QuEChERS for the determination of selected pharmaceuticals in vegetables using matrixes matched analysis.

Compound	Range (µg/g)	R^2	Equation	LOD (µg/g)	LOQ (µg/g)
Caffeine	0.5-10	0.9401	y = 14.741x+26.453	0.010	0.030
Ciprofloxacin	0.5-10	0.9558	y = 24.94x+41.693	0.005	0.014
Norfloxacin	0.5-10	0.9973	y = 33.78x+30.535	0.001	0.003
Theophylline	0.5-10	0.9342	y = 16.348x+28.51	0.006	0.018
Trimethoprim	1-10	0.9434	y = 8.286x+6.153	0.003	0.009

4.4.2.2. Precision

The precision of the optimized method was investigated by calculating the relative standard deviation (% RSD) in terms of reproducibility (between-day) and repeatability (within-day) to

cabbage sample spiked with two concentration levels. In each case, three replicate spiked vegetable (cabbage) samples were analyzed in the same optimized parameters. The precision result reports repeatability was obtained and reproducibility which was satisfactory for all selected pharmaceutical compounds Table 4.4.3.

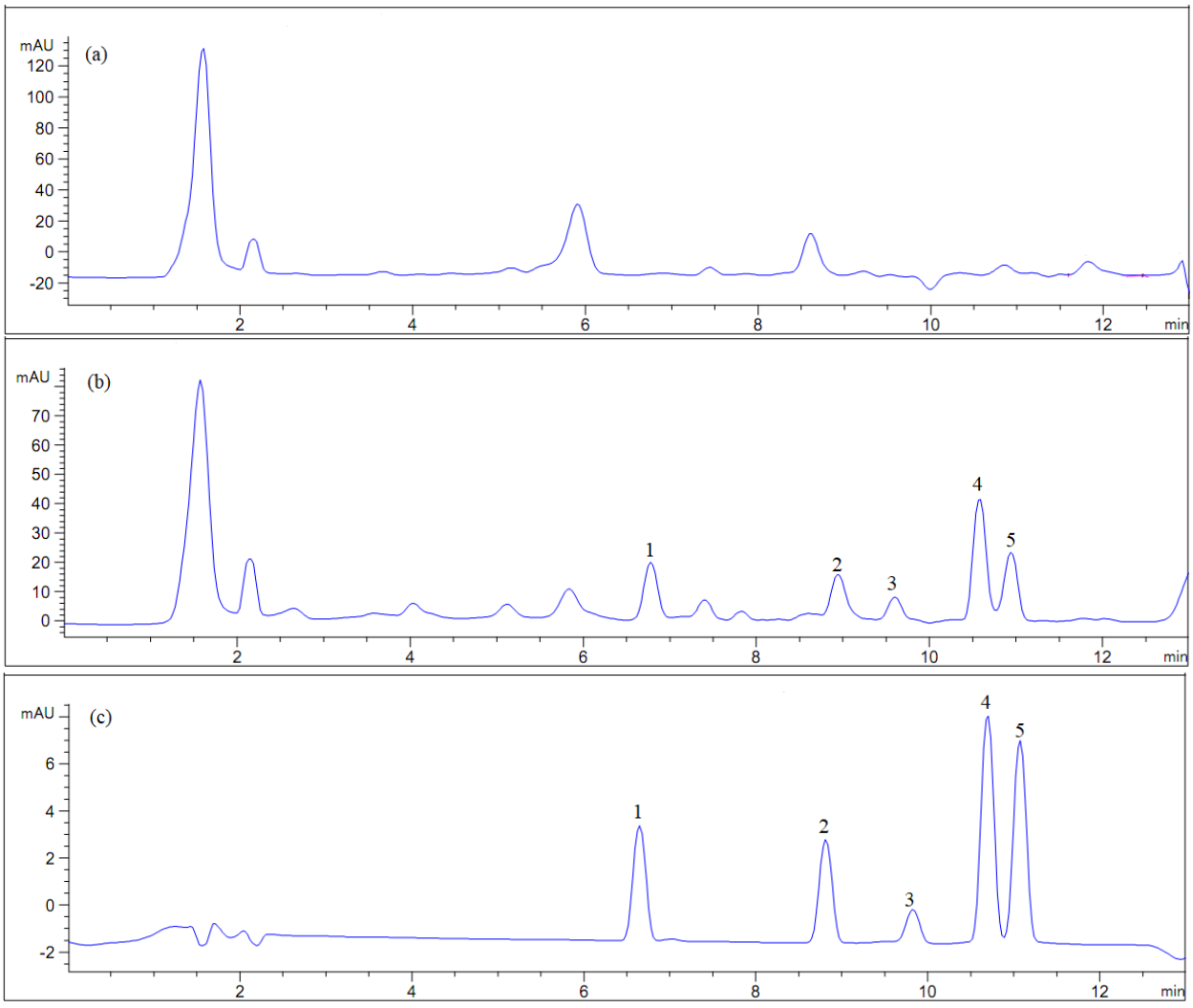
Table 4.4. 3. Analytical performance precision of the optimized QuEChERS for the determination of pharmaceuticals from vegetables

pharmaceuticals	**Intra-day,% RSD (n=3)**		**Inter-day,% RSD (n=6)**	
	Level 1	**Level 2**	**Level 1**	**Level 2**
Theophylline	2.83	4.56	5.405	8.45
Caffeine	1.63	5.66	4.86	4.39
Trimethoprim	0.64	6.44	4.43	6.31
Norfloxacin	4.60	6.56	5.85	11.33
Ciprofloxacin	0.78	11.81	6.85	8.73

(Level 1= 1 µg/g, Level 2=5 µg/g)

4.4.2.3. Selectivity

The selectivity of the method was evaluated by analyzing extracted cabbage, which was spiked previously by a mixture of the standard solution. The selectivity study was used to observe the interference compound in the vegetable matrix. The chromatogram in Figure 4.4.12 (a), (b) and (c) shows about high selectivity of the optimized method towards the selected compound which was promising for further analysis of the vegetable sample.



(1- Theophylline, 2- Caffeine, 3- Trimethoprim, 4-Norfloxacin and 5- Ciprofloxacin)

Figure 4.4.12. Chromatogram of the selected compound and vegetable (a) cabbage extract, (b) spiked cabbage and extract using optimized QuEChERS and (c) standard mixture solution.

4.4.2.4. Matrix effects evaluation

Matrix effects that can cause suppression or enhancement of analytical signals are frequently observed in the chemical analysis field. This phenomenon is due to matrix compounds that are eluted with the same retention time as the target compounds. Matrix effects depend on the nature of the matrix and the efficiency of the sample preparation step. Therefore, the sample preparation step should eliminate interfering compounds while retaining the target analytes (Miossec et al., 2018; Stuber and Reemtsma, 2004; Yang et al., 2005). To study this phenomenon, solvent,

estuarine and marine sediments were spiked by adding 1 µg/g of the target compounds and the optimized QuEChERS procedure Figure 4.4.13 (a) and (b).

$$\text{Matrix effect (\%)} \quad \left(\frac{\text{A(Spiked)} - \text{A(Blank)}}{\text{A(Solvent)}} - 1\right) \text{x } 100 \qquad (13)$$

where: A (matrix) is the area in the spiked matrix, A (blank) is the area in the non-spiked matrix and A (solvent) is the area in the spiked solvent without sample matrix.

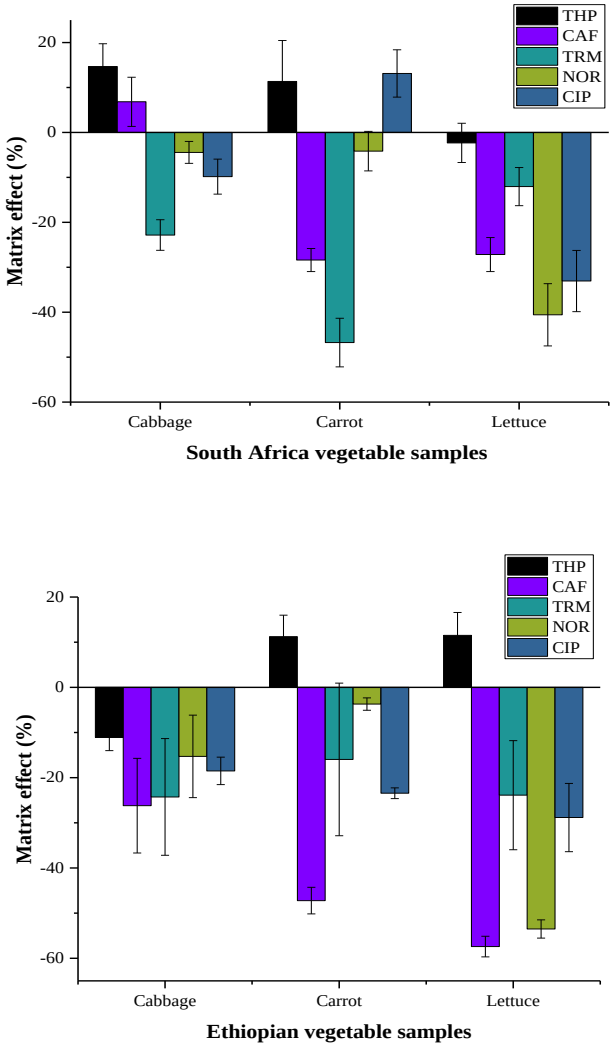


Figure 4.4.13. Matrix effect (%) calculated for all target analytes in vegetable samples from South Africa and Ethiopia.

The calculated matrix effect results showed variability among the targeted compounds and also among the different types of vegetable samples and between the sampling areas. Matrix effect, signal suppression has been observed to be higher in the vegetable lettuce followed by carrot and cabbage in both sampling countries. Matrix effects values ranged from −46.7% to + 14% in the South Africa sample, while values ranged from −57.4% to + 16.9% in the Ethiopian vegetable sample. The addition of deuterated internal standards is therefore recommended to correct matrix effects.

4.4.2.5. Application and recovery studies

Vegetables commercially available (cabbage, carrot and lettuce) were considered for application to the proposed method. The selected analytes were not detected in any of the studied vegetable samples. To evaluate the accuracy of the proposed method, recovery studies were carried out with spiking at two different concentration level using vegetable samples (cabbage, carrot and lettuce) collected from two countries (Ethiopia and South Africa) from the main vegetable site and the result obtained is presented in Table 4.4.4. In both concentration levels, the recoveries obtained with the current method were in the range of 33.5 to 115%. The lower and the higher recovery values were obtained from South Africa, Johannesburg vegetable samples.

Table 4.4. 4. Analytical performance recovery (% R) and relative standard deviation (% RSD) for each selected pharmaceutical compounds in vegetable (n = 3)

Vegetable sample		Spiked (µg/g)	Theophylline		Caffeine		Trimethoprim		Norfloxacin		Ciprofloxacin	
			% R	% RSD	% R	% RSD	% R	% RSD	% R	% RSD	% R	% RSD
South Africa	Cabbage	0	-	-	-	-	-	-	-	-	-	-
		1	115	5.1	106	5.5	77.2	3.4	95.6	2.4	90.2	3.90
		5	93.8	4.1	33.5	6.8	88.8	11.9	97.7	12.0	81.5	9.8
	Carrot	0	-	-	-	-	-	-	-	-	-	-
		1	111	9.1	71.6	2.6	53.3	5.4	95.8	4.4	113	5.3
		5	113	11.8	69.9	7.1	66.1	11.1	79.5	12.6	77.9	0.8
	Lettuce	0	-	-	-	-	-	-	-	-	-	-
		1	97.7	4.4	72.8	3.8	85.9	4.2	59.4	6.9	66.9	6.8
		5	90.9	10.9	87.9	14.7	94.2	12.1	52.3	2.2	63.1	2.5
Ethiopia	Cabbage	0	-	-	-	-	-	-	-	-	-	-
		1	88.9	2.9	73.8	10.5	75.7	12.9	84.7	9.1	81.5	3.1
		5	75.2	13.5	65.0	10.8	58.5	8.5	75.5	7.5	84.5	5.0
	Carrot	0	-	-	-	-	-	-	-	-	-	-
		1	111	3.4	52.8	2.1	84.0	11.9	96.3	0.9	76.6	0.8
		5	94.6	3.2	40.4	2.9	57.5	1.2	65.5	3.4	47.2	1.7
	Lettuce	0	-	-	-	-	-	-	-	-	-	-
		1	107	5.1	40.9	2.3	67.6	12.1	45.1	2.0	65.8	7.6
		5	113	3.9	51.9	8.0	42.9	4.6	72.4	1.1	66.4	5.3

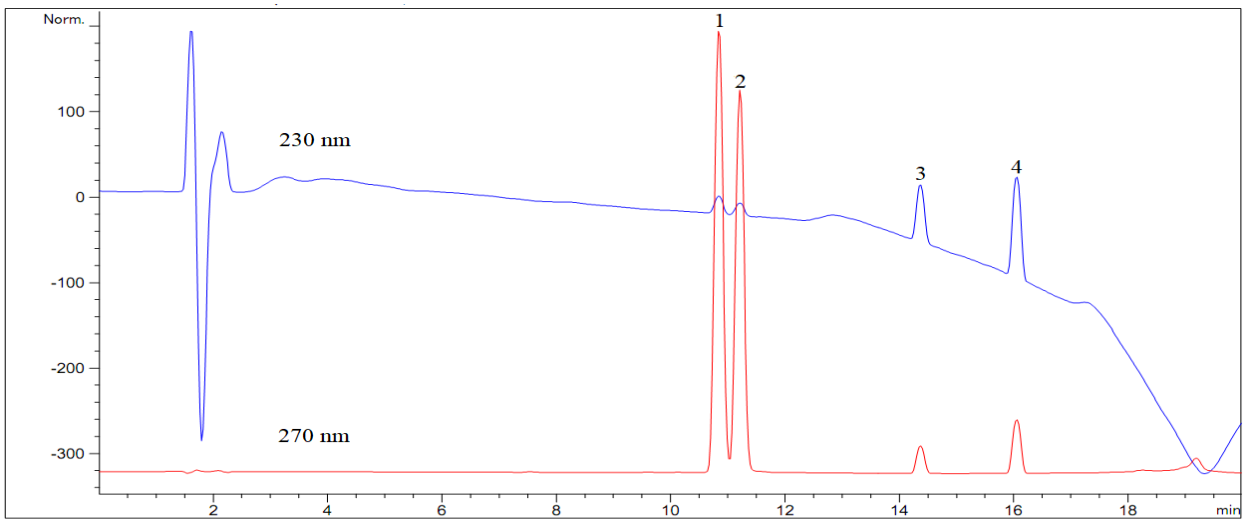
(Level 1 = 1 µg/g, Level 2 = 5 µg/g)

4.5. Synthesis and characterization of molecularly imprinted polymer for adsorption studies of pharmaceuticals from water

A preliminary experiment to select suitable pharmaceuticals for the adsorption study was done from the targeted compound for this study. The selection experiment was done using the synthesized imprinted polymer. A mixture solution of thirteen selected pharmaceutical compounds in the concentration of 1 mg/L was prepared and the peak area was recorded and then mixed with 30 mg of the imprinted polymer and left in the refrigerator overnight. On the following day, the mixture was centrifuged and the supernatant solution was analyzed with the method optimized and presented in section 3.1. The concentration before adsorption and after adsorption used to evaluate the performance of the polymer for adsorption of pharmaceuticals. From thirteen pharmaceutical compounds exposed to the imprinted polymer for adsorption, only three compounds show good adsorption and were selected for further study.

4.5.1. Chromatographic separation conditions

Liquid chromatography with diode array detector and Kromasil C_{18}, 5 µm (4.6 mm x 150 mm, 5 µm) column was used for separation for adsorption study. The detector settings were adjusted to 230 nm for venlafaxine and albendazole and 270 nm for norfloxacin and ciprofloxacin and the chromatogram is shown in Figure 4.5.1. Binary mobile phase acetonitrile (A) and water with 0.1% (v/v) formic acid (B) were used throughout for separation. The flow rate was set at 1.0 mL/min and the injection volume was 10 µL in ambient column temperature.



(1- norfloxacin; 2-ciprofloxacin ; 3-venlafaxine; 4-albendazole)

Figure 4.5. 1. Chromatogram of selected pharmaceutical compounds for adsorption study

4.5.2. Characterization of the synthesized polymer

4.5.2.1. FT-IR analysis of MIP and NIP

The FT-IR characterization of the polymers was done to identify the functional groups present. The results in Figure 4.5.2 (b) and (c) of MIP and NIP show that the shape and position of the bands are identical indicating that they were from the same compound and similarly synthesized. However, the result in Figure 4.5.2 (a) showed extra peaks that indicate some changes occurred after the adsorption of the target pharmaceuticals on the surface of the imprinted polymer. Absorption peak around 2,920 cm^{-1} can be assigned to the stretching vibration of the C–H groups and at 1,747, 1325 and 1,130 cm^{-1} may be associated with the presence of C=O and C–O and C-N, respectively, which result from the compound in preparation. However, a strong broad band absorption peak ≈ of 3360 cm^{-1} was due to O-H stretching, a weak peak around 2530 cm^{-1} shows the presence of the S-H group and C-H around 2000 cm^{-1} indicates the adsorption of the target analyte on the MIP surface.

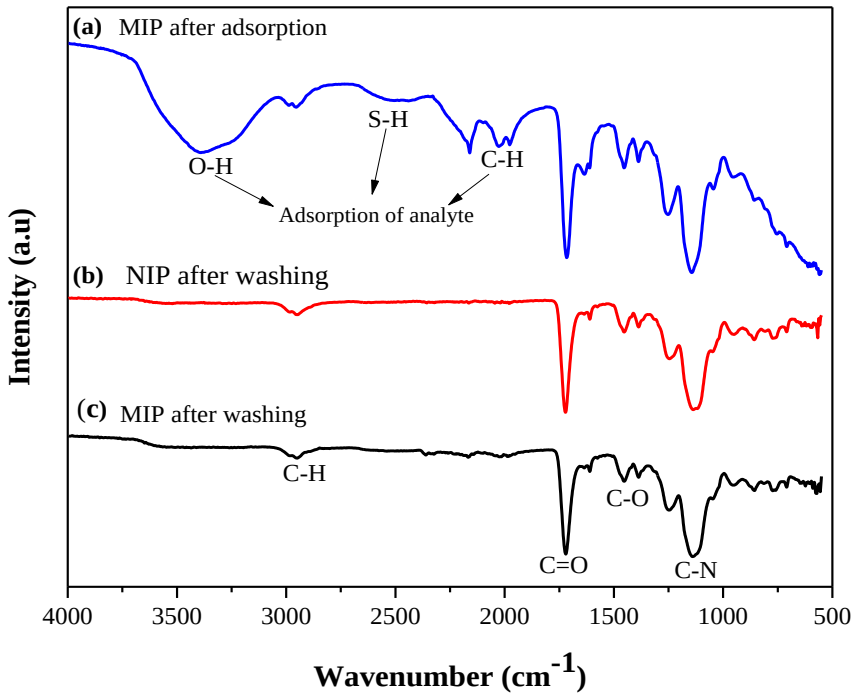


Figure 4.5. 2 FT-IR spectra of molecularly imprinted polymer (MIP), non-imprinted polymer (NIP) and after adsorption on MIP.

4.5.2.2. Thermogravimetric analysis (TGA)

Thermogravimetric analysis was used to evaluate the thermal stability of the polymer. As shown in (Figure 4.5.3.) the polymer exhibited no weight loss with an increase in temperature from 100 – 300 ^{o}C. However, beyond this temperature, MIP starts to decompose faster than the NIP this might be due to the structural difference caused with template removal (Madikizela and Chimuka, 2016). At temperatures ranging from 350 ^{o}C to 500 ^{o}C, a rapid weight loss occurred; indicating the decomposition of the material and at a temperature above 600 ^{o}C the polymer decomposes. Therefore, the synthesized polymer (MIP and NIP) showed good thermal stability at temperatures lower than 320 ^{o}C.

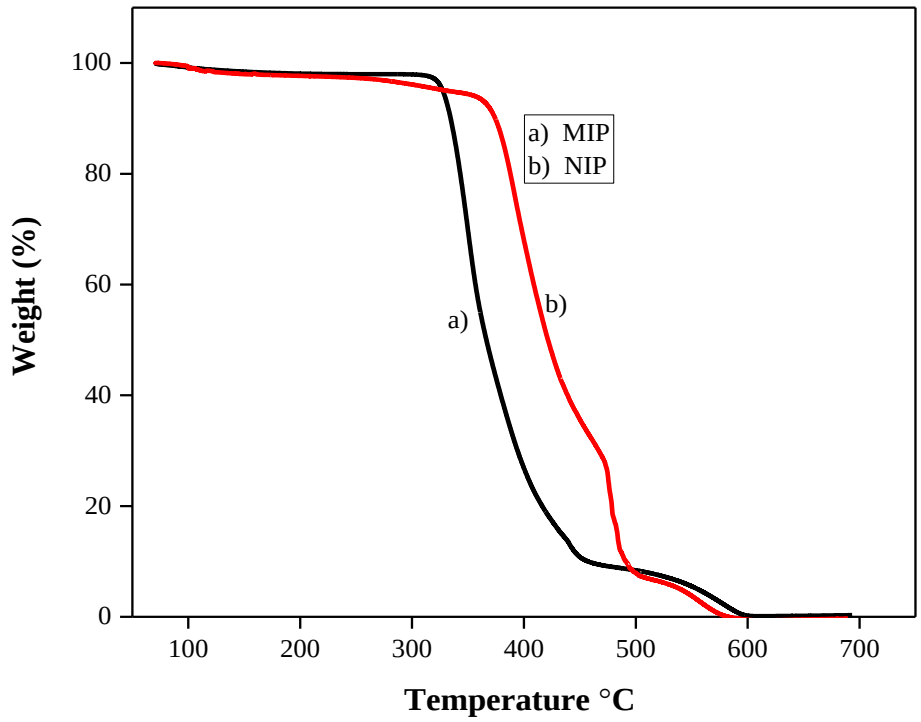


Figure 4.5. 3. Thermal stability of the synthesized polymer (MIP and NIP)

4.5.2.3. X-ray diffraction (XRD)

The X-ray diffraction result gives information about the crystallinity of the homogenized powder sample. From the result (Figure 4.5.4), it can be noted that MIP and NIP show similarity in the pattern which indicates that they are constructed from the same structural backbone. Besides, no X-ray diffraction peak shows the absence of crystallinity and the amorphous nature of the polymer. This result agrees with the study that showed the lack of crystallinity for multi-template MIP synthesized using naproxen, ibuprofen and diclofenac as a template and NIP (Madikizela et al., 2017a).

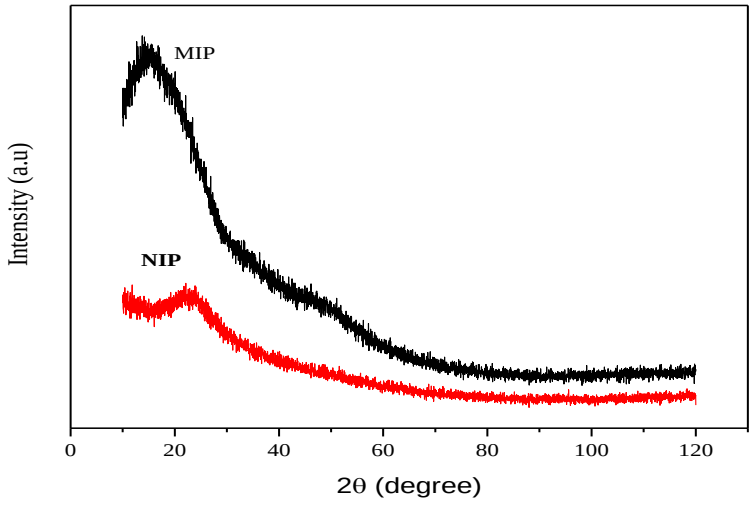


Figure 4.5. 4. X-ray diffraction (XRD) result for a molecularly imprinted polymer and non-imprinted polymer

4.5.2.4. Scanning electron microscopy (SEM)

The results from scanning electron microscopy (SEM) images were used to study the polymer morphology (Sundar et al., 2010). As is shown in Figure 4.5.5, the surface of the MIP is rougher and has large pore sizes compared to the NIP, resulting in the availability of more surface area for adsorption. The morphology difference in the image of MIP is due to the presence of a templet during the synthesis of the polymer and washing after to form such a rough surface which is not in the NIP.

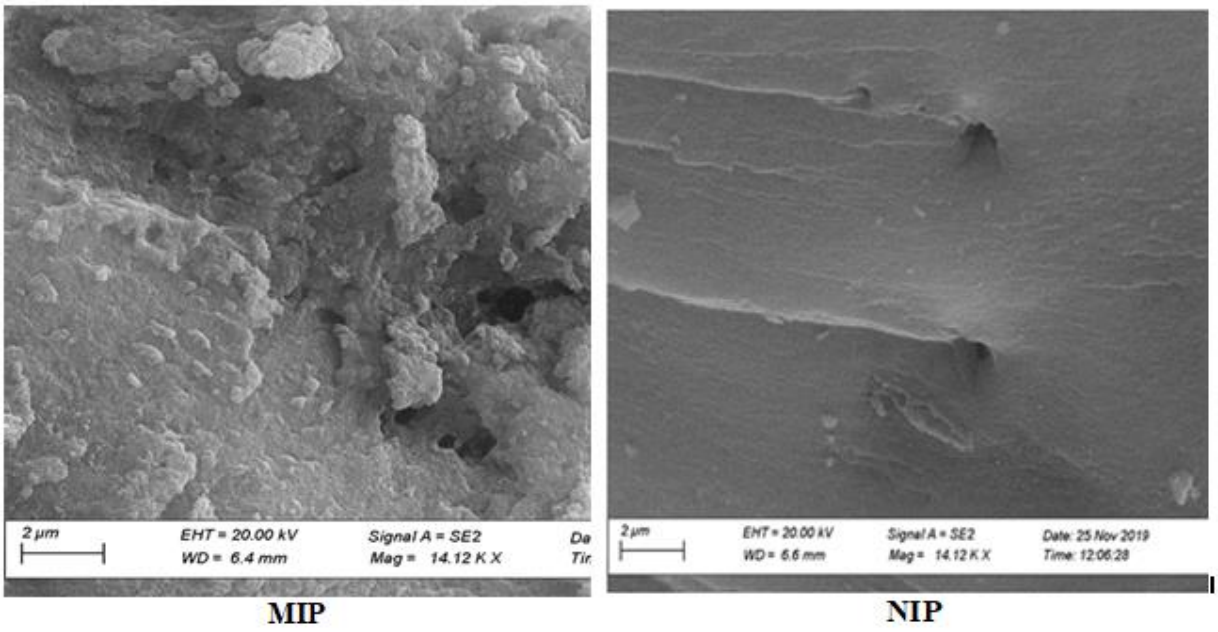


Figure 4.5. 5. Scanning electron microscope images for a molecularly imprinted polymer and non-imprinted polymer

4.5.2.5. Brunauer, Emmett and Teller (BET) analysis

Brunauer, Emmett and Teller (BET) results show a larger surface area for the MIP than the NIP. As the molecularly imprinted polymer has templets during synthesis which is removed during washing, it leaves behind the surface of many pores which is not in the non-imprinted polymer. As it is reported in (Table 4.5.1) surface area and total pore volume in the MIP are larger than the NIP which is expected and result in a good adsorption capacity of MIP than NIP and agrees with the reported study (Madikizela et al., 2018d). The higher surface area of the MIP can reduce diffusion resistance and facilitates mass transfer through it (Dai et al., 2012). The pore diameter for both MIP and NIP is greater than 50 nm indicating the surface of the polymer was macro-porous (Cormack and Elorza, 2004).

Table 4.5. 1. Brunauer, Emmett and Teller (BET) results of MIP and NIP

Polymer	**Surface area (m^2/g)**	**Total pore volume (cm^3/g)**	**Pore diameter (nm)**
MIP	7.95	0.34	163.8
NIP	5.85	0.29	204.1

4.5.3. Optimization of adsorption parameter

4.5.3.1. Effect of polymer mass on adsorption

The effect of the polymer amount used for the batch adsorption was investigated by varying the mass of the MIP from 10 to 60 mg while the other experimental conditions were kept constant. The adsorption increases as the mass of the polymer adsorbent increases however for albendazole and venlafaxine polymer a mass 20 mg was found to be optimum but not for ciprofloxacin and norfloxacin (Figure 4.5.6). The polymer mass was further increased to obtained maximum adsorption for all of the compounds under study. As the polymer mass increases the adsorption also increases up to the mass of 50 mg and further increments to 60 mg does not show a significant change in adsorption. Therefore, the polymer mass of 50 mg was selected as an optimum mass of adsorbent for the simultaneous adsorption of selected pharmaceutical compounds.

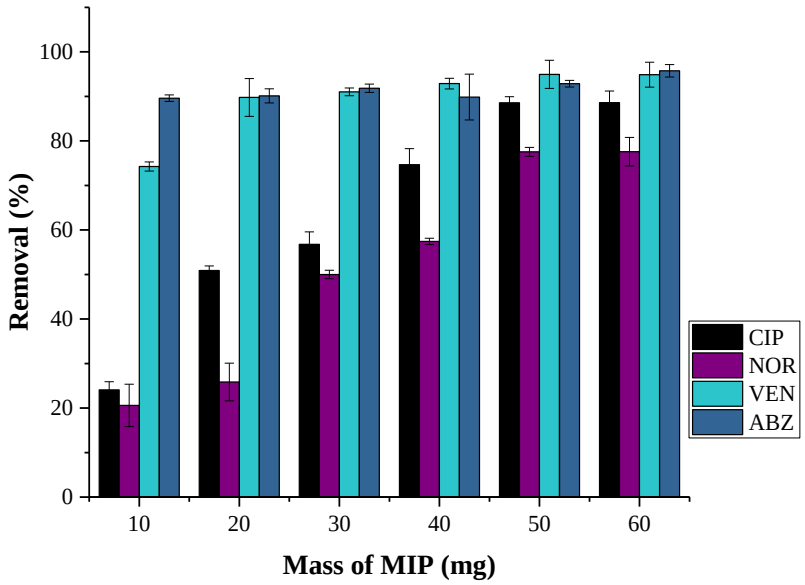


Figure 4.5. 6. Effect of polymer amount on the adsorption of pharmaceuticals

4.5.3.2. Effect of sample pH on adsorption

The effect of the pH of the sample solution is important to obtain the maximum adsorption of target compounds. The pH of the solution affects the extraction process of pharmaceutical molecules by affecting both the adsorbate chemistry and surface binding-sites of the adsorbent (Madrakian et al., 2013). The effect of the pH of the sample solution was investigated from 3-9 while the other parameters were kept constant Figure 4.5.7. The adsorption for all pharmaceuticals studied was greatly affected by sample pH. High adsorption efficiency > 75% was reported at the basic condition of pH 9 when compounds are in its zwitterionic form. When sample pH is in the acidic condition the compounds will undergo protonation (positively charged) and beyond pH 9, the basic conditions lead to deprotonation (negatively charged). On the other hand, the acidic monomer 4-vinyl benzoic acid (pK_a = 4.24) in the polymer is negatively charged at pH greater than the pK_a and positively charged at pH less than the pK_a. Therefore, an electrostatic repulsion takes place, which explains the poor adsorption in the same regions. At pH 9 however, where the compounds are in its zwitterionic form and most acidic groups present in the MIP are deprotonated, a favorable interaction occurs between the positively charged part of the compounds and the negative charge of the adsorbents. Consequently, at pH greater than 9, the adsorption efficiency of the compound dropped sharply as a result of

pharmaceuticals having a negative charge and 4-vinyl benzoic acid also negatively charged. Thus at pH greater than 9, the electrostatic repulsive interaction is the only driving force.

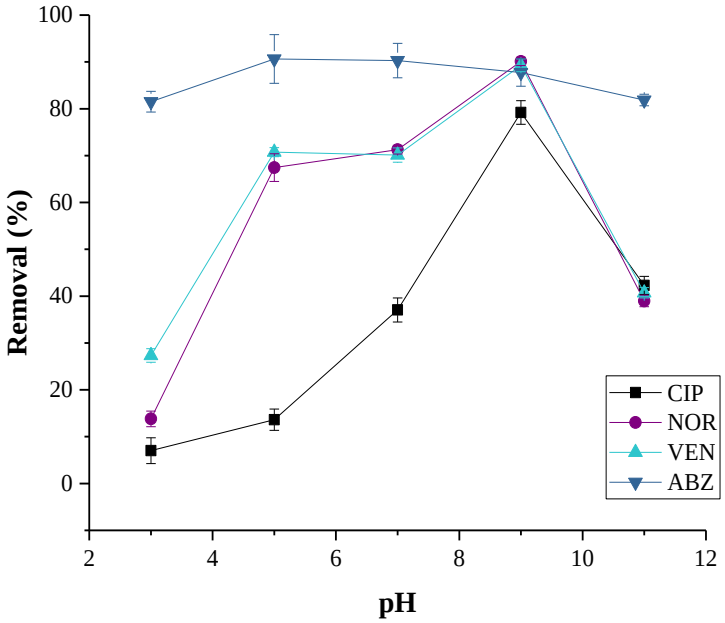


Figure 4.5. 7. Effect of pH in the adsorption of pharmaceuticals by MIP

4.5.3.3. Effect of contact time on adsorption

The effect of contact time was found to be an important parameter to ensure maximum adsorption for removal. The contact time for adsorption was varied from 10–50 min (Figure 4.5.8). The adsorption efficiency increases from 10 to 20 min for all compounds and starts decreasing from 20 to 40 min except for albendazole and then shows a slight insignificant incremental. As the time goes from 10 to 20 min the interaction of the compounds to the porous adsorbent increases and adsorption increases as excess cavities exist. However, as time goes beyond 20 min the analyte binds to the inner pores of the adsorbent and attaches strongly which slows and decreases the adsorption of the compounds. This trend agreed on the study reported for optimization of contact time using MIP as an adsorbent for the compound norfloxacin and enrofloxacin (Lu et al., 2019). However, albendazole remains the same throughout the study and 20 min contact time was selected as the optimum time for adsorption. Adsorption capacity versus pH and contact time respectively are shown in (Appendix C1)

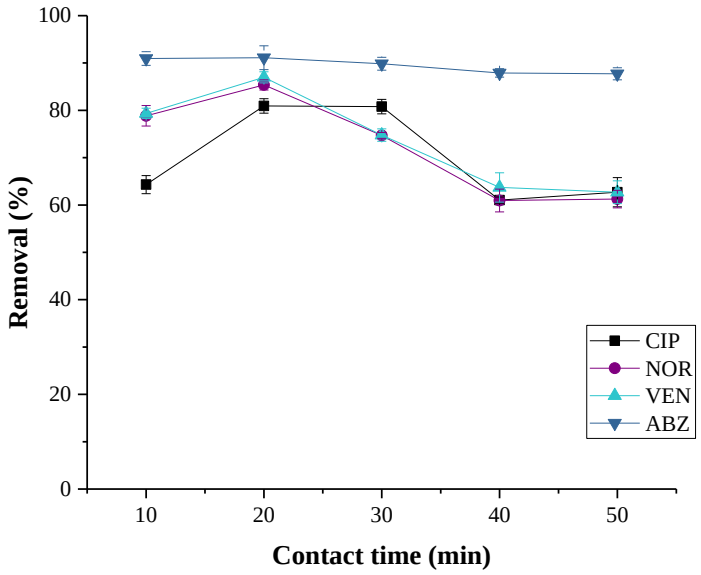


Figure 4.5. 8. Effect of contact time on the adsorption of pharmaceuticals

4.5.3.4. Effect of the initial concentration on adsorption

The effect of the initial concentration of the target analyte on the adsorption capacity of the polymer was carried out to understand how different concentration affects the adsorbent capacity. The experiment was conducted by varying the concentration of the target analyte while keeping the other parameters, polymer amount (50 mg), pH of the solution pH (9), contact time (20 min) and sample volume (10 mL) were constant. The adsorption capacity increased linearly as a function of initial concentration and this was observed until 50 mg/L thereafter the curves slightly flattened once equilibrium was reached as shown in Figure 4.5.9. The concentration was not increased beyond 60 mg/L as these compounds are expected to be not beyond these levels in the environmental water sample. The adsorption capacity of the pharmaceuticals at equilibrium was obtained as, 45.2, 54.2, 40.5 and 35.8 (mg/g) which is for venlafaxine, albendazole, ciprofloxacin and norfloxacin, respectively.

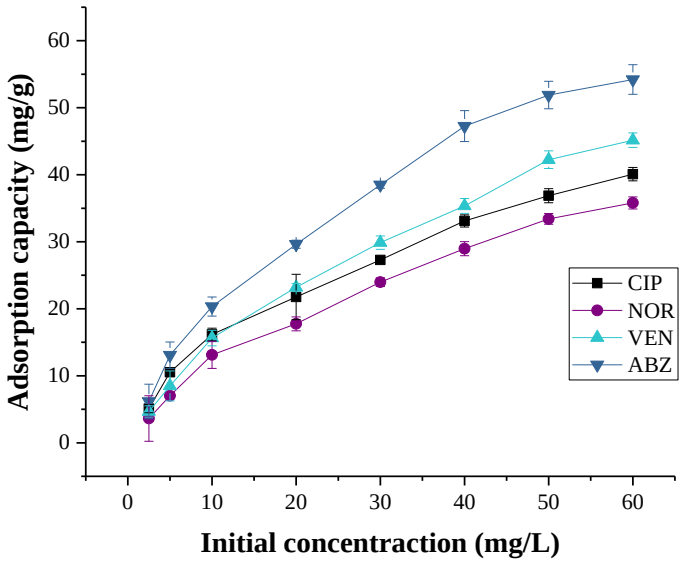


Figure 4.5. 9. Effect of initial concentration on the adsorption capacity of MIP

4.5.4. Elution solvent selection

The result of the solvents considered in this study is presented in Figure 4.5.10. 9(a) and (b) for both molecularly imprinted and non-imprinted polymers. The best solvent for elution was 2% formic acid in water with methanol (1:1, v/v) as ciprofloxacin is not soluble in pure methanol however the addition of water is acceptable. The same solvent worked well in eluting compounds from both polymers for all selected pharmaceuticals. However, the peak area was found high in the molecularly imprinted polymer than in the non-imprinted polymer which implies the imprinted polymer can adsorb more due to the cavity formed by the template removed during preparation which is not in the non-imprinted polymer. The use of acetonitrile as an elution solvent was attempted but the solvent was found to be incompatible with some analytes because of poor solubility. Therefore, the mixture of 2% formic acid in water and methanol (1:1, v/v) was used to elute from the polymer after equilibrium adsorption time. This mixture of the solvent was also used for the regeneration of the MIP for their reusability study.

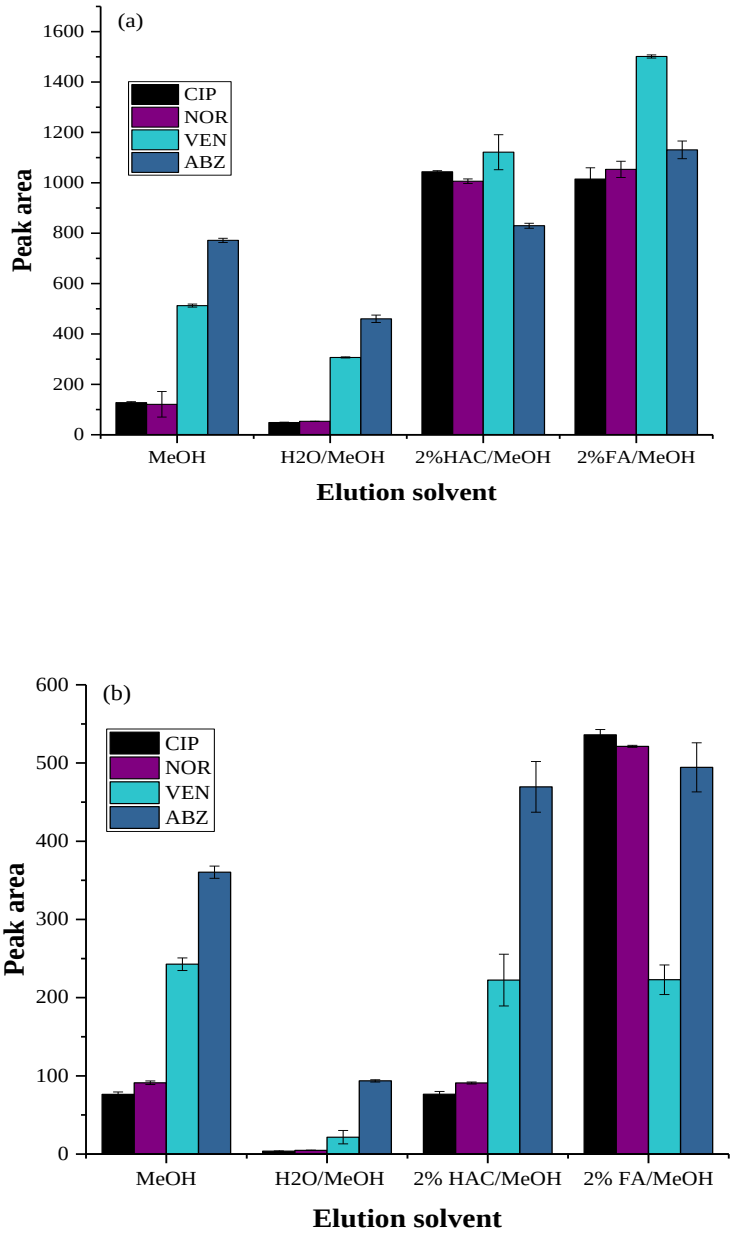


Figure 4.5. 10. Elution solvent selection using MIP (a) and NIP (b)

4.5.5. Adsorption isotherms and kinetics

To gain an understanding of the adsorption mechanism of pharmaceuticals on to the adsorbent, two adsorption isotherm models, Langmuir and Freundlich were used to fit the experimental data. As the result depicted in Table 4.5.2, the R^2 values from the adsorption models show the fitness of the experimental data in the models used were generally good for

both isotherm models. However, the Freundlich model provided the best correlation for all the pharmaceuticals as compared to the Langmuir model. Moreover, the n-value which is greater than 1 and higher kf value indicates adsorption is favourable (Madikizela et al., 2018d). The fitting of experimental data further confirms the adsorption was taking place in the multilayer heterogeneous surface.

Table 4.5. 2. Adsorption isotherm and calculated parameters

		Freundlich			**Langmuir**		
Polymer	**Compound**	**R^2**	**k_f**	**n**	**R^2**	**K_L (L/mg)**	**q_{max} (mg/g)**
MIP	ABZ	0.9902	3.8	1.5	0.9878	0.03	84.0
	CIP	0.9848	3.4	1.6	0.9747	0.04	55.6
	NOR	0.9970	1.9	1.4	0.9809	0.02	62.5
	VEN	0.9926	2.7	1.4	0.9792	0.03	72.5
NIP	ABZ	0.9974	1.1	1.2	0.9465	0.01	62.9
	CIP	0.9889	1.3	1.2	0.9603	0.01	48.1
	NOR	0.9954	1.6	1.2	0.9478	0.01	44.6
	VEN	0.9941	1.3	1.2	0.8783	0.01	61.2

On the other hand, to estimate the rate of adsorption different adsorption kinetics models could be used (Li et al., 2010; Mironyuk et al., 2019). Kinetic models, such as pseudo-first order and pseudo-second-order models were employed to predict the rate of reaction and reaction mechanisms. As is reported in Table 4.5.3, pseudo-second-order fitted well with the experimental data based on the correlation coefficient (R^2) value and suggested a chemisorption nature adsorption process. The plotted curve for both isotherms and kinetics model considered in this study were presented in (Appendix C2) part of the thesis.

Table 4.5. 3. Kinetic models and calculated parameters

Polymer	Compounds	Pseudo-first-order		Pseudo-second-order		
		R^2	K_1	R^2	K_2	q_{max} (mg/g)
MIP	ABZ	0.6078	0.059	0.9997	0.637	41.5
	CIP	0.0199	0.019	0.9631	0.117	12.5
	NOR	0.8972	0.052	0.9829	0.097	11.0
	VEN	0.7995	0.038	0.9932	0.102	12.0
NIP	ABZ	0.2051	0.014	0.9998	4.155	37.6
	CIP	0.3397	0.022	0.9902	0.827	13.0
	NOR	0.7826	0.045	0.9977	0.200	10.5
	VEN	0.7419	0.742	0.9963	0.267	11.4

4.5.6. Cross-selectivity study

The selectivity of the imprinted and non-imprinted polymer towards the four selected pharmaceuticals was investigated using the partition coefficient and imprinting factor. As it is reported in Table 4.5.4, the selectivity of the polymer, based on partition coefficient (K_D) and imprinting factor (α) value, was in the order of albendazole > venlafaxine > norfloxacin > ciprofloxacin. Among the pharmaceuticals, albendazole was found to be a pharmaceutical that highly competes with the template towards the MIP cavity and showed high selectivity.

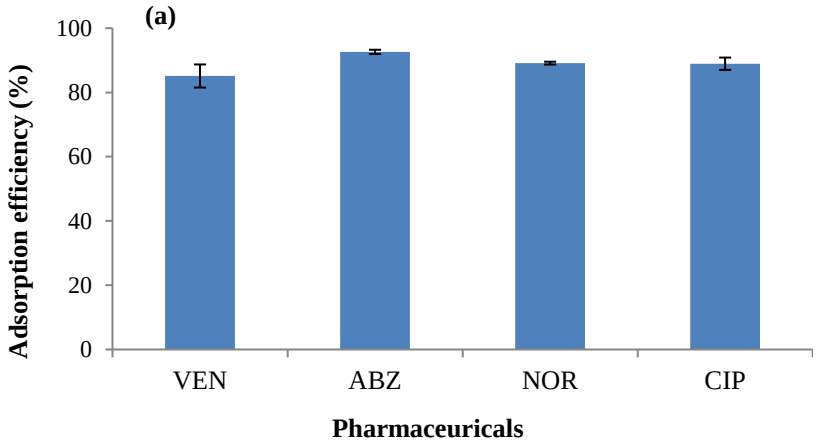
Table 4.5. 4. Imprinting factor of MIP and NIP towards the pharmaceuticals

Pharmaceuticals	K_D (MIP)	K_D (NIP)	α
ABZ	10.2	4.0	2.6
CIP	4.2	2.5	1.7
NOR	5.8	2.5	2.3
VEN	6.7	2.8	2.4

This could be related to the number of groups responsible for hydrogen bonding with the polymer and similarity in size which is many in albendazole than venlafaxine and other compounds.

4.5.7. Adsorption and recovery of venlafaxine, albendazole, ciprofloxacin and norfloxacin from river water on MIPs

The synthesized MIP feasibility was evaluated by the adsorption efficiency and extraction recovery of selected four pharmaceuticals using river water. The result in Figure 4.5.11 (a) and (b) clearly shows the adsorption efficiency and extraction recovery of selected compounds in river water which ranges from 85-92% and 40-65%, respectively. The adsorption efficiency observed in the result reveals that the matrix of the real water has less effect on the adsorption of the pharmaceuticals in the MIP. On the other hand, extraction recovery was found to be lower relative to what is adsorbed. This difference can be explained in terms of the non-specific nature of MIP particles which is due to the backbone polymer that tends to adsorb compounds on the surface. This nonspecific adsorption leads to washing away of some target compounds during the washing step before elution. The order of extraction recovery in Figure 4.5.11b is in line with the imprinting factors found in Table 4.5.4. Thus extraction recovery represents the target compounds selectively adsorbed by the target MIPs. The extraction recovery though is on the lower side still indicates that these compounds can be selectively extracted simultaneously using the prepared MIPs.



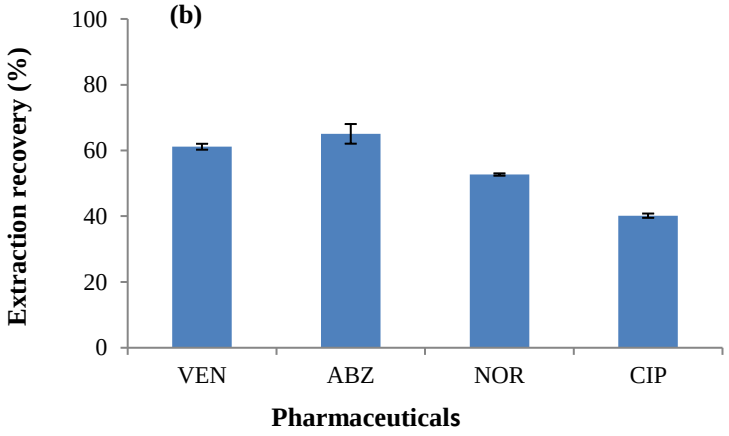


Figure 4.5. 11. Adsorption efficiency (a) and extraction recovery (b) of pharmaceuticals from river water using MIP

Figure 4.5. 12. Extraction of selected pharmaceuticals using MIP as an adsorbent in SPE cartridge

4.5.8. Regeneration and re-use of the MIP

The results of the reusability studies are shown in Figure 4.5.13. In this study, the compounds were eluted by an optimized solvent which is 1 mL of 2% formic in water with methanol (1:1, v/v)). Thereafter the MIP particles were regenerated by 10 mL of pure methanol to desorb unwanted compounds. The results in Figure 4.5.15 shows that MIP particles were still good even after the 6^{th} cycle reuse. The result of regeneration studies over several sorption and desorption cycles shows the well-known advantage of MIPs over most conventional sorbents that are used once and thrown away. This is important as it lowers the cost and excess material usage.

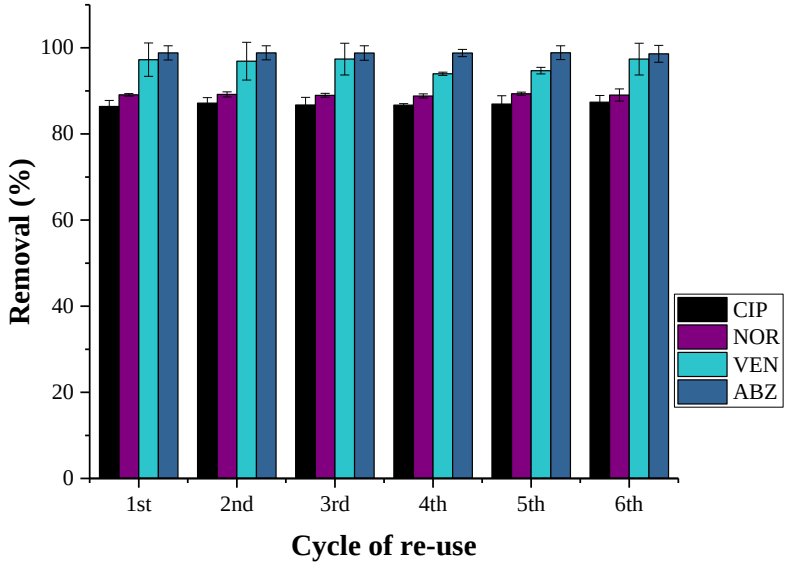


Figure 4.5. 13. Re-usability of the MIP

4.5.9. Comparison of the developed method with other studies

There is no study reported in the literature about the simultaneous adsorption and extraction of venlafaxine, ciprofloxacin, norfloxacin and albendazole using MIP in an aqueous environment to compare with the present work. However, Table 4.5.5 shows some recent studies about the adsorption and extraction of the target pharmaceuticals considered for this study using MIP in an aqueous solution. The values used to compare with the reported study were adsorption capacity (mg/g), adsorption time (min) and extraction recovery (%). As it is presented in the table the value obtained in this study was found to be comparable and even better than most of the studies reported. The extraction recovery of the pharmaceutical evaluated with the extraction of a single compound at a time like norfloxacin (Li et al., 2019), ciprofloxacin (Lian and Wang, 2016; Zhu et al., 2019), albendazole (Cacho et al., 2009) and venlafaxine (Miranda et al., 2016) reported higher value of recovery. However, for simultaneous extraction of norfloxacin and ciprofloxacin (Benito-Pena et al., 2008) the values found in this study are higher. The optimum extraction time in this study is also reasonable compared to some other studies in Table 4.5.5.

Table 4.5. 5. Comparison of the present study with reported studies using MIP.

Pharmaceutical compounds	**Adsorption capacity (mg/g)**	**Adsorption time (min)**	**Extraction recovery (%)**	**References**
Norfloxacin	0.66	15	77.6	(Li et al., 2019)
Norfloxacin	---	---	26.0	(Benito-Pena et al., 2008)
Ciprofloxacin	3.50	480	90.5	(Lian and Wang, 2016)
Ciprofloxacin	19.6	350	97.8	(Zhu et al., 2019)]
Ciprofloxacin	---	---	29.0	(Benito-Pena et al., 2008)
Albendazole	---	---	83.6	(Cacho et al., 2009)
Venlafaxine	---	---	84.0	(Miranda et al., 2016)
Venlafaxine	45.2		61.1	
Albendazole	54.2	20	65.1	This study
Ciprofloxacin	40.1		52.7	
Norfloxacin	35.8		40.2	

4.6. Removal of pharmaceuticals from water using *Moringa oleifera* seed

Moringa oleifera plant seed parts and extracts (water-soluble protein, seed cake and seed husk) were tested for the removal of selected pharmaceuticals (MET, AMOX, MTZ, TRM, THP, NOR, CIP, CAF, DOXH and ALB) in water. These parts and extracts of *Moringa* undergo different characterization to understand how they are suitable for adsorption. The factors that affect pharmaceuticals sorption capacity of the adsorbent including contact time, initial pharmaceutical concentration and pH were investigated. All the experiments were carried out in triplicate and average values are presented. The adsorbent efficiency for the removal of selected pharmaceuticals also evaluated by contaminated real river water.

4.6.1. Characterization of *Moringa* seed part for removal

4.6.1.1. Study of surface morphology

The surface morphology of the *Moringa oleifera* seed part was studied to characterize its surface characteristics using scanning electron microscopy (SEM) image to evaluate for adsorption of selected pharmaceuticals from water. Figure 4.6.1(a), (b) and (c) shows the SEM image of the water-soluble protein, seed husk and seed cake powders respectively. The surface morphology of the powders confirms its heterogeneous and porous nature. Thus the surface morphology was expected to facilitate the adsorption of the target compounds as previously indicated by another study (Kebede et al., 2019b).

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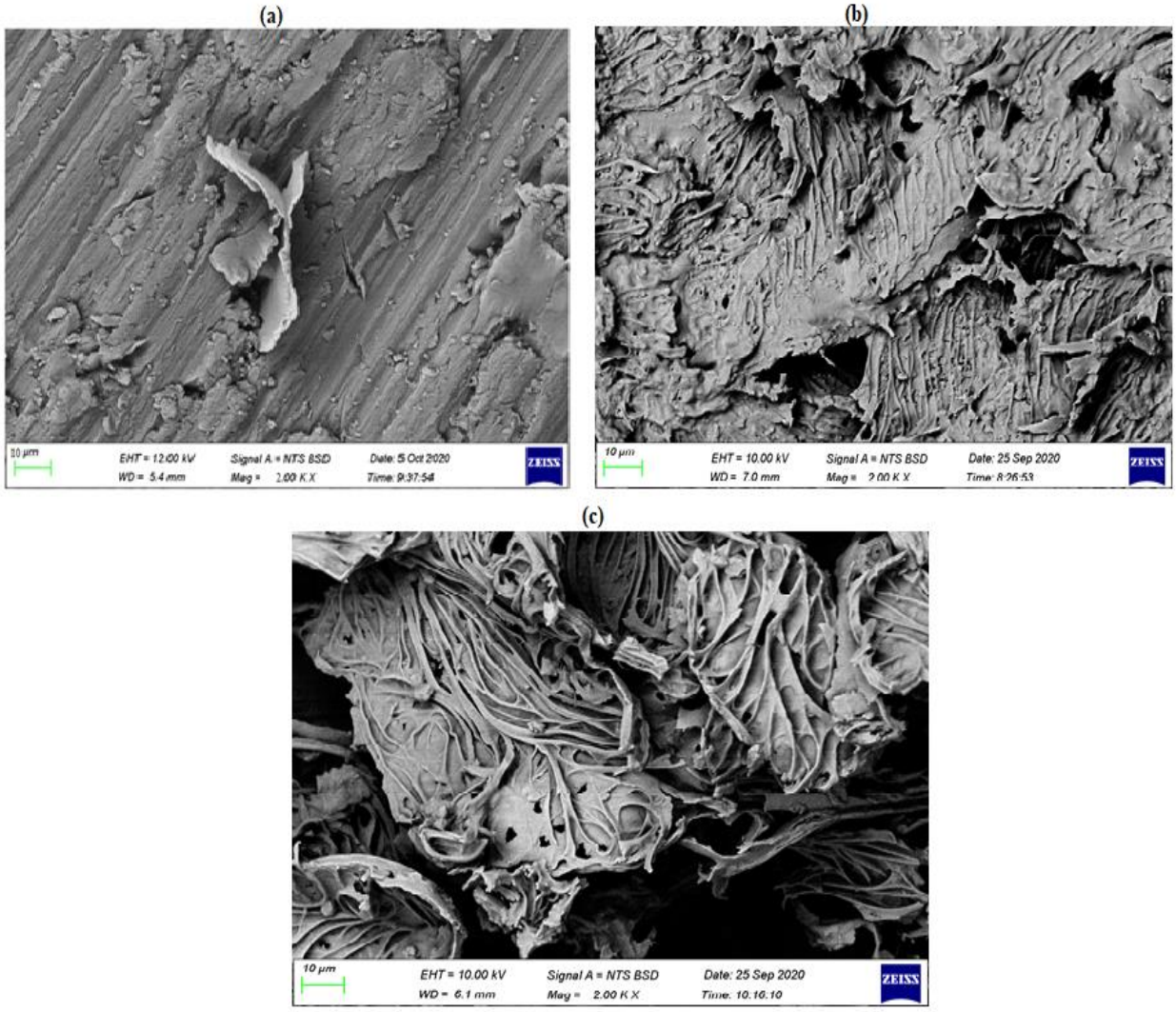


Figure 4.6. 1. Scanning electron microscopy (SEM) images of water-soluble protein powder (a), seed cake (b) and husk (c) of *Moringa oleifera* seed

4.6.1.2. FT-IR characterization before and after removal

The FT-IR technique was used to study the main functional groups of the *Moringa oleifera* seed parts intended for the removal of pharmaceuticals. The FT-IR spectra acquired for seed part water-soluble protein, seed husk and seed cakes are shown in Figure 4.6.2. As indicated in the FT-IR spectra water-soluble protein powder contains amine and amide functional groups at wavenumbers 1646, 1531, 1451 and 1415 cm^{-1}associated with C=O stretch amide I, NH amide II, NH amide I band, and C-N stretch amide III, respectively, which agrees with that stated in the study (Kebede et al., 2019a). A band position shift from wavenumbers 3287 to 3149 cm^{-1} and

1646 to 1508 cm^{-1} and also new peaks appeared at wavenumbers 2783 and 786 cm^{-1}. The results obtained confirmed that interaction between pharmaceuticals and protein powder had occurred, which facilitated the removal process.

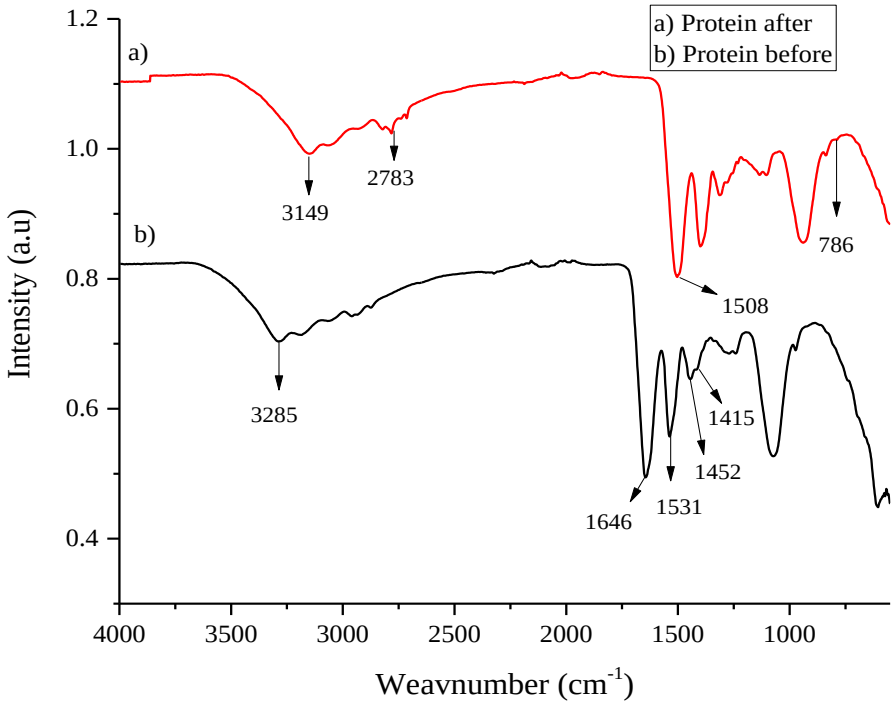


Figure 4.6. 2 FT-IR spectra of *Moringa oleifera* seed water-soluble protein powder before and after the removal of pharmaceuticals

FT-IR spectra shown below in Figure 4.6.3 is for *Moringa oleifera* seed cake. A broad band centered at 3273 cm^{-1} assigned to the O–H stretching band of the hydroxyl group and N-H stretching band of the amide group from the protein and fatty acid structure (Ravikumar and Udayakumar, 2020). The two peaks at 2918 and 2850 cm^{-1} are attributed to the asymmetric and symmetric stretching which corresponds to the C–H in the CH_2 functional groups, respectively. The two peaks at 1728 and 1628 cm^{-1} could be attributed to the C=O carbonyl group stretching (Araujo et al., 2018). The band at 1534 cm^{-1} is $N–H_{deformation}$ and 1217 cm^{-1} is $C–N_{stretching}$ spectra indicated the presence of protein and lipid content *Moringa oleifera* seed cake (Zaid and

Ghazali, 2019). The appearance of a new peak and disappearance of the peak was observed after adsorption which confirms the interaction of the compound with the adsorbent.

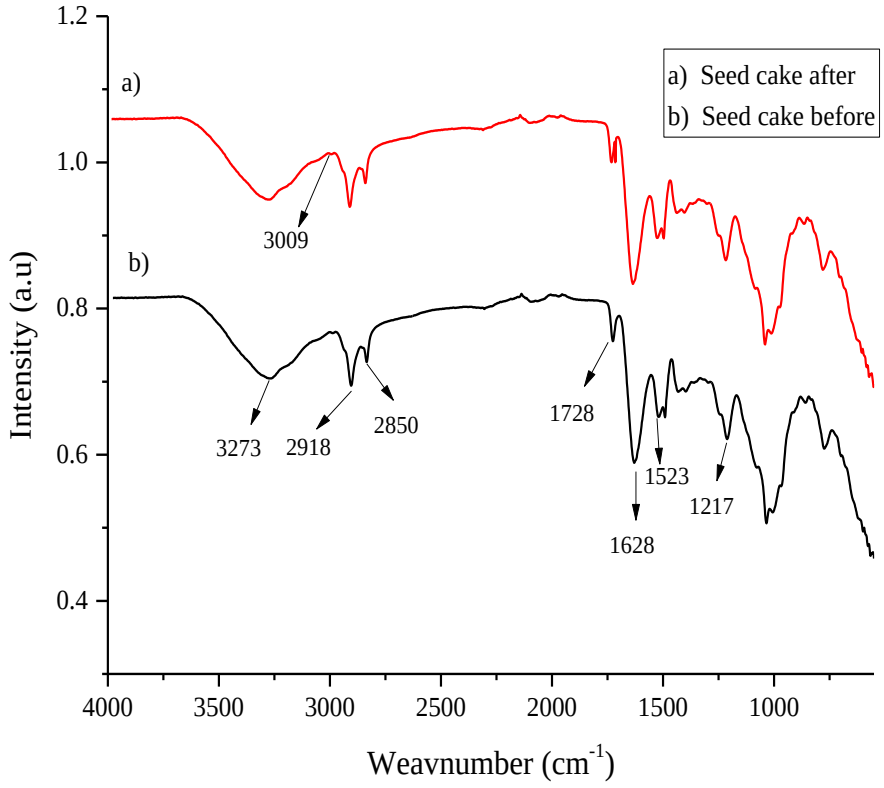


Figure 4.6. 3 FT-IR spectra of *Moringa oleifera* seed water-soluble protein powder before and after the removal of pharmaceuticals.

The FT-IR spectra of *Moringa* seed husk is given in Figure 4.6.4 shows, a broadband and predominate peak centered at 3306 cm^{-1} can be assigned to O–H stretching. Due to the high content of protein in the seed husk, there is also a contribution in this region from the N–H stretching of amide groups (Alves and Coelho, 2013; Okoya et al., 2020). The peaks at 2916 and 2837 cm^{-1} are assigned to symmetrical and asymmetrical C–H stretching of the CH_2 group present in fatty acids (Alves and Coelho, 2013). The presence of this band confirms the protein structure of the Moringa husks. In the 1800 to 1500 cm^{-1} region, there are several overlapping bands between 1750 and 1630 cm^{-1}. This set can be attributed to the C=O connected with stretching (Araujo et al., 2018). Owing to the heterogeneous type of seed, the carbonyl group may be linked to different neighborhoods.

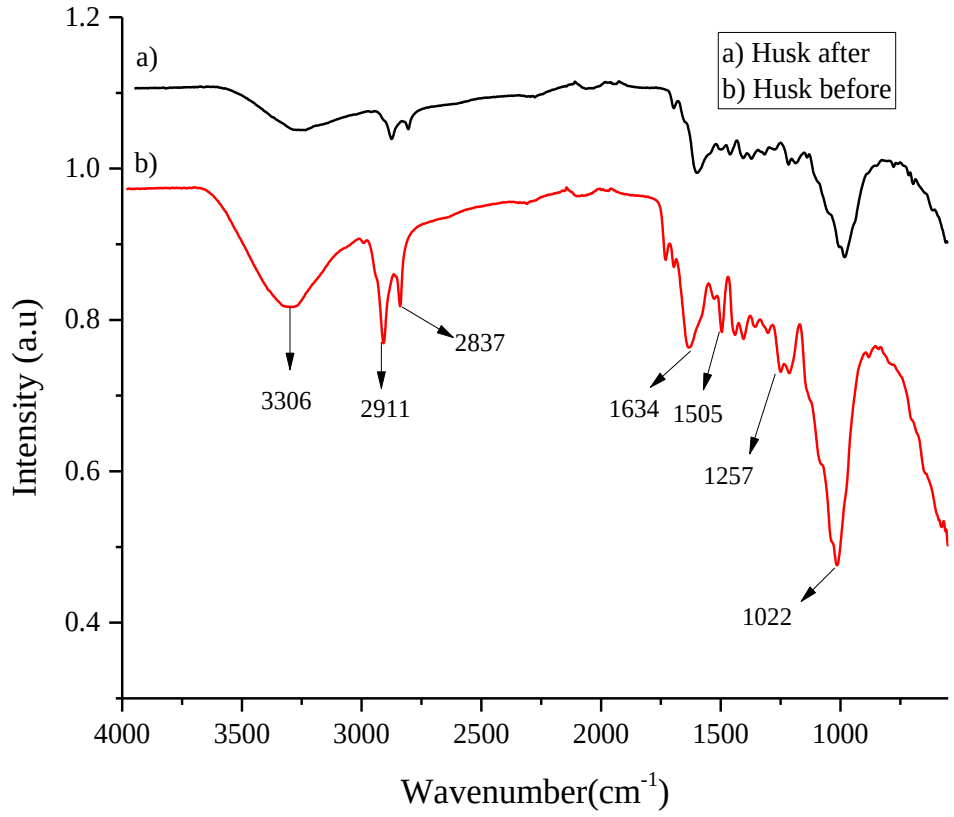


Figure 4.6. 4 FT-IR spectra of *Moringa oleifera* seed husk powder before and after removal of pharmaceuticals.

4.6.1.3. Determination of pH of point zero charge (pHpzc)

To understand the adsorption mechanism, it is necessary to determine the point of zero charges (pH_{PZC}) of the adsorbents. Adsorption of cations is favored at pH > pH_{PZC}, while the adsorption of anions is favored at pH < pH_{PZC}. The specific adsorption of cations shifts pH_{PZC} towards lower values, whereas the specific adsorption of anions shifts pH_{PZC} towards higher values. Figure 4.6.5 shows that for all the concentrations of KNO_3, the zero value of ΔpH lies at the pHi value of 6, which is considered as the pH of point zero charge of *Moringa* adsorbents. The adsorbents surface will be positively charged when the solution pH is below 6 and negatively charged when the solution pH is above 6. The interaction of the adsorbent and the adsorbent, therefore, is

because of electrostatic interaction between the positively charged adsorbent surface and ionized pharmaceuticals compounds.

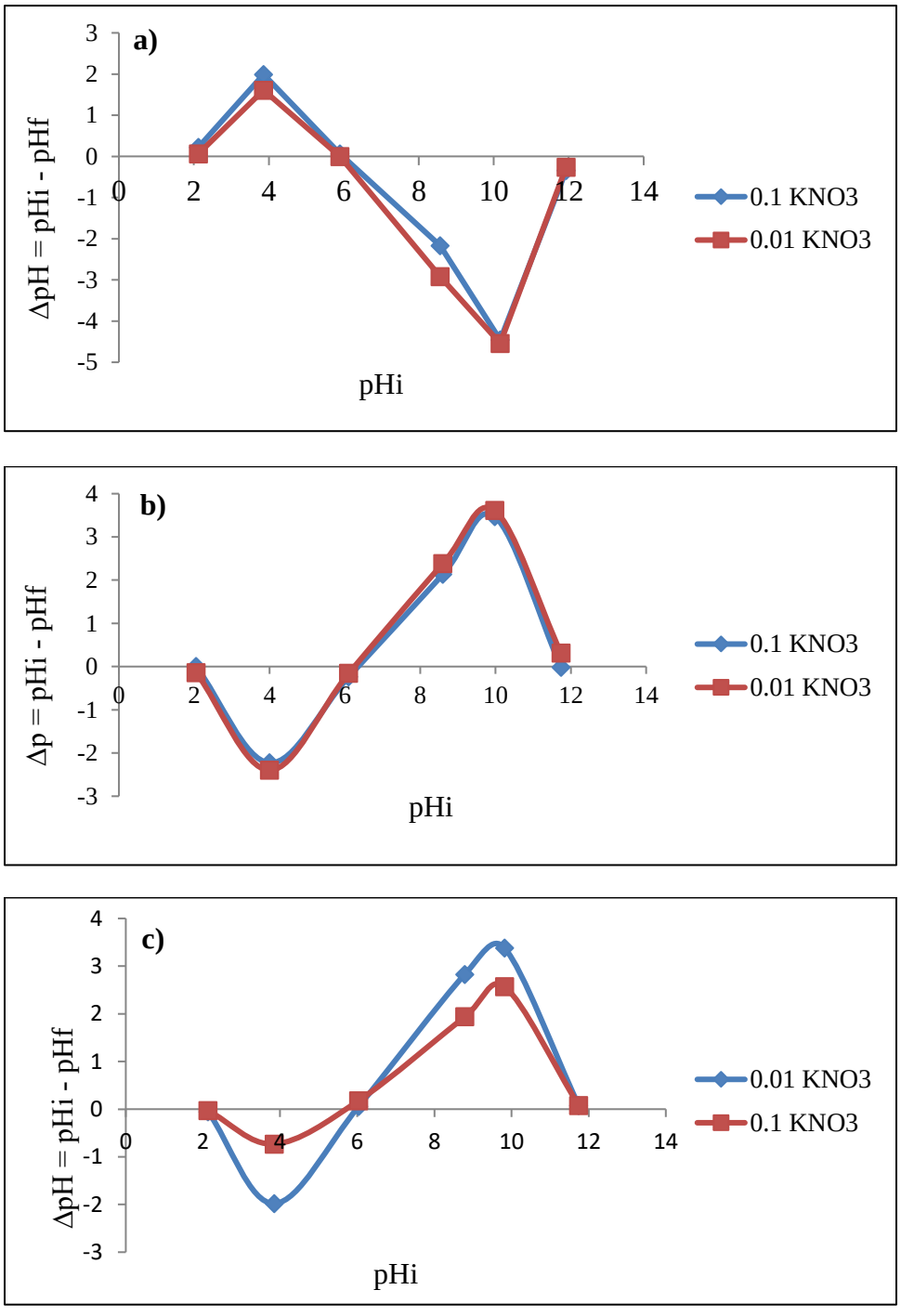


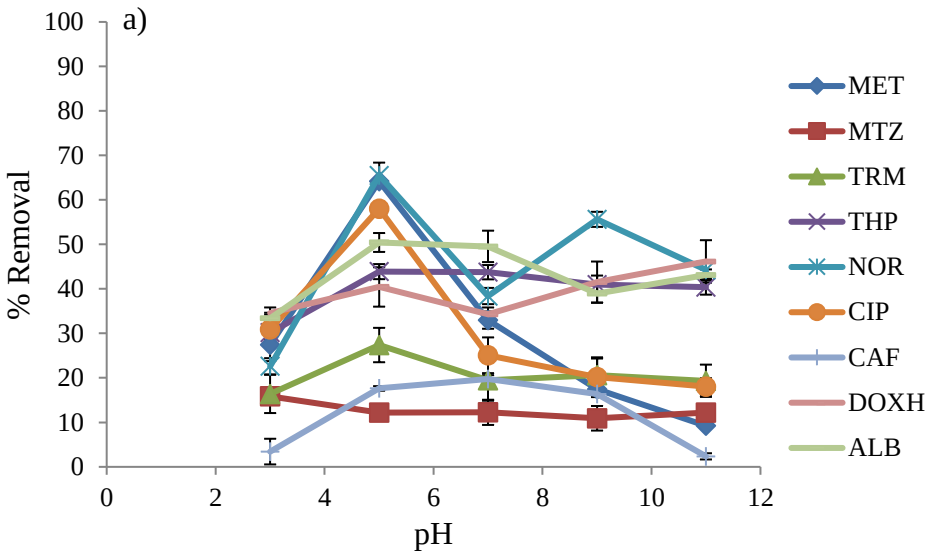
Figure 4.6. 5. Point of zero charges of *Moringa* adsorbent a) seed cake, b) husk and c) water-soluble protein

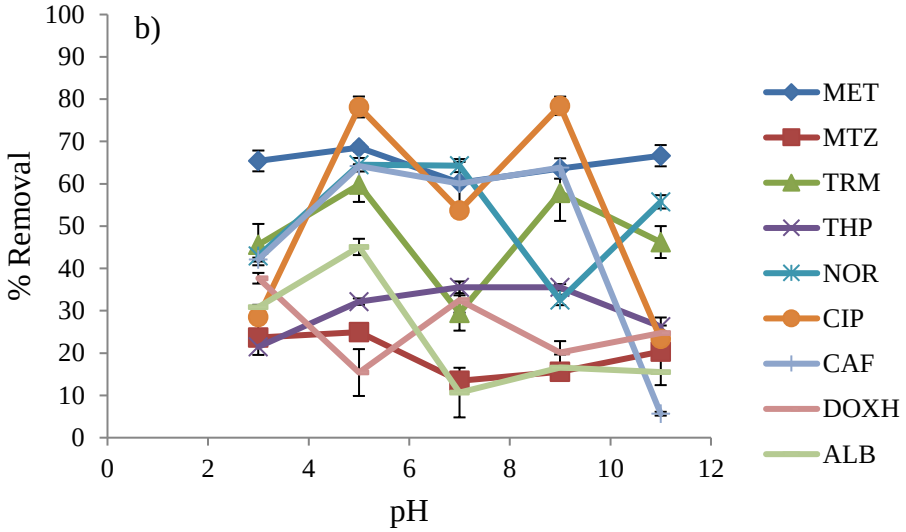
4.6.2. Removal of pharmaceuticals using *Moringa oleifera*

The part of *Moringa oleifera* seed; water-soluble protein, seed husk and seed cake were used for the removal of selected pharmaceuticals from water. The amines and the amides were found as dominant functional groups responsible for adsorption implies the seed contains proteins. Different parameters that affect the efficiency of *Moringa* adsorbent to remove pharmaceuticals, such as adsorption time, initial analyte concentration and pH were studied and the optimum parameter conditions for each were selected.

4.6.2.1. Effect of pH

As shown in (Figure 4.6.6), the effect of solution pH on the removal of multi-class pharmaceuticals from aqueous solution was studied by varying pH values (3-11) while keeping all other parameters constant (adsorbent dosage of 40 mg, analyte concentration of 0.5 mg L^{-1}, the volume of 30 mL, contact time 60 min and room temperature). The amount of pharmaceuticals removed at different sample pH besides being pH dependent was also affected by the type of Moringa biosorbent. Overall, sample pH of 5 showed highest removal efficiencies for most pharmaceuticals. Optimum sample pH of 5 was more pronounced on Moringa seed cake as an adsorbent. Other Moringa biosorbent, the trend on the effect on sample pH was not very pronounced. Sample pH 5 and 9 seem to have given close to optimum removal efficiencies for the other two with more pharmaceuticals slightly still removed pH 5. This could be attributed to varying functional groups present in these biosorbents (Kebede et al., 2019b). The study reported by Araujo et al., (2018) on the removal of pharmaceuticals using Moringa as an adsorbent, pH of 5 sample solution was an optimum pH leads relatively high adsorption on the adsorbent. The other reported study also shows that the maximum removal was attained at pH 5 and pH 6 of the sample solution (Kebede et al., 2019b). The sample pH effects studies are important as they affect both the chemistry of the analytes and that of the bio sorbents. Solution pH of 5 was selected as an optimum for all the biosorbents as a compromise.


a)
% Removal
pH
MET
MTZ
TRM
THP
NOR
CIP
CAF
DOXH
ALB


b)
% Removal
pH
MET
MTZ
TRM
THP
NOR
CIP
CAF
DOXH
ALB

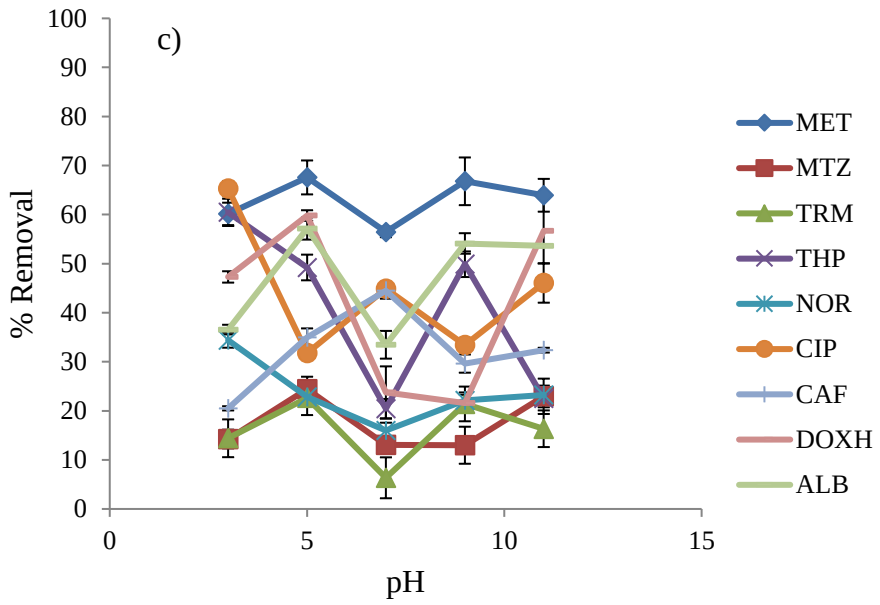
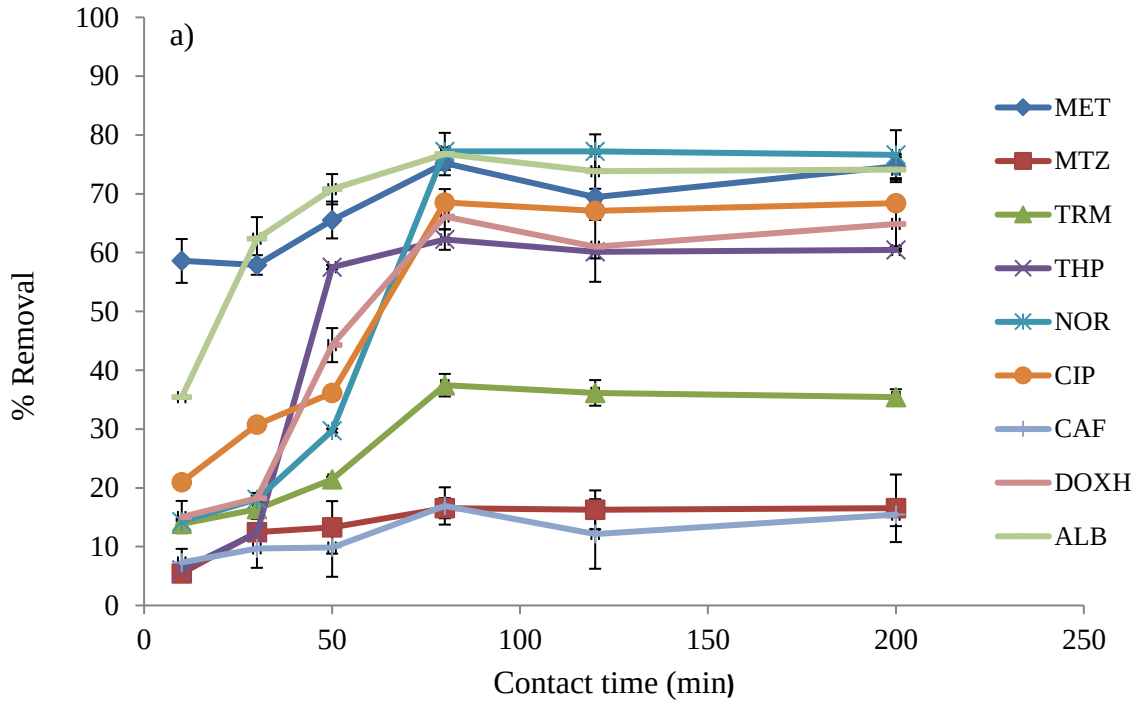
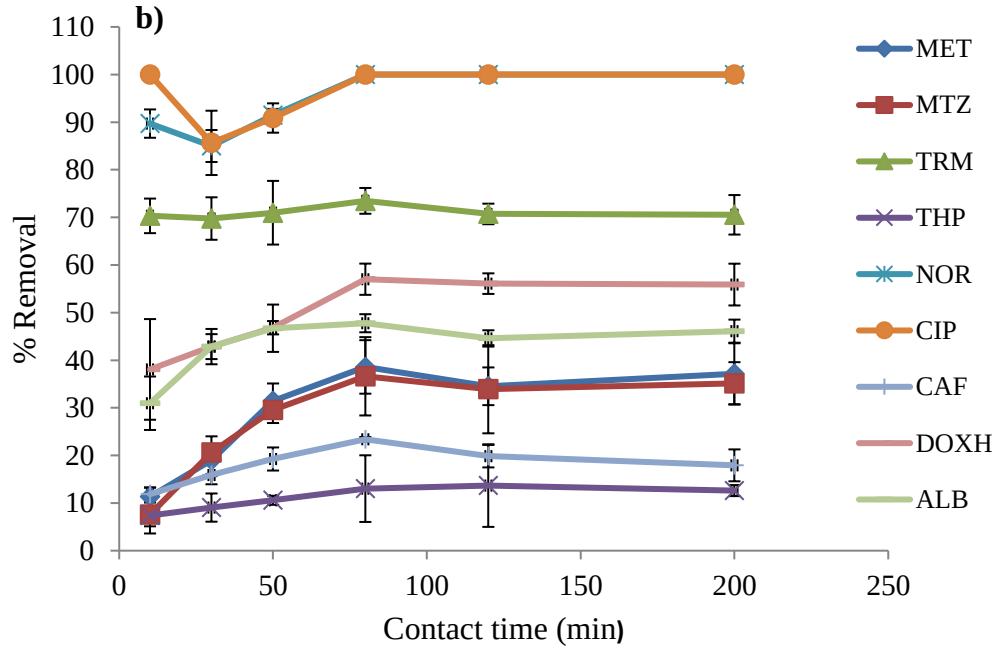


Figure 4.6. 6. Effect of pH on the removal of pharmaceuticals by a) seed cake, b) husk and c) water-soluble protein

4.6.2.2. Effect of contact time

The effect of contact time on the removal of pharmaceuticals was studied by varying the time from 10 to 200 min while keeping other parameters constant (adsorbent dosage of 40 mg, the volume of 30 mL, agitation speed of 250 rpm, initial concentration 0.5 mg L^{-1}, pH at 5 and room temperature). Results (Figure. 4.6.7) indicate that the adsorption of the analyte on the adsorbents was very fast and most of the interaction took place within the first 30 min. This phenomenon could be due to the presence and availability of several active functional groups on the surface of the *Moringa* adsorbent at the initial stages of removal. On increasing the contact time up to 80 min an increase in the removal efficiency was observed and it remained constant as the contact time increased further to 200 min, showing that it had reached the saturation point. The equilibrium was achieved after 80 min for most of the analytes. However, *Moringa oleifera* water-soluble protein adsorbent shows that time variation in the range considered in this study does not affect the removal of pharmaceuticals. Adsorption capacity (mg/g) versus contact time (min) shown in (Appendix E1).


a)
% Removal
Contact time (min)
MET
MTZ
TRM
THP
NOR
CIP
CAF
DOXH
ALB


b)
% Removal
Contact time (min)
MET
MTZ
TRM
THP
NOR
CIP
CAF
DOXH
ALB

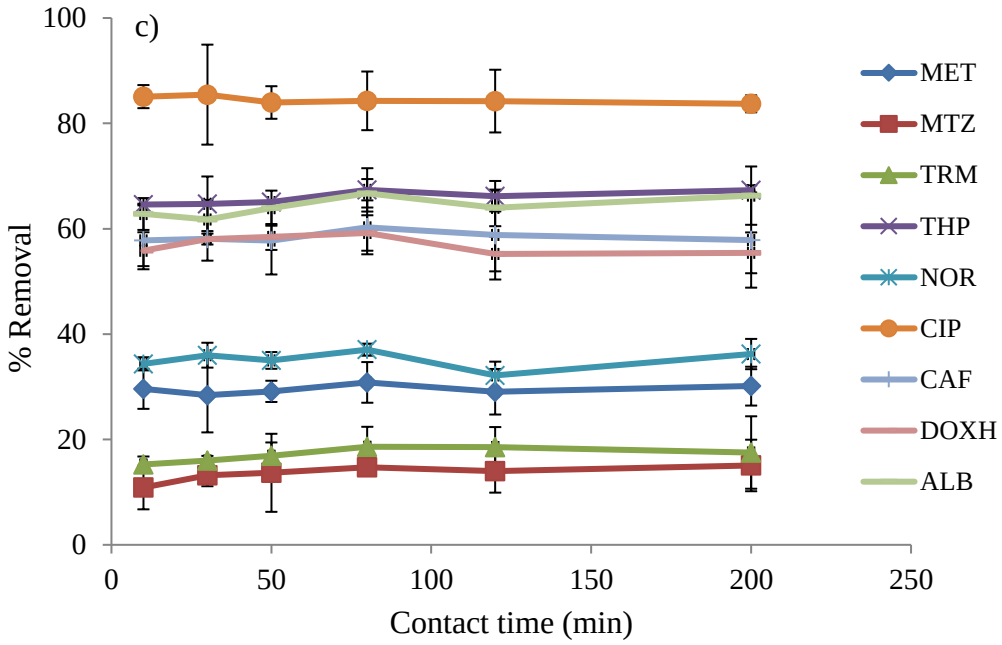
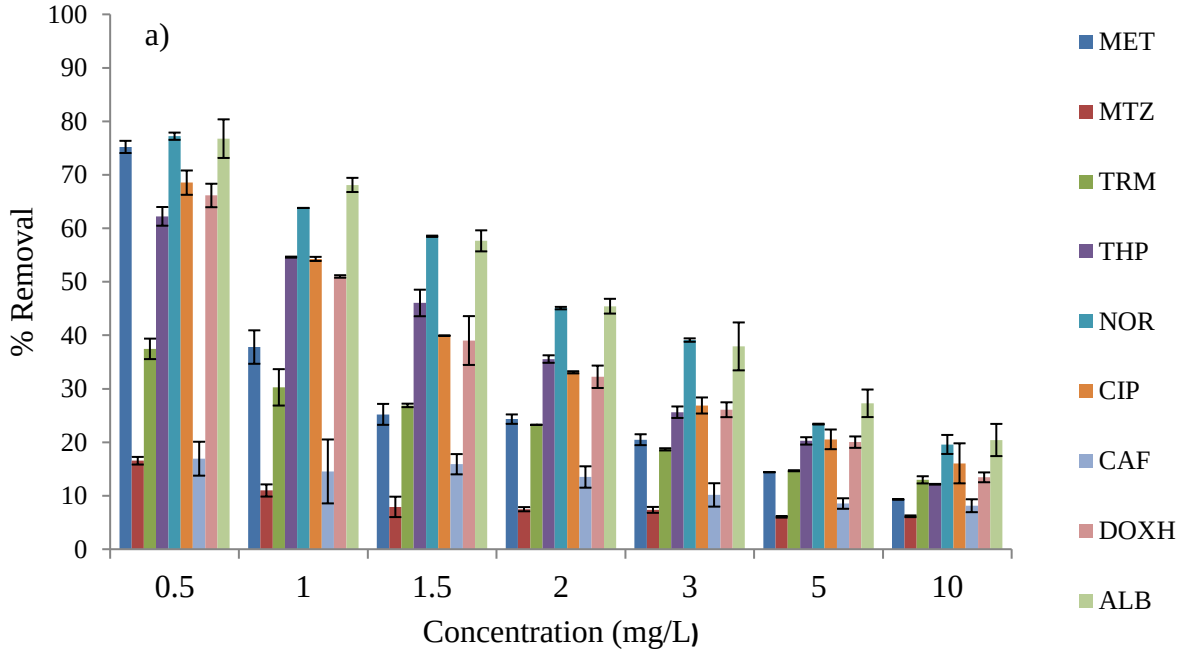
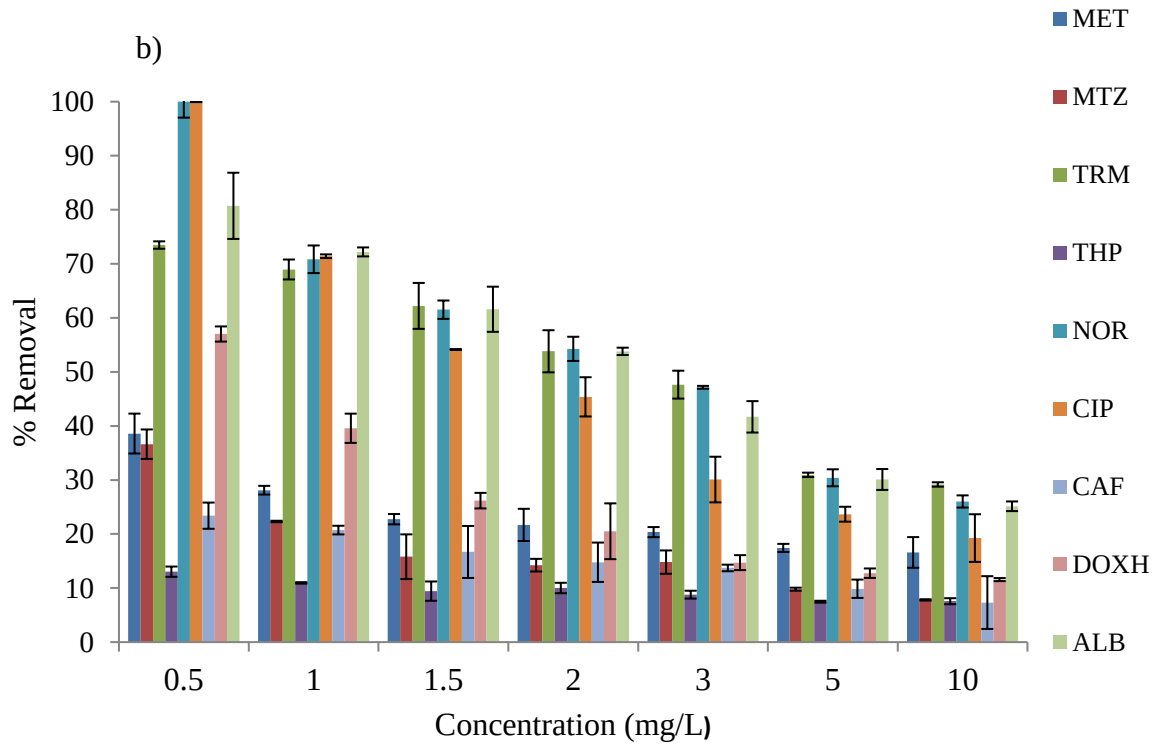


Figure 4.6. 7. Effect of contact time on the removal of pharmaceuticals by a) seed cake, b) husk and c) water-soluble protein

4.6.2.3. Effect of the initial concentration of pharmaceuticals

The effect of initial concentration was investigated by varying the concentration from 0.5-10 mg L^{-1} while all other parameters were maintained constant (i.e. adsorbent dosage of 40 mg, the volume of 30 mL, agitation speed of 250 rpm, the contact time of 80 min, pH at 5.5 at room temperature). Figure 4.6.7 shows that an increase in the adsorbate concentration (0.5 to 10 mg L^{-1}) resulted in the rapid drop in percentage removal. The sudden decrease observed could be explained by the reduction of active sites on the *Moringa* adsorbent. However, it was observed that the number of pharmaceuticals adsorbed per unit mass of adsorbent (adsorption capacity) in (mg kg^{-1}) was increased as the corresponding concentration increased from 0.5 to 10 mg L^{-1}. This tread was found similar to the reported studies done using *Moringa stenopetala* seeds water-soluble protein as a biosorbent for the removal of antibiotics and sulphonamides (Kebede et al., 2019a; Kebede et al., 2019b). The adsorption capacity for the Moringa biosorbents, seed cake ranged from 3-311, for the seed husk ranged from 7-413 and for water-soluble protein was 2-326 mg/g. The result implies seed husk has the highest adsorption capacity compared to other biosorbents. Adsorption capacity (mg/g) versus concentration (mg/L) shown in (Appendix E2).


a)
% Removal
100
90
80
70
60
50
40
30
20
10
0
0.5
1
1.5
2
3
5
10
Concentration (mg/L)
MET
MTZ
TRM
THP
NOR
CIP
CAF
DOXH
ALB


b)
% Removal
100
90
80
70
60
50
40
30
20
10
0
0.5
1
1.5
2
3
5
10
Concentration (mg/L)
MET
MTZ
TRM
THP
NOR
CIP
CAF
DOXH
ALB

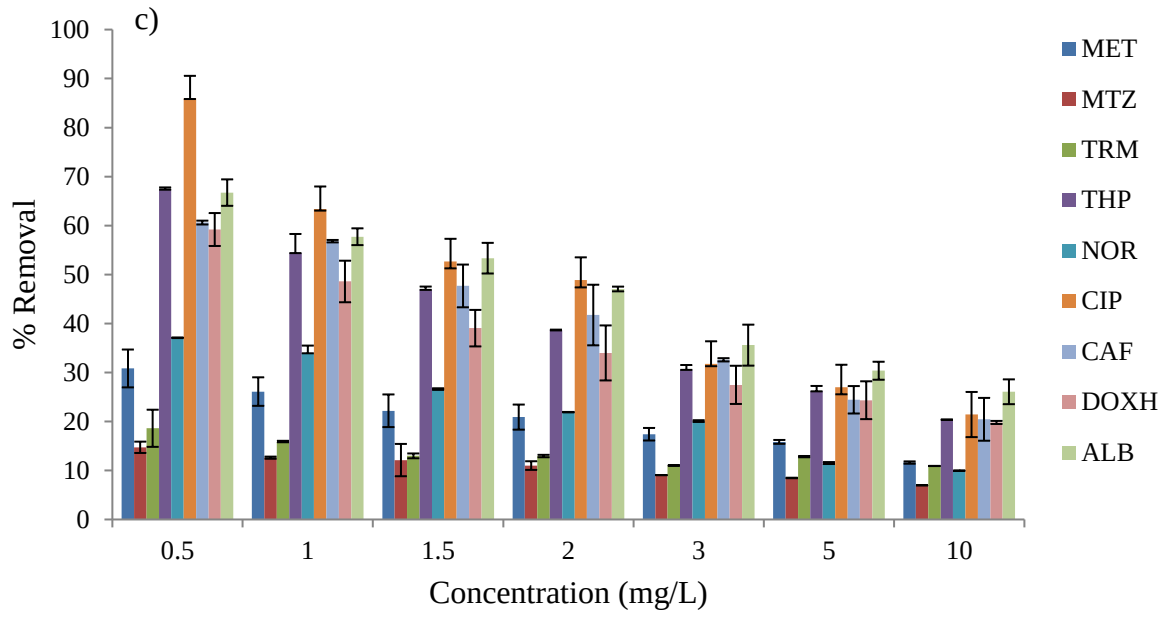


Figure 4.6. 8. Effect of initial concentration on the removal of pharmaceuticals by a) seed cake, b) husk and c) water-soluble protein

4.6.3. Adsorption isotherms and kinetics

4.6.3.1. Adsorption isotherms

Experiments for adsorption isotherms were carried out by varying adsorbate concentrations. Langmuir and Freundlich adsorption isotherm models were used to fit the experimental adsorbates with adsorbents interaction and the data is presented in Table 4.6.1. The Langmuir parameters, q_{max} and K_L, were calculated from the intercepts and slopes, respectively, of the linear plot of C_e/q_e versus C_e. Similarly, Freundlich parameters (n, k_f) were calculated from the slope and intercept, respectively, of the linear plot of log q_e versus log C_e based on what is explained in (Section 2.8.6.3). The results show that the Langmuir dimensionless equilibrium constant (R_L) is less than one and greater than zero, which indicates the favourability of the adsorption. The value of n (heterogeneity factor) which is greater than one indicates that the Freundlich isotherm is indeed favourable for the adsorption of multiclass pharmaceuticals using *Moringa oleifera* seed adsorbent. Based on the R^2 values from the adsorption models the fitness of the experimental data in the models used were generally good for both isotherm models. However, the Freundlich model provided the best correlation for all the multiclass

pharmaceuticals as compared to Langmuir. The fitting of the experimental data in the Freundlich isotherm further confirms that adsorption was taking place in the multilayer heterogeneous surface of the adsorbent.

Table 4.6. 1. Adsorption isotherms parameters

Adsorbent	Analyte	Langmuir parameters				Freundlich parameters		
		q_{max} (mg/g)	K_L (L/mg)	RL (L/mg)	R^2	K_f ((mg/g)/(mg/L)$^{1/n}$)	n	R^2
Seed cake	MET	54.6	0.33	0.55	0.9332	15.4	2.31	0.9379
	MTZ	44.8	0.12	0.62	0.8900	3.94	1.07	0.916
	TRM	114.9	0.20	0.59	0.8919	20.9	1.72	0.9897
	THP	158.7	0.58	0.48	0.9899	59.2	2.44	0.9246
	NOR	370.4	0.33	0.55	0.8860	100	2.14	0.9428
	CIP	285.7	0.42	0.52	0.9516	91.3	2.38	0.9741
	CAF	116.3	0.11	0.62	0.8031	11.9	1.39	0.9859
	DOXH	53.5	0.57	0.48	0.9850	16.3	1.94	0.9702
	ALB	53.5	0.57	0.48	0.9600	32.4	1.81	0.9853
Seed husk	MET	111.1	0.11	0.62	0.9013	10.8	1.23	0.9885
	MTZ	76.3	0.15	0.61	0.8627	8.30	1.50	0.9181
	TRM	256.4	0.21	0.58	0.8766	46.1	1.64	0.9677
	THP	6.3	0.75	0.44	0.8191	12.9	1.26	0.9944
	NOR	526.3	0.25	0.57	0.8623	117.8	1.98	0.9733
	CIP	312.5	0.57	0.48	0.9218	123.8	2.92	0.9370
	CAF	83.3	0.22	0.58	0.9705	15.4	1.66	0.9924
	DOXH	49.0	0.26	0.57	0.8131	11.9	2.19	0.9234
	ALB	192.3	0.21	0.58	0.9082	34.8	1.67	0.9771
Water-soluble protein	MET	112.4	0.09	0.63	0.9531	9.60	1.28	0.9931
	MTZ	65.4	0.07	0.64	0.7248	4.40	1.06	0.9785
	TRM	131.6	0.08	0.63	0.8332	10.4	1.21	0.9926
	THP	333.3	0.19	0.59	0.9345	56.1	1.58	0.9600
	NOR	192.3	0.26	0.57	0.7819	44.6	1.83	0.9003
	CIP	400.0	0.34	0.54	0.9453	104.4	1.93	0.9211
	CAF	243.9	0.18	0.60	0.9284	4.6	1.50	0.9622
	DOXH	106.4	0.16	0.60	0.9318	14.8	1.45	0.9667
	ALB	256.4	0.11	0.62	0.8685	28.8	1.35	0.9765

4.6.3.2. Adsorption kinetics

The values of pseudo-first and pseudo-second-order kinetic model of the rate constants (K_1, K_2, q_e (cal) and q_e (exp)) are determined from the intercept and slope of the curves as a function of time and presented in Table 4.6.2. The correlation coefficient (R^2) of the plotted curve was in the range of 0.0260–0.9518 for the pseudo-first-order kinetics model, while it was in the range 0.9083-0.9991 for the pseudo-second-order kinetics model. The best fit of these models was also compared using the lowest possible value, sum of error squared (SSE). The SSE values of the two models are listed in Table 4.6.2. From the results, it was observed that the pseudo-second-order kinetics model gives the lowest SSE values than the pseudo-first-order kinetics model. The results obtained further proved the fitness of the pseudo-second-order kinetics model to describe the adsorption process of multiclass pharmaceuticals on *Moringa* adsorbent. Therefore, chemisorption could be the rate-limiting step in the adsorption of multiclass pharmaceuticals with *Moringa oleifera* seed.

Table 4.6. 2. Adsorption kinetics parameters

Adsorbent	Analyte	Pseudo-first order					Pseudo-second order			
		q_e (mg/g) exp	q_e (mg/g) cal	k_1(min^{-1})	R^2	SSE	R^2	k^2 (min^{-1})	q_e (mg/g) cal	SSE
Seed cake	MET	13.1	4.43	0.018	0.9275	3.25	0.9965	0.079	13.2	0.06
	MTZ	3.08	1.33	0.015	0.9068	0.66	0.9714	0.017	7.78	0.64
	TRM	13.6	8.09	0.016	0.6989	2.06	0.9491	0.016	15.4	0.7
	THP	36.9	15	0.017	0.6022	8.27	0.9242	0.012	45.9	3.41
	NOR	64.7	45.2	0.026	0.7754	7.38	0.9664	0.013	80.0	5.77
	CIP	63.3	7.87	0.034	0.8950	20.9	0.9387	0.118	7.87	1.82
	CAF	7.08	3.77	0.011	0.7721	1.24	0.9694	0.026	7.25	0.06
	DOXH	11.9	8.91	0.020	0.9494	1.11	0.9083	0.009	12.8	0.36
	ALB	20.2	5.04	0.013	0.6705	5.72	0.9971	0.074	20.4	0.07
Seed husk	MET	6.71	4.17	0.015	0.9248	0.96	0.9785	0.020	7.39	0.26
	MTZ	6.84	4.1	0.015	0.9068	1.04	0.9714	0.017	7.78	0.35
	TRM	26.6	1.12	0.001	0.1053	9.62	0.9997	0.982	25.6	0.37
	THP	7.73	3.35	0.013	0.9518	1.66	0.9906	0.053	8.06	0.13
	NOR	83.8	9.76	0.014	0.6594	27.9	0.9991	0.138	85.5	0.63
	CIP	92.4	4.32	0.008	0.2214	33.3	0.9989	0.159	94.3	0.74
	CAF	9.78	2.73	0.003	0.1519	2.66	0.9824	0.392	7.77	0.76
	DOXH	8.49	3.05	0.017	0.8188	2.06	0.9967	0.065	8.72	0.08
	ALB	21.2	7.98	0.016	0.6766	5.10	0.9967	0.038	22.3	0.41
Water-soluble protein	MET	5.33	1.66	0.008	0.3024	1.39	0.9677	0.051	5.12	0.08
	MTZ	2.75	1.3	0.012	0.7135	0.55	0.9826	0.039	2.82	0.03
	TRM	6.73	3.69	0.008	0.5890	1.15	0.9149	0.017	6.73	0.03
	THP	39.9	37.5	0.033	0.9011	0.92	0.9749	0.026	43.9	0.75
	NOR	31.1	5.56	0.002	0.0260	9.64	0.9709	0.047	18.1	4.89
	CIP	77.8	1.36	0.007	0.1332	28.9	0.9997	0.653	78.1	0.10
	CAF	25.2	1.05	0.001	0.0527	9.12	0.9996	0.769	24.3	0.34
	DOXH	8.81	3.4	0.003	0.2505	2.05	0.9991	0.269	8.19	0.24
	ALB	17.6	1.01	0.005	0.9508	6.25	0.9957	0.099	14.7	1.08

4.6.3.3. Application of the adsorbent on real river water samples

The optimized method for the *Moringa* adsorbents (adsorbent mass of 40 mg, pH 5, analyte concentration of 0.5 mg L^{-1}, volume of 30 mL, contact time of 80 min and room temperature) developed for the removal of multiclass pharmaceutical compounds from the standard mixture solution was applied to river water collected from Kebena River (R1) and Akaki River (R2) Addis Ababa, Ethiopia. None of the analytes were detected before spiking possible because they might be in low concentrations and therefore need sample concentrations. Percent of removal of the adsorbents were evaluated after spiking 30 mL of River water sample with 0.5 mg L^{-1}standard mixture solutions of the target pharmaceuticals. In (Table 4.6.3), it is observed that the method developed and optimized using a standard mixture solution containing a known concentration of analytes was applicable for the removal of pharmaceuticals from the real water sample. The maximum removal obtained was in the range of 10.4-77.5%, 8.5-75.4% and 10.5-88.5% for *Moringa* seed cake, husk and water-soluble protein, respectively and more than half of the pharmaceutical compound shows > 50% removal. There was a slight decrease in the removal of the pharmaceuticals in the real water sample compared to the removal obtained for the standard mixture solution containing known concentrations of the analyte. This decrease might be because of the presence of various competitor ions within the water sample which can probably be adsorbed at the accessible active sites on the adsorbents.

Table 4.6. 3. Removal efficiency of *Moringa oleifera* seed adsorbent from real water samples

Analyte	**Rt**	**Seed cake**			**Husk**			**Protein**		
		Pure water	**R1**	**R2**	**Pure water**	**R1**	**R2**	**Pure water**	**R1**	**R2**
MET	1.94	75.2	62.4	58.7	38.6	35.4	33.5	30.8	28.5	30.1
MTZ	5.38	16.5	10.6	12.5	36.6	38.5	40.5	14.7	11.5	10.5
TRM	6.02	37.5	33.5	25.6	73.5	70.9	66.4	18.6	20.4	19.4
THP	6.39	62.2	65.3	55.7	13.0	10.4	8.5	67.4	59.8	65.4
NOR	7.07	77.2	68.4	70.5	98.7	82.5	76.4	37.0	30.5	38.5
CIP	7.27	68.5	64.3	50.8	96.5	87.5	70.5	86.0	80.5	88.5
CAF	7.34	16.9	11.4	10.4	23.4	20.3	25.4	60.6	56.6	60.4
DOXH	11.74	66.1	59.7	49.6	57.0	49.5	40.6	59.2	56.7	60.4
ALB	13.83	76.8	77.5	70.5	80.7	75.4	70.5	66.7	65.4	60.4

Chapter 5 – CONCLUSIONS AND FUTURE RECOMMENDATION

5.1. Conclusions

In this study, a simple, accurate, precise and robust HPLC-UV method has been developed for simultaneous estimation of thirteen selected pharmaceutical compounds in pure and tablet dosage form. The developed method was validated with ICH guidelines and proved to be suitable for the intended application. This method is also used for the optimization and application of solid-phase extraction techniques for the determination of pharmaceuticals from water samples. Optimized solid-phase extraction parameters like type of sorbent, pH of the sample solution, elution solvent, load volume and addition of EDTA were investigated. The method uses HLB cartridges for SPE simultaneous extraction of thirteen different therapeutic classes of pharmaceutical compounds. Antibiotic trimethoprim; anthelmintic albendazole and central nervous system stimulant caffeine were quantified and antibiotic norfloxacin were detected in hospital wastewater using the developed method.

As pharmaceuticals are in low concentrations in the aquatic environment, UHPLC-MS was optimized and used for the quantification of pharmaceuticals in the water samples collected from different contamination sources in Addis Ababa, Ethiopia. The concentration and detection frequency of the nine pharmaceuticals ranged from not detected to 48.8 µg/L and 25 to 100%, respectively. Amoxicillin was the pharmaceutical compound considered not detected in all sampling points and caffeine was the pharmaceutical compound found in high concentration. Among the contamination sources that were sampled for this study, the sewerage treatment plant contributes a higher percentage of the total pharmaceuticals detected in the water samples.

QuEChERS method of sample preparation was optimized using selected pharmaceutical compounds. Different cleanup sorbents were evaluated for their matrix effect removal after extraction and a combination of d-SPE sorbent $MgSO_4$, PSA, C_{18} and DE were found to be selective cleanup sorbent for the targeted compounds. The addition and amount of diatomaceous earth and EDTA during the cleanup and extraction steps were also examined and optimized. Solvent type and composition, salt type and extraction time were also optimized. The method was successfully applied to different vegetable samples collected from Addis Ababa, Ethiopia and Johannesburg, South Africa (carrot, cabbage and lettuce) and none of the target analytes

were found in the sample investigated. The matrix effect study on vegetable samples collected was found to be very high that suppressed the signal during analysis.

Molecularly imprinted polymer was synthesized using bulk polymerization and characterized for adsorption and extraction study of venlafaxine, ciprofloxacin, norfloxacin and albendazole from aqueous environment. Parameters affecting adsorption efficiency was optimized for simultaneous adsorption and the results indicate that the synthesized polymer exhibited good selectivity for the selected four pharmaceutical compounds. In the adsorption isotherm, the Freundlich model was best fitted with the experimental data indicating the heterogeneous nature of binding sites in the adsorbent and pseudo-second-order kinetic model indicating chemisorption during adsorption. The polymer synthesized demonstrated a high selectivity towards albendazole than the template venlafaxine and was followed by ciprofloxacin and norfloxacin. The polymer was used to adsorb the target analyte from river water that shows a promising result for the removal and extraction of selected pharmaceuticals other than the template. Hence, the developed method is a promising alternative for the simultaneous adsorption and extraction of these compounds in aqueous samples.

The removal efficiency of *Moringa oleifera* (seed husk, seed cake and water-soluble protein) as a natural adsorbent for the removal of multi-class pharmaceuticals from water was also studied. The studied adsorbents have promising adsorption capacities for the removal of multi-class pharmaceuticals. It was found that the adsorption process for removal in the current study was dependent on pH, contact time and initial pharmaceutical concentration. The adsorption of the analytes was found to fit the Freundlich adsorption model indicating monolayer sorption with a heterogeneous energetic distribution of active sites. The kinetic study also revealed that the adsorption process in the present study obeyed the pseudo-second-order model indicating chemisorption as the rate-limiting step. Finally, these Moringa adsorbents were used to remove the studied pollutant from real water and were found to be a more effective, efficient, economic alternative adsorbent.

5.2. Future Recommendation

In general, this study attempted to identify for the first time the occurrences of multi-class pharmaceuticals in Ethiopian water samples identified as a potential contamination source. To

give an idea of the status of these and other compounds in the Ethiopian aquatic environments a future recommendation should be designed. An interesting approach is recommended for future work to monitor concentration of pharmaceuticals together with their metabolites in different water samples in different seasons. Furthermore, studies on the detection of pharmaceuticals and metabolites in different vegetable samples in different sampling seasons and in different area will be important. It is also recommended to work on the surface modification in Moringa adsorbents to increase its removal efficiency.

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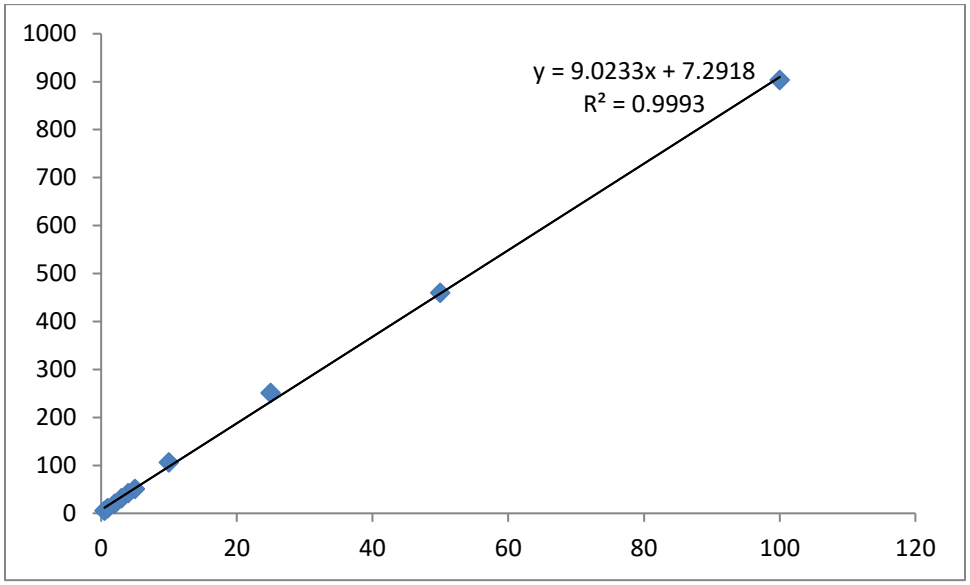
7. ANNEX

List of published papers and manuscripts

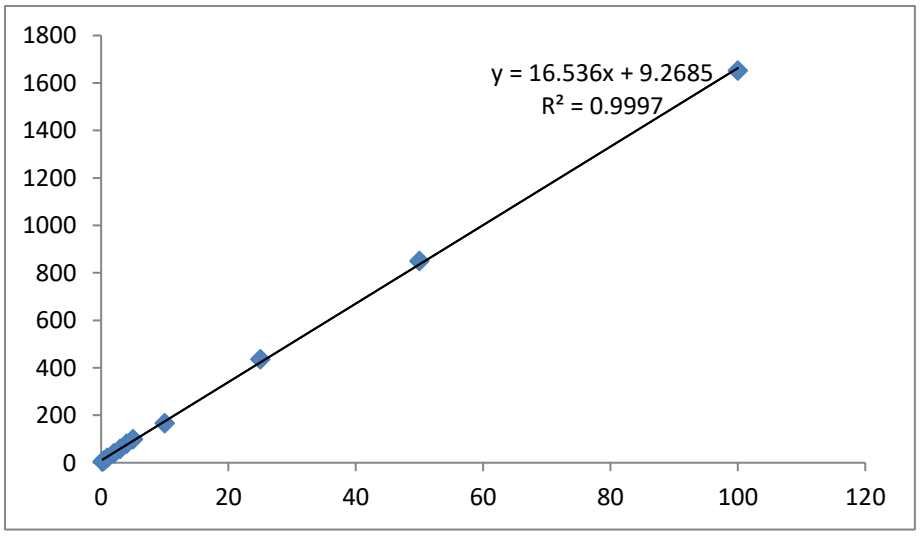
1. Gezahegn T., **Tegegne B**., Zewge F., Chandravanshi B.S. (2019) Salting-out assisted liquid-liquid extraction for the determination of ciprofloxacin residues in water samples by high performance liquid chromatography-diode array detector. *BMC Chem* 13(28). **DOI:** https://doi.org/10.1186/s13065-019-0543-5.
2. **Bisratewongel Tegegne** Luke Chimuka, Bhagwan Singh Chandravanshi, Feleke Zewge. (2021). Molecularly imprinted polymer for adsorption of venlafaxine, albendazole, ciprofloxacin and norfloxacin in aqueous environment. *Separation Science and Technology*, 56(13), 2217–2231. **DOI:** https://doi.org/10.1080/01496395.2020.1819323.
3. **Bisratewongel Tegegne**, Bhagwan Singh Chandravanshi, Feleke Zewge, Luke Chimuka. (2021) Development and validation of a single HPLC method for the determination of thirteen pharmaceuticals in bulk and tablet dosage form. *Bulletin of Chemical Society of Ethiopia*, 35(1), 17-31. **DOI:** https://dx.doi.org/10.4314/bcse.v35i1.2.
4. **Bisratewongel Tegegne**, Bhagwan Singh Chandravanshi, Feleke Zewge, Luke Chimuka. Solid-phase optimisation for simultaneous determination of thirteen pharmaceuticals in Ethiopian water samples with HPLC-DAD detection: an initial assessment. 193, Article number: 310. **DOI:** https://doi.org/10.1007/s10661-021-08999-y
5. **Bisratewongel Tegegne**, Bhagwan Singh Chandravanshi, Feleke Zewge, Luke Chimuka Optimization of Modified QuEChERS method for extraction of selected pharmaceuticals from vegetable samples. **Manuscript** under preparation.
6. **Bisratewongel Tegegne**, Bhagwan Singh Chandravanshi, Feleke Zewge, Luke Chimuka Occurrence of pharmaceutical residue in potential source of water samples from Addis Ababa, Ethiopia. **Manuscript** ready to be submitted in Journal of Chemosphere.
7. **Bisratewongel Tegegne**, Bhagwan Singh Chandravanshi, Feleke Zewge, Luke Chimuka. *Moringa olifera* seed as an adsorbent for potential removal of multi-class pharmaceuticals from water. **Manuscript** ready to be submitted in Journal of Hazardous Materials.

8. APPENDIXES

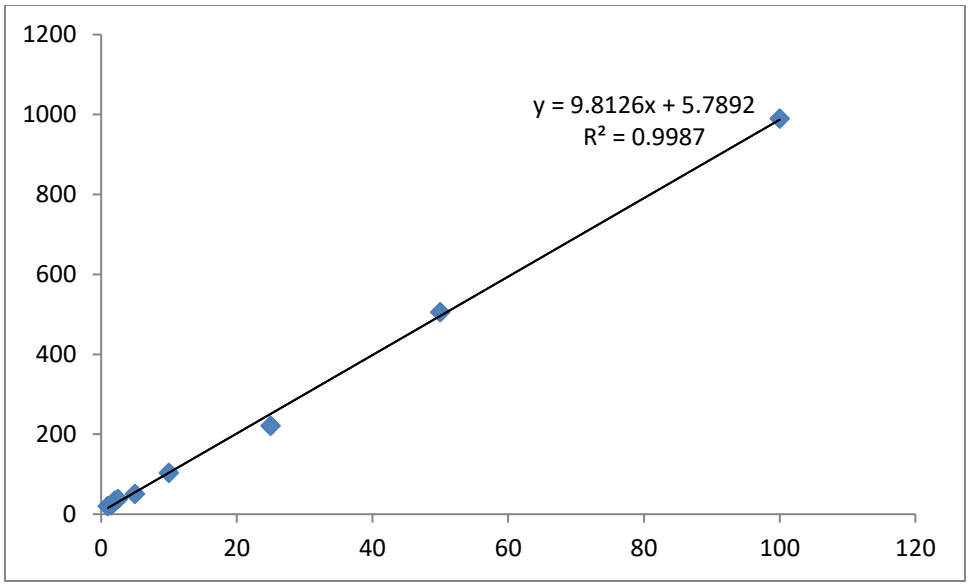
Appendix A. Calibration curve for thirteen pharmaceutical compounds (Y axis peak area, X axis concentration)



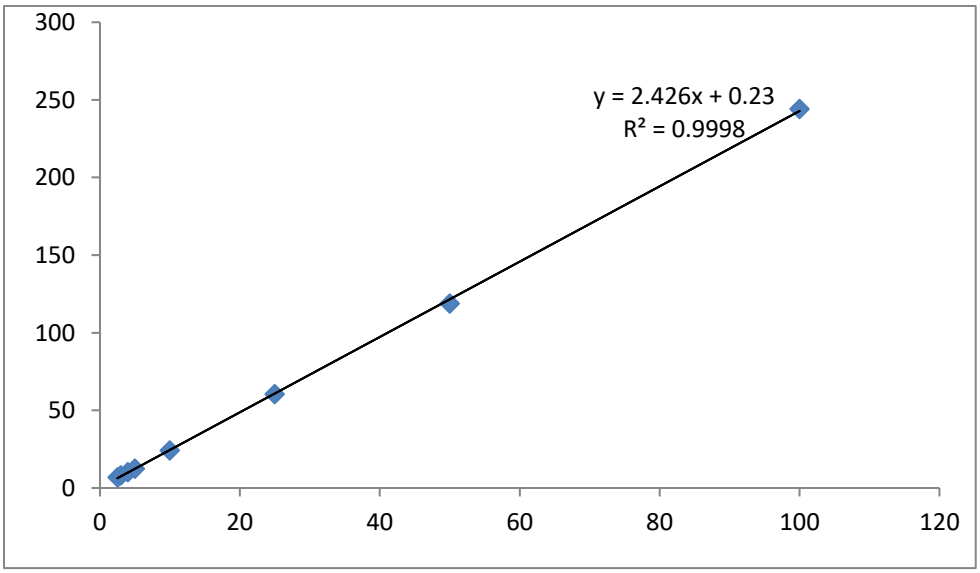
Appendix A1. Calibration curve of metformin.



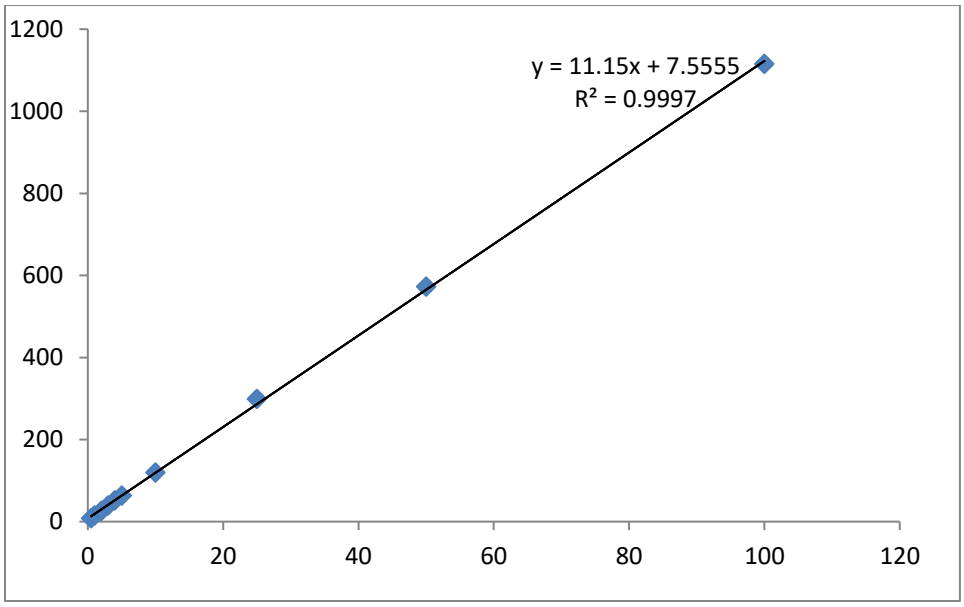
Appendix A2. Calibration curve of trimephrim.



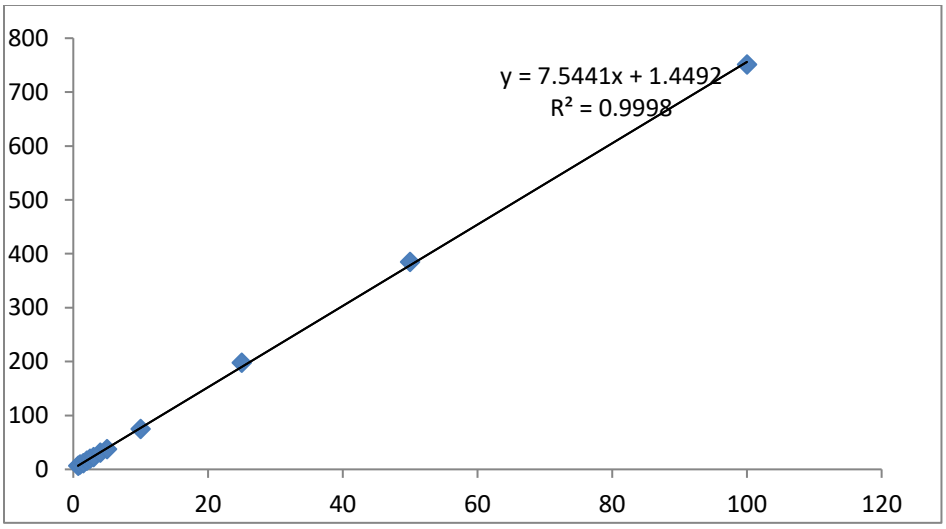
Appendix A3. Calibration curve of ciprofloxacin.



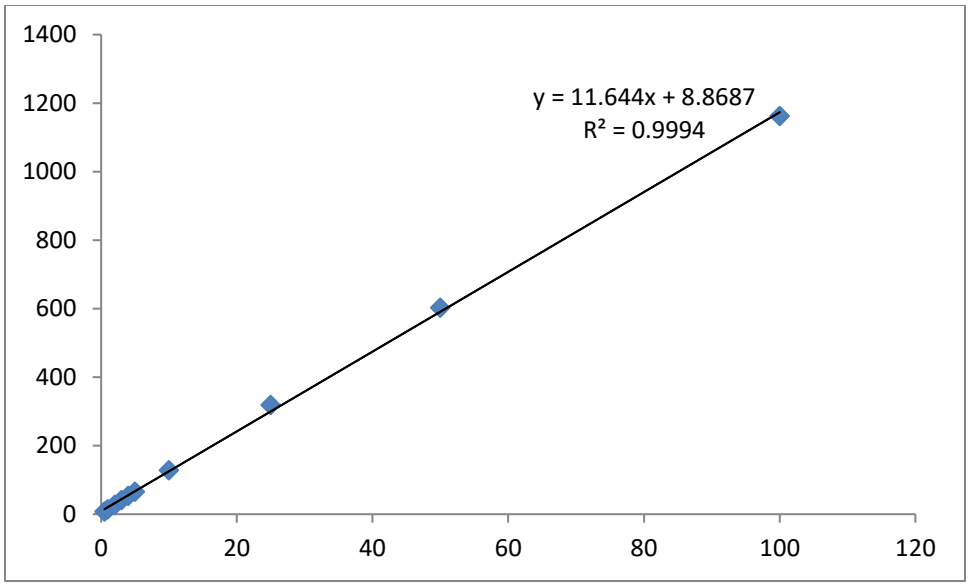
Appendix A4. Calibration curve of amoxicillin.



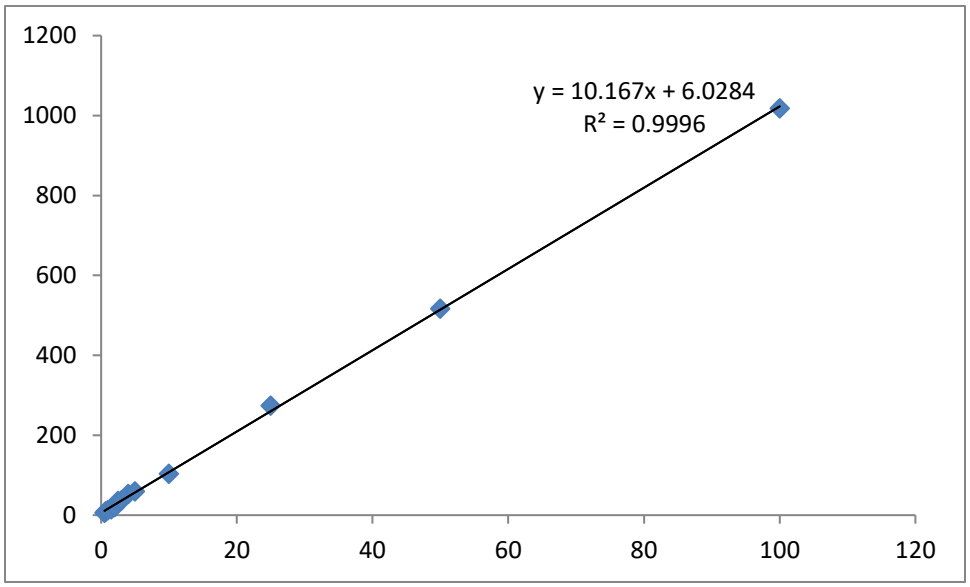
Appendix A5. Calibration curve of chloroquine.



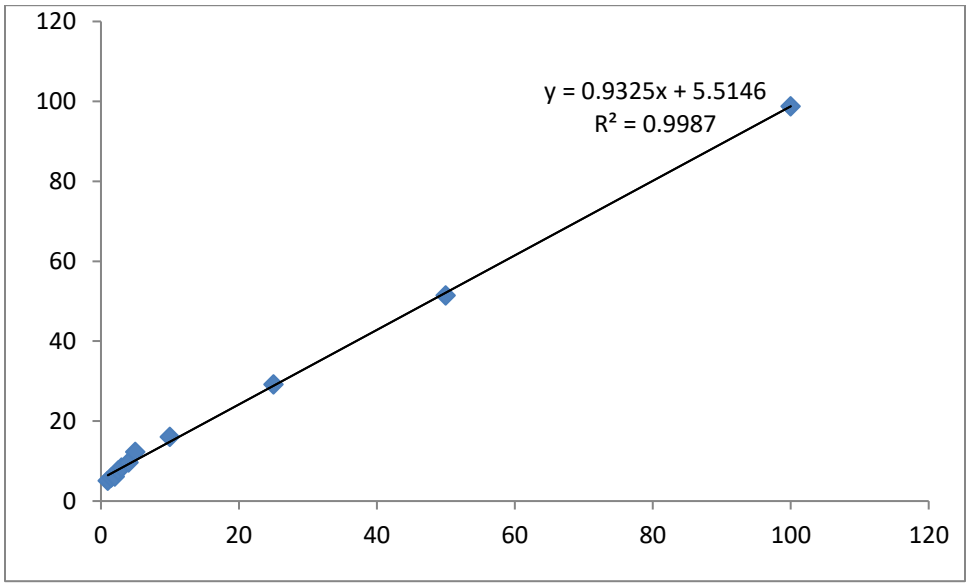
Appendix A6. Calibration curve of norfloxacin.



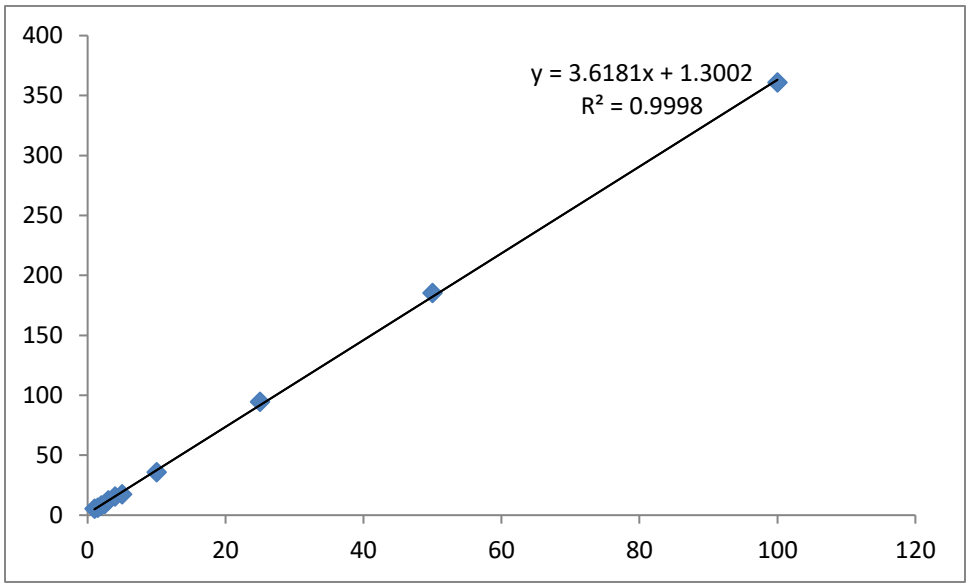
Appendix A7. Calibration curve of theophylline.



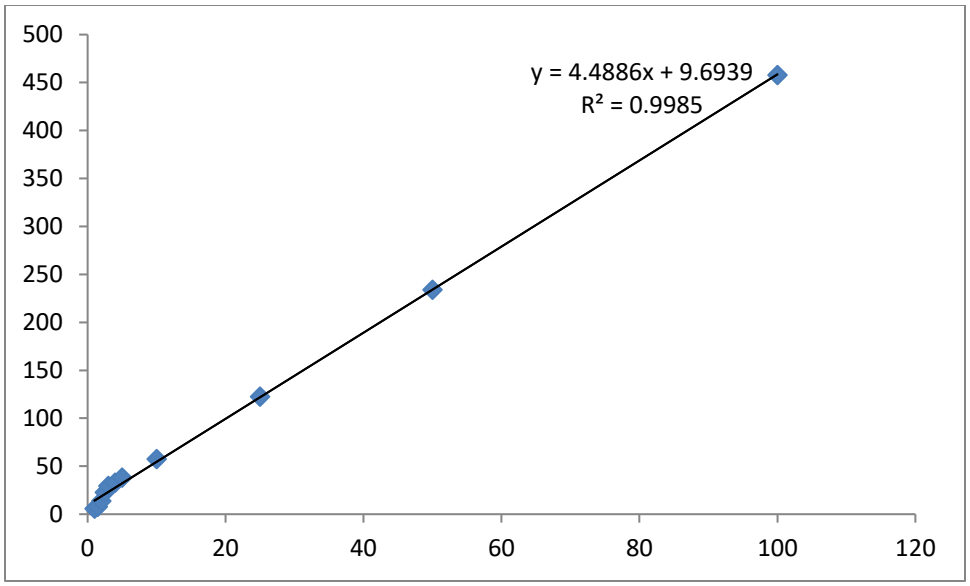
Appendix A8. Calibration curve of caffeine.



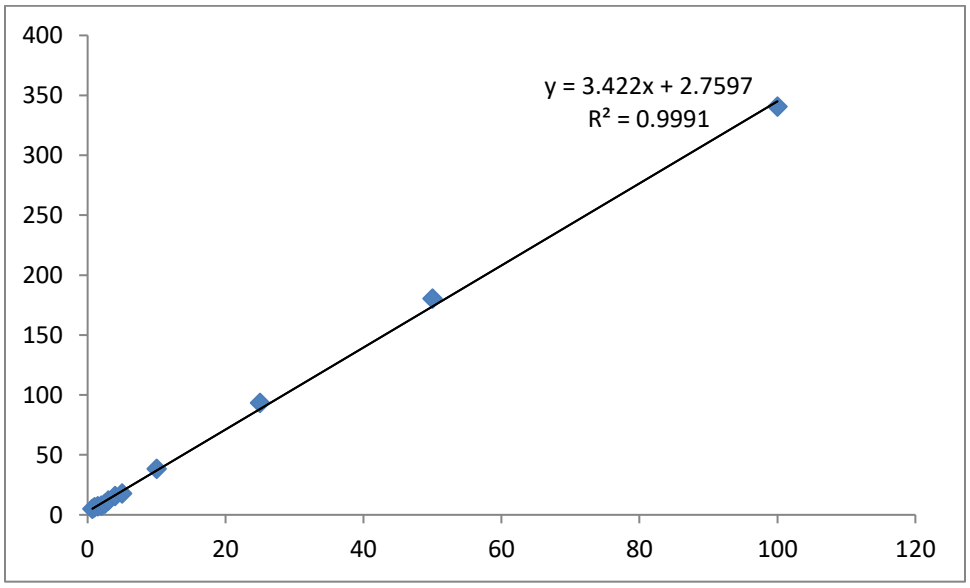
Appendix A9. Calibration curve of doxycycline hyclate.



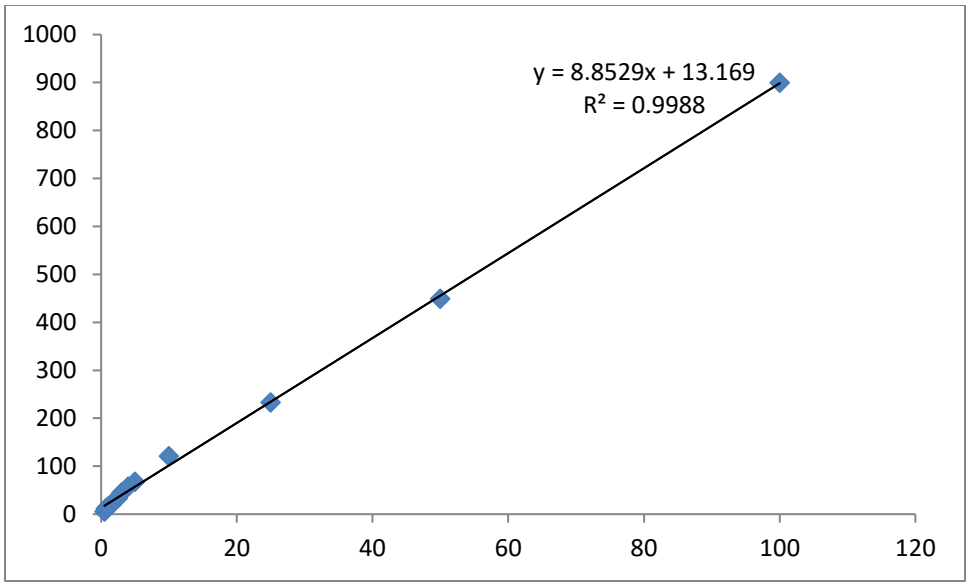
Appendix A10. Calibration curve of acetyl salicylic acid.



Appendix A11. Calibration curve of metronidazole.

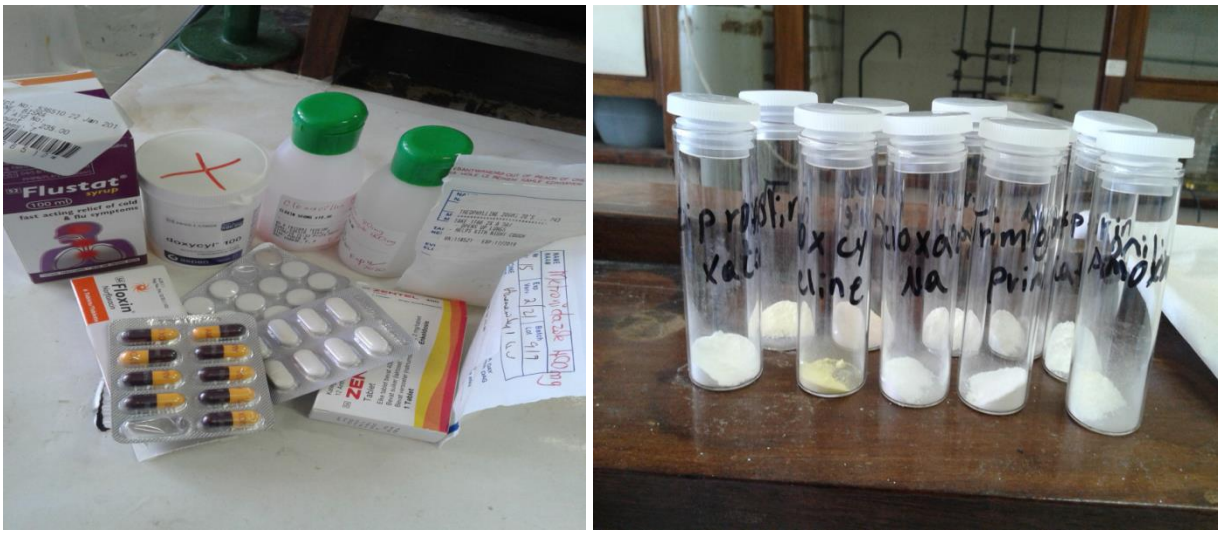


Appendix A12. Calibration curve of cloxacilin.



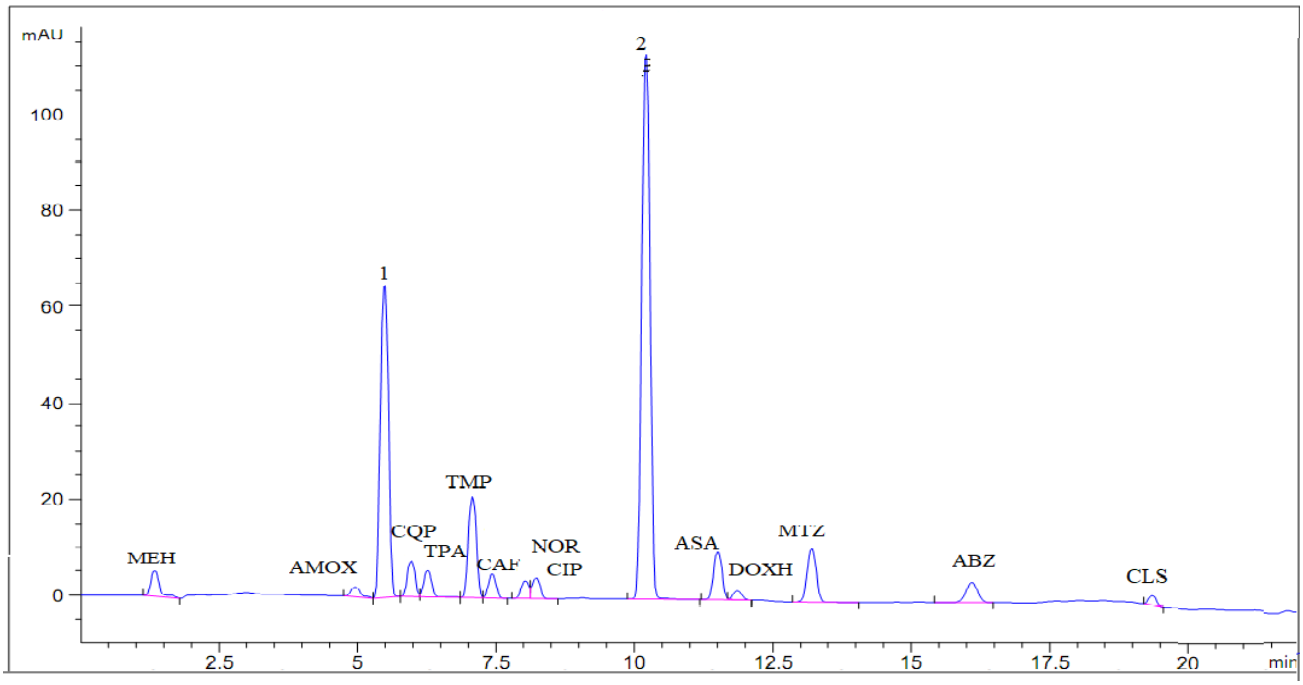
Appendix A13. Calibration curve of albendazole.

Appendix B: Pharmaceutical tablet and the chromatogram



(a) (b)

Appendix B1. Commercially available pharmaceuticals (a) and its powder (b) for recovery study of the developed method.

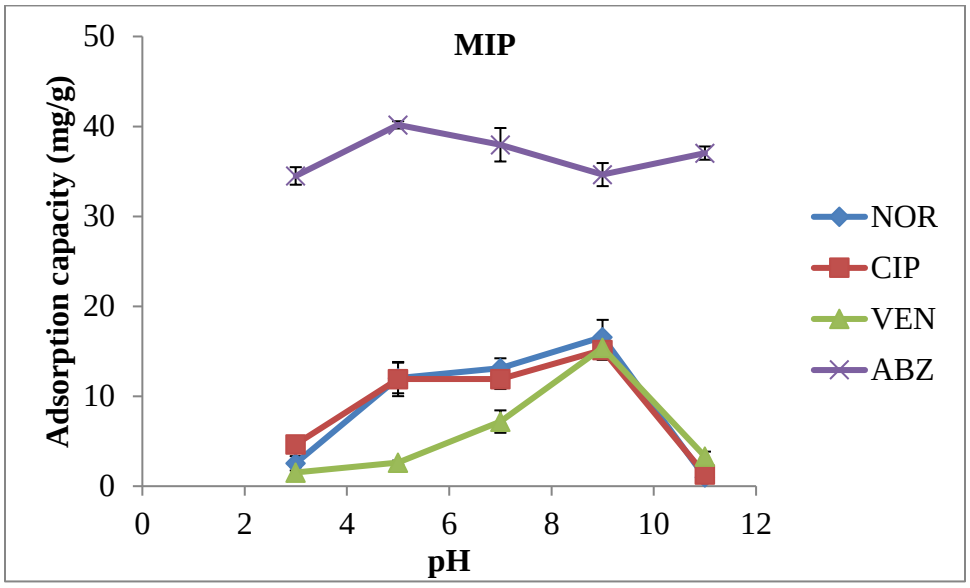


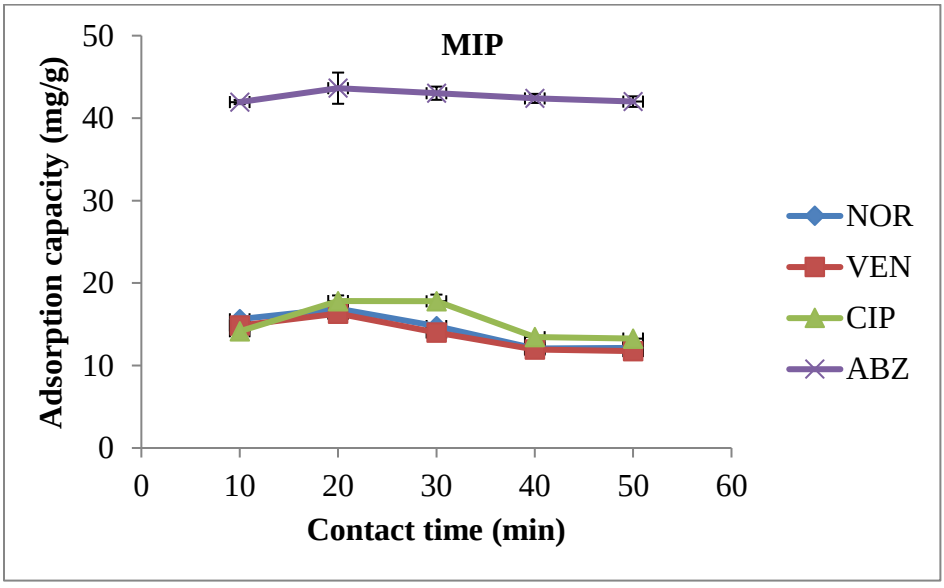
MEH (Metformin hydrochloride), AMOX (Amoxicillin trihydrate), CQP (Chloroquine phosphate), TPA (Theophylline anhydrous), TMP (Trimethoprim), CAF (Caffeine), NOR (Norfloxacin), CIH (Ciprofloxacin), ASA (Acetyl salicylic acid), DOX (Doxycycline hyclates), MTZ (Metronidazole), ABZ (Albendazole), CLS (Cloxacillin Na) (1 and 2 is unknown compound with in the tablet)

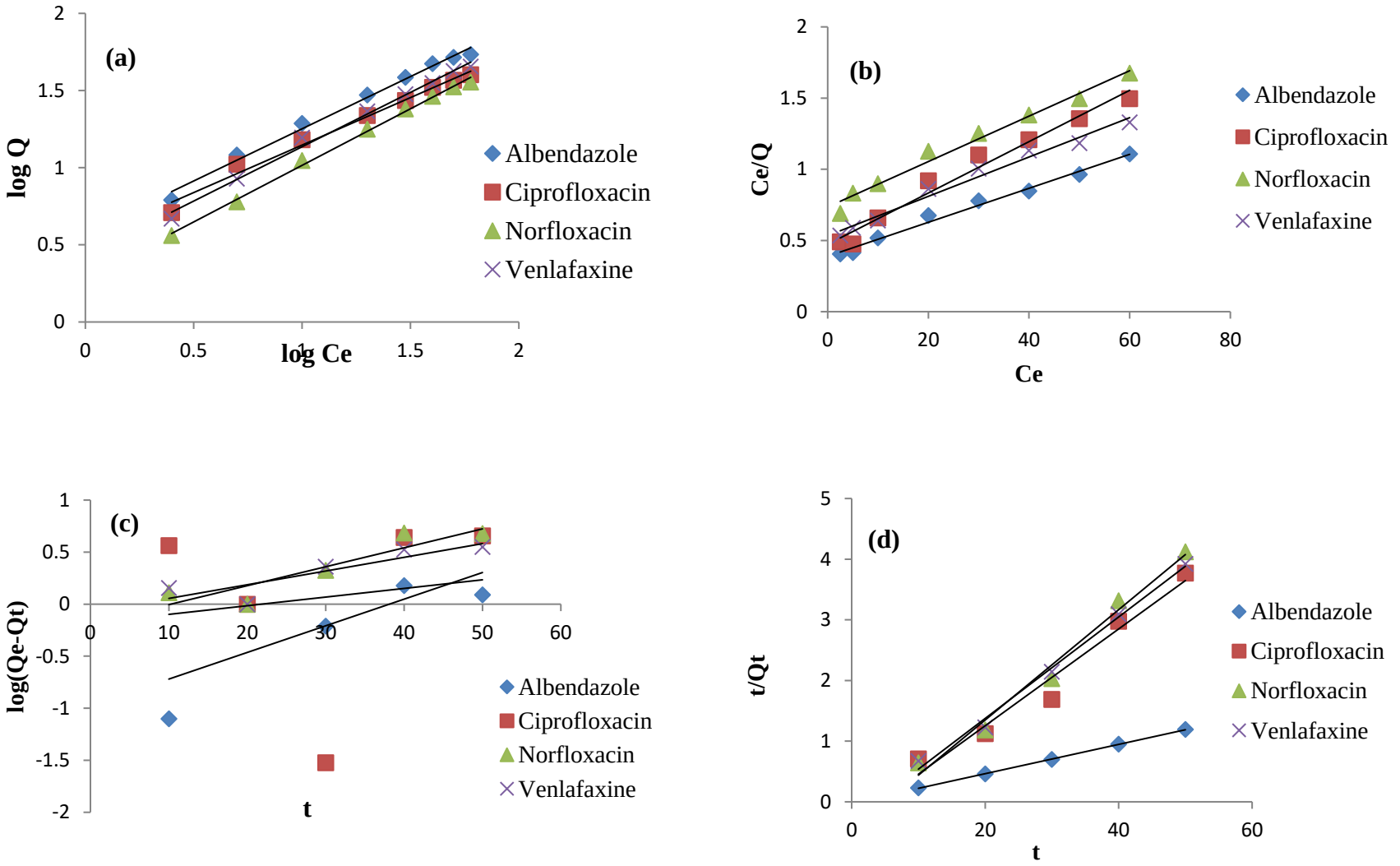
Appendix B2. Complete chromatogram showing the peaks of 13 selected pharmaceuticals from tablet dosage.

Appendix C: Adsorption capacity, adsorption isotherm and kinetic models

Appendix C1. Adsorption capacity versus pH and contact time respectively

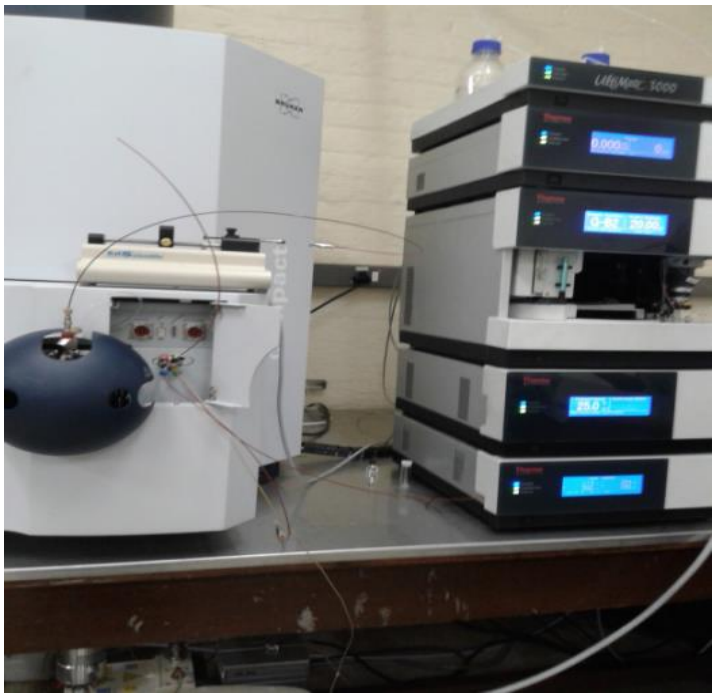




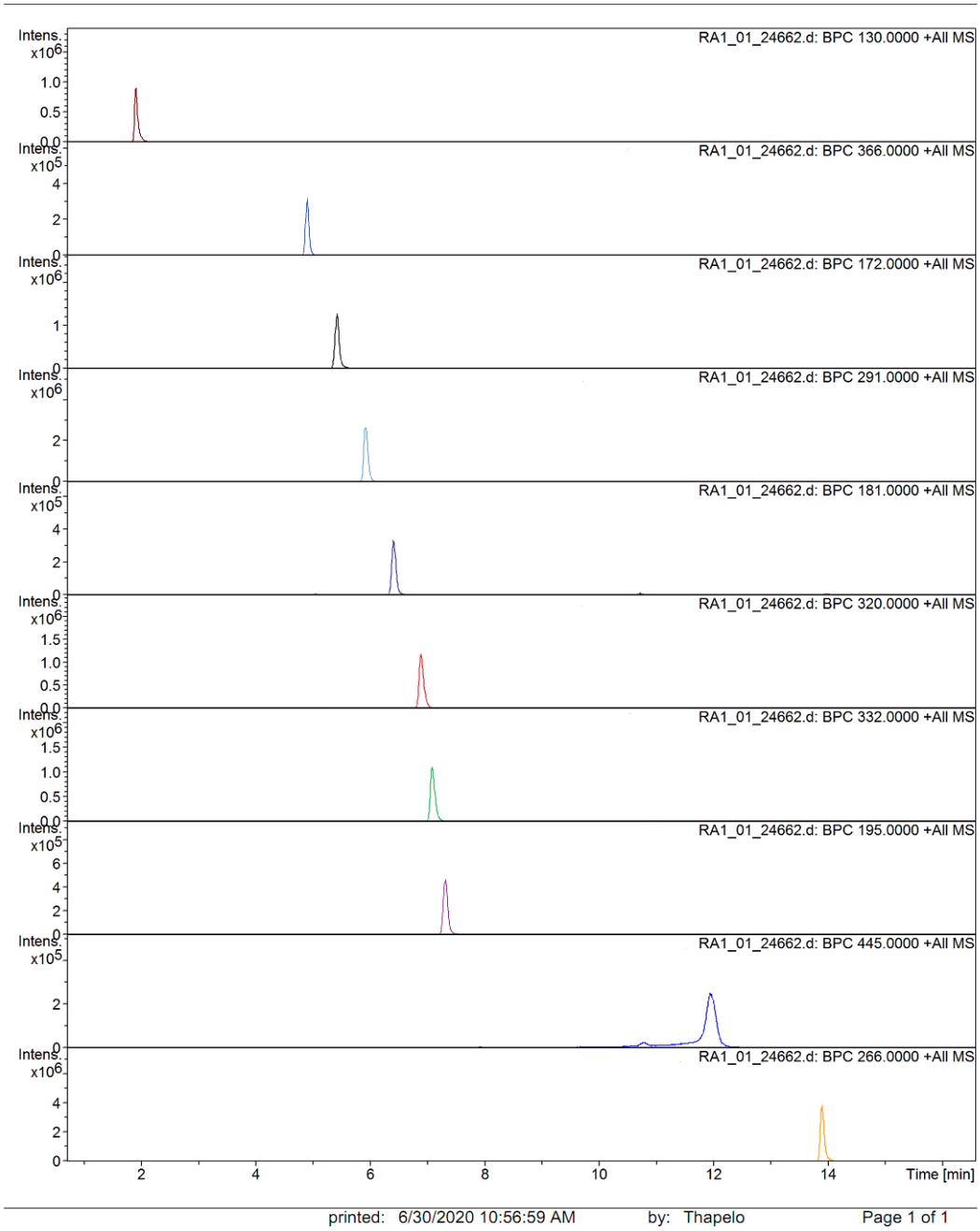


Appendix C2. Adsorption isotherm and kinetic models; **(a)** Freundlich model, **(b)** Langmuir model, **(c)** pseudo first order model and (**d)** pseudo second order model.

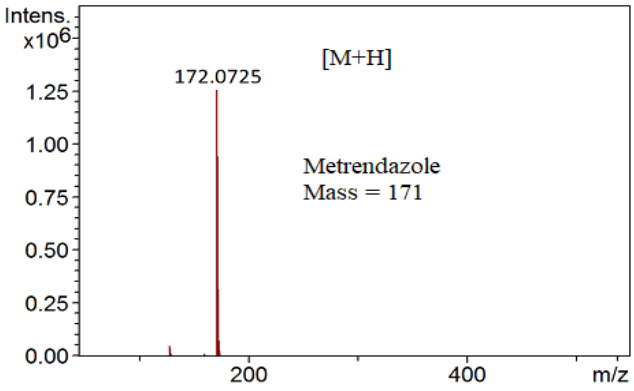
Appendix D: UHLC-MS

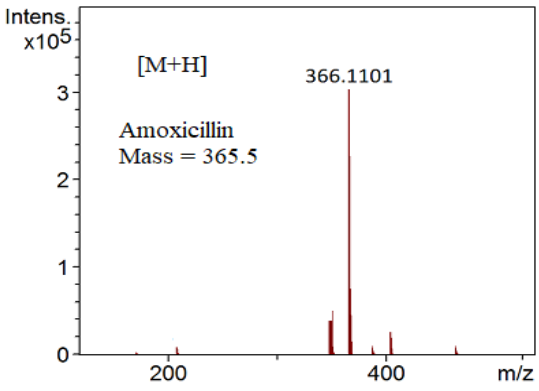


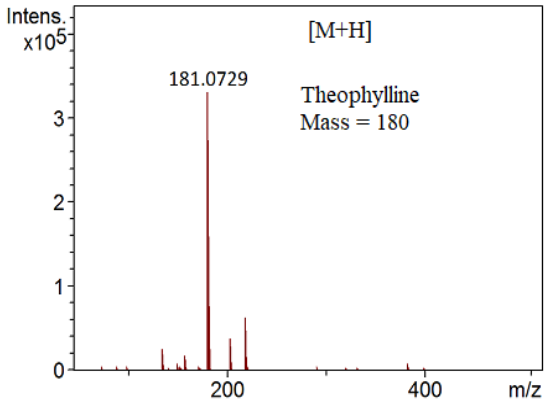
UHPLC-MS used for analysis

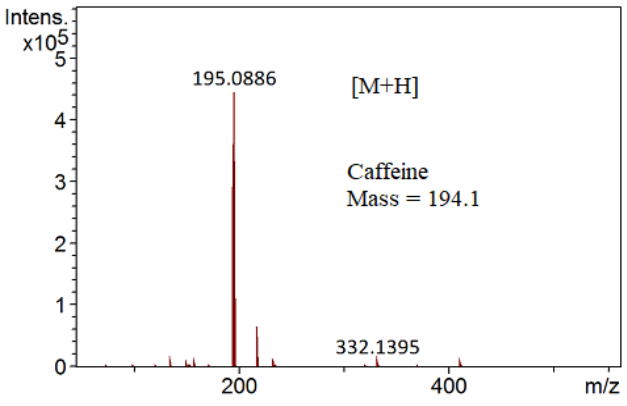


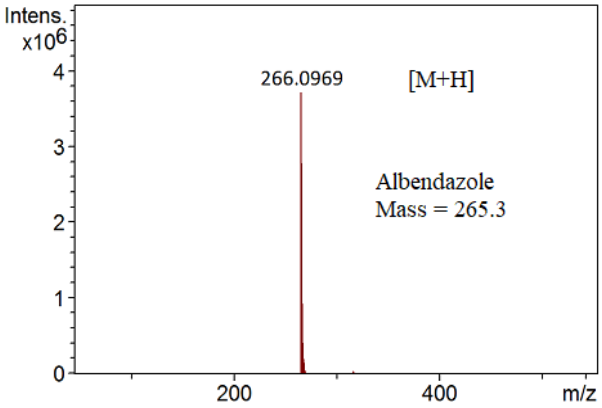
Appendix D1. Total chromatogram of all compounds considered in this study (TIC).

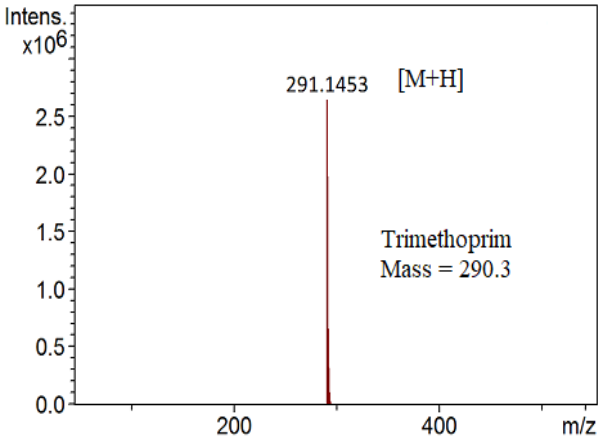

Intens.
x10^6
1.25
1.00
0.75
0.50
0.25
0.00
172.0725
[M+H]
Metrendazole
Mass = 171
200
400
m/z

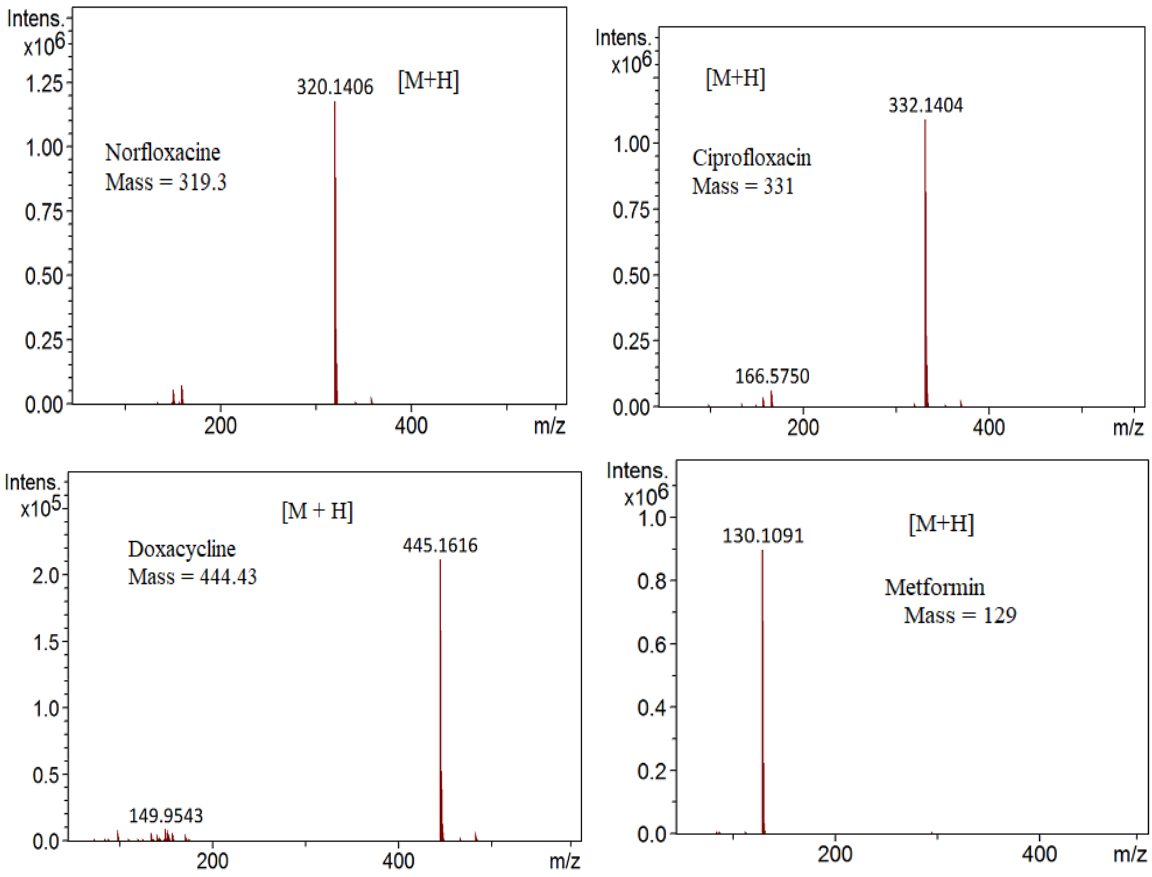

Intens.
x10^5
3
2
1
0
[M+H]
366.1101
Amoxicillin
Mass = 365.5
200
400
m/z


Intens.
x10^5
3
2
1
0
[M+H]
181.0729
Theophylline
Mass = 180
200
400
m/z


Intens.
x10^5
5
4
3
2
1
0
195.0886
[M+H]
Caffeine
Mass = 194.1
332.1395
200
400
m/z

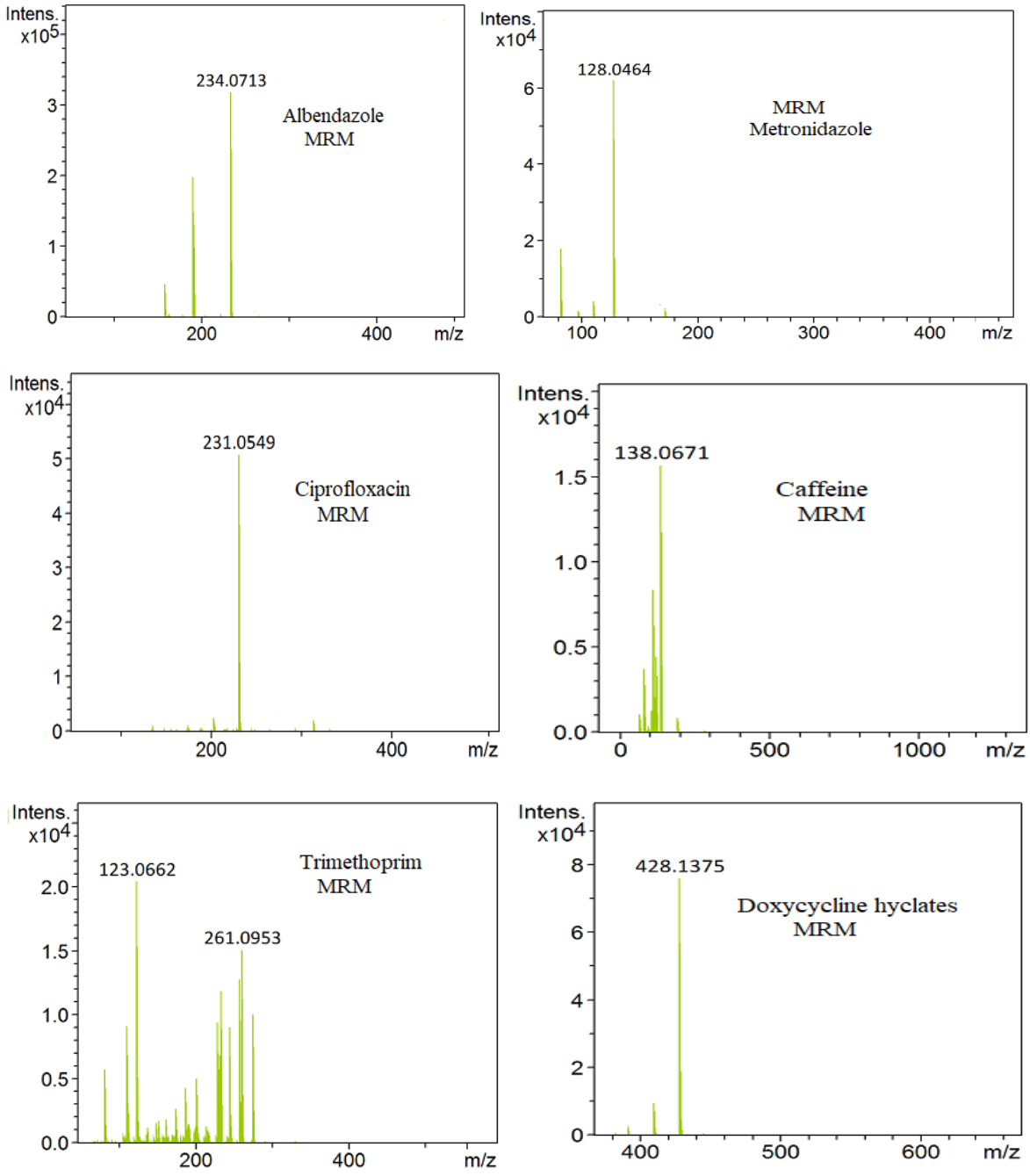

Intens.
x10^6
4
3
2
1
0
266.0969
[M+H]
Albendazole
Mass = 265.3
200
400
m/z

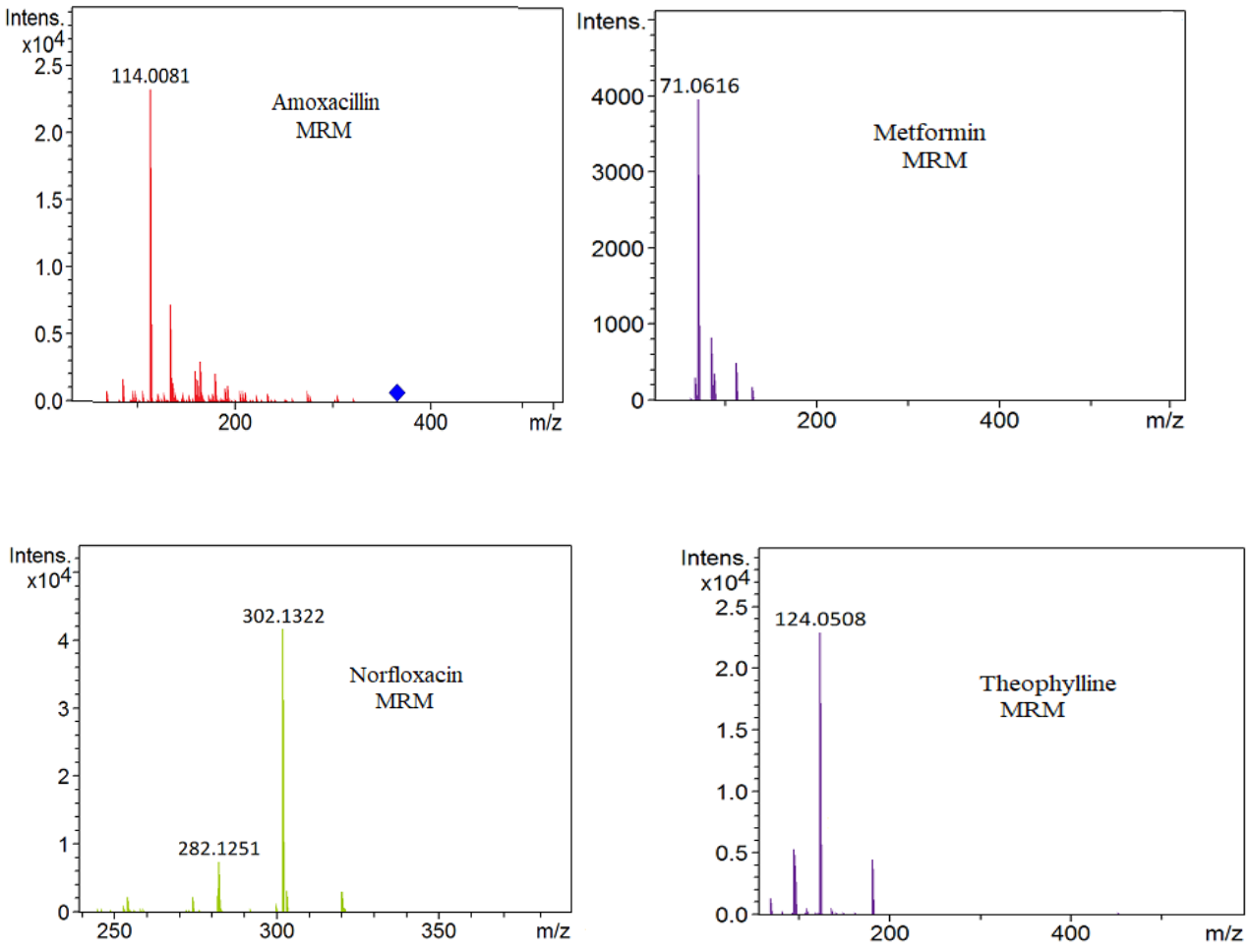

Intens.
x10^6
2.5
2.0
1.5
1.0
0.5
0.0
291.1453
[M+H]
Trimethoprim
Mass = 290.3
200
400
m/z



Appendix D2. The precursor ion (m/z) in the multiple reaction monitoring (MRM) mode.

MRM (product ion)





Appendix D3. The fragment ions in the multiple reaction monitoring (MRM) mode.

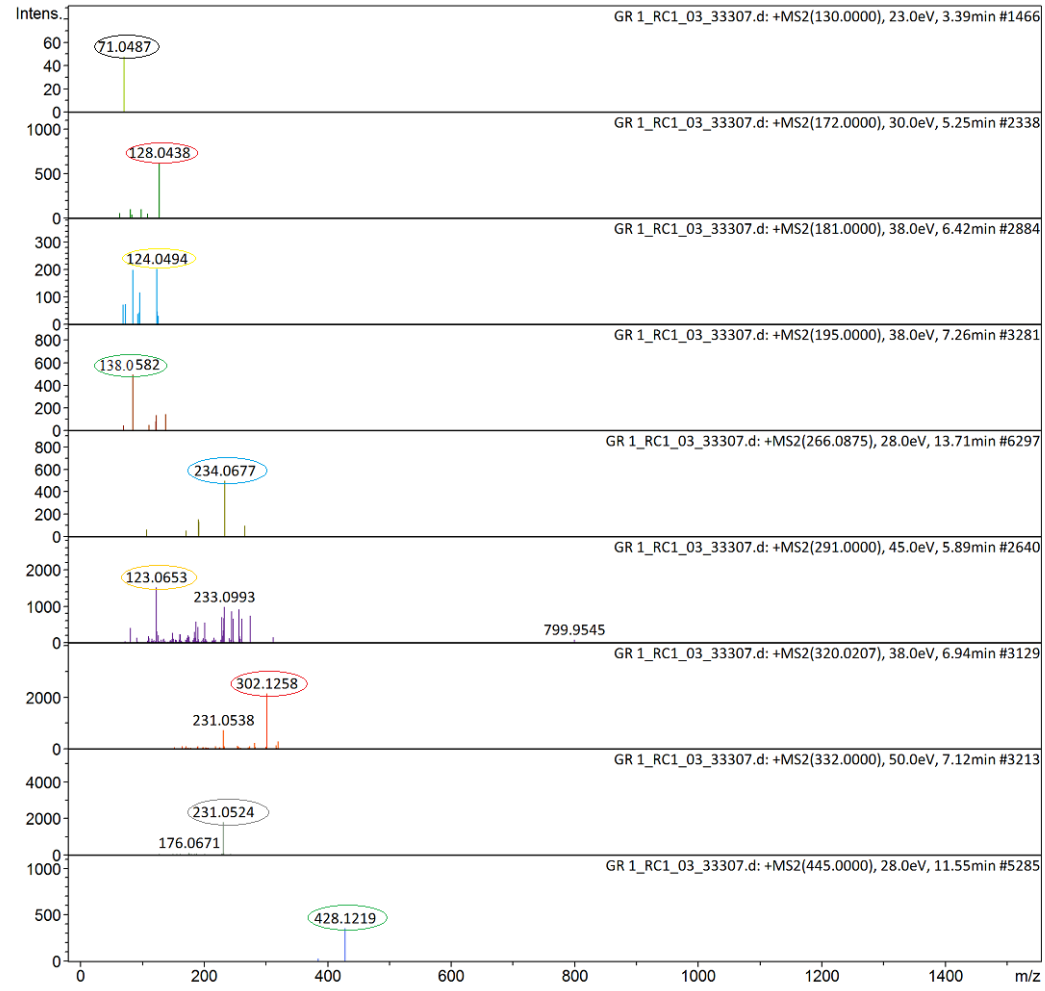
Display Report

Analysis Info

Analysis Name	D:\Data\runs\bisrat\26-06-20\GR 1_RC1_03_33307.d	Acquisition Date	6/29/2020 11:26:39 PM
Method	MRM 10 com 4 Calibration.m	Operator	Thapelo
Sample Name	GR 1	Instrument	compact 8255754.20116
Comment			

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	220 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	9.0 l/min
Scan End	1300 m/z	Set Charging Voltage	2000 V	Set Divert Valve	Waste
		Set Corona	0 nA	Set APCI Heater	0 °C



GR 1_RC1_03_33307.d

Bruker Compass DataAnalysis 4.3 printed: 8/6/2020 10:12:50 AM by: Thapelo Page 1 of 1

Appendix D4. The fragment ions in MRM mode for water sample collected from Gefersa River.

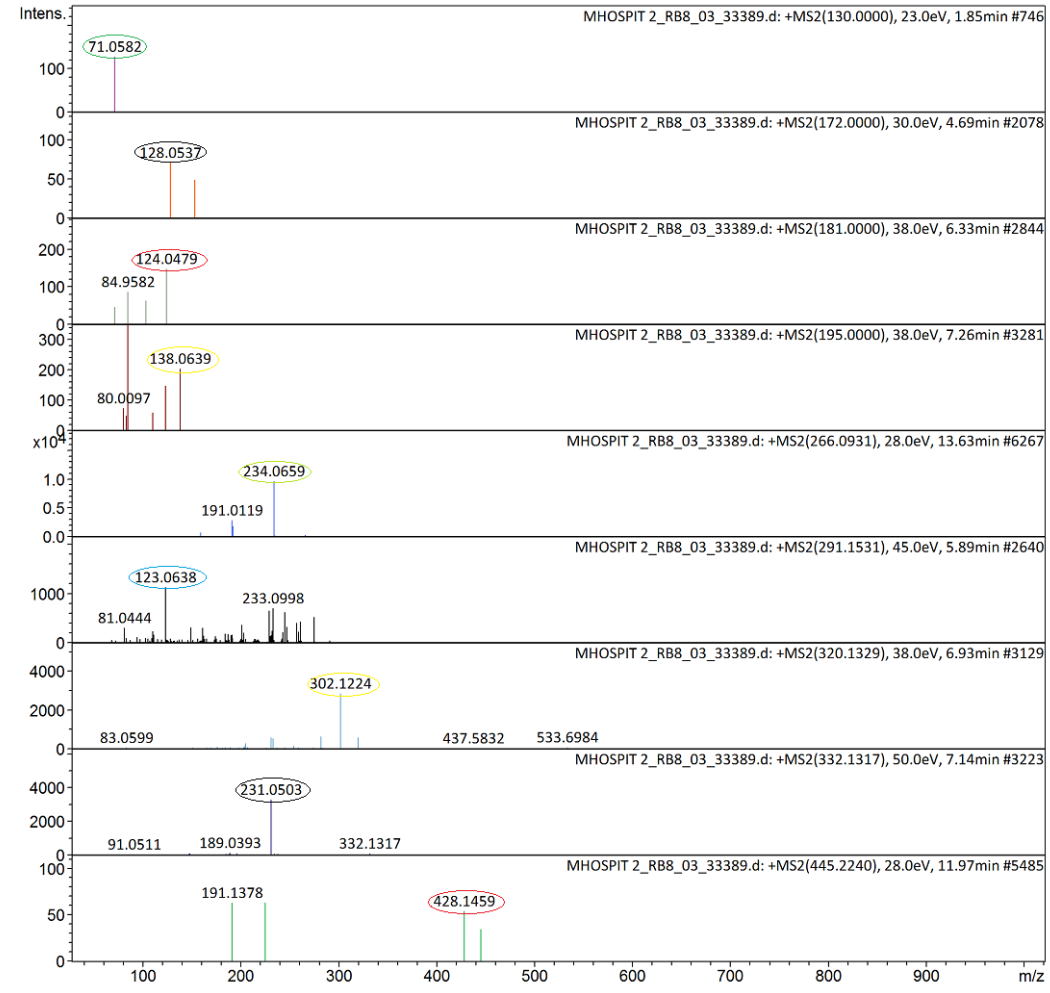
Display Report

Analysis Info

Analysis Name	D:\Data\runs\bisrat\30-06-20\MHOSPIT 2_RB8_03_33389.d	Acquisition Date	6/30/2020 11:37:17 PM
Method	MRM 10 com 4 Calibration.m	Operator	Thapelo
Sample Name	MHOSPIT 2	Instrument	compact 8255754.20116
Comment			

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	220 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	9.0 l/min
Scan End	1300 m/z	Set Charging Voltage	2000 V	Set Divert Valve	Waste
		Set Corona	0 nA	Set APCI Heater	0 °C



MHOSPIT 2_RB8_03_33389.d

Bruker Compass DataAnalysis 4.3 printed: 8/7/2020 10:30:16 AM by: Thapelo Page 1 of 1

Appendix D5. The fragment ions in MRM mode for water sample collected from Menilik Hospital.

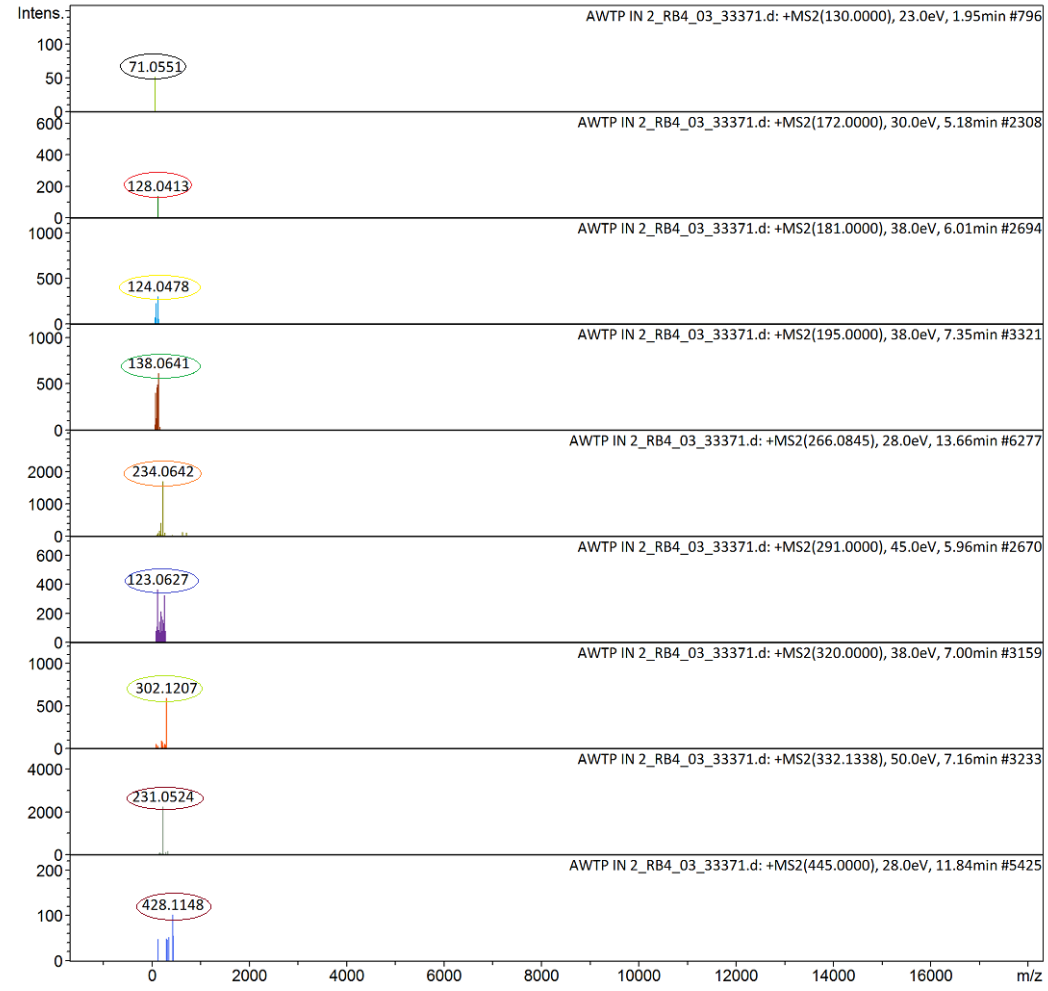
Display Report

Analysis Info

Analysis Name	D:\Data\runs\bisrat\30-06-20\AWTP IN 2_RB4_03_33371.d	Acquisition Date	6/30/2020 6:23:16 PM
Method	MRM 10 com 4 Calibration.m	Operator	Thapelo
Sample Name	AWTP IN 2	Instrument	compact 8255754.20116
Comment			

Acquisition Parameter

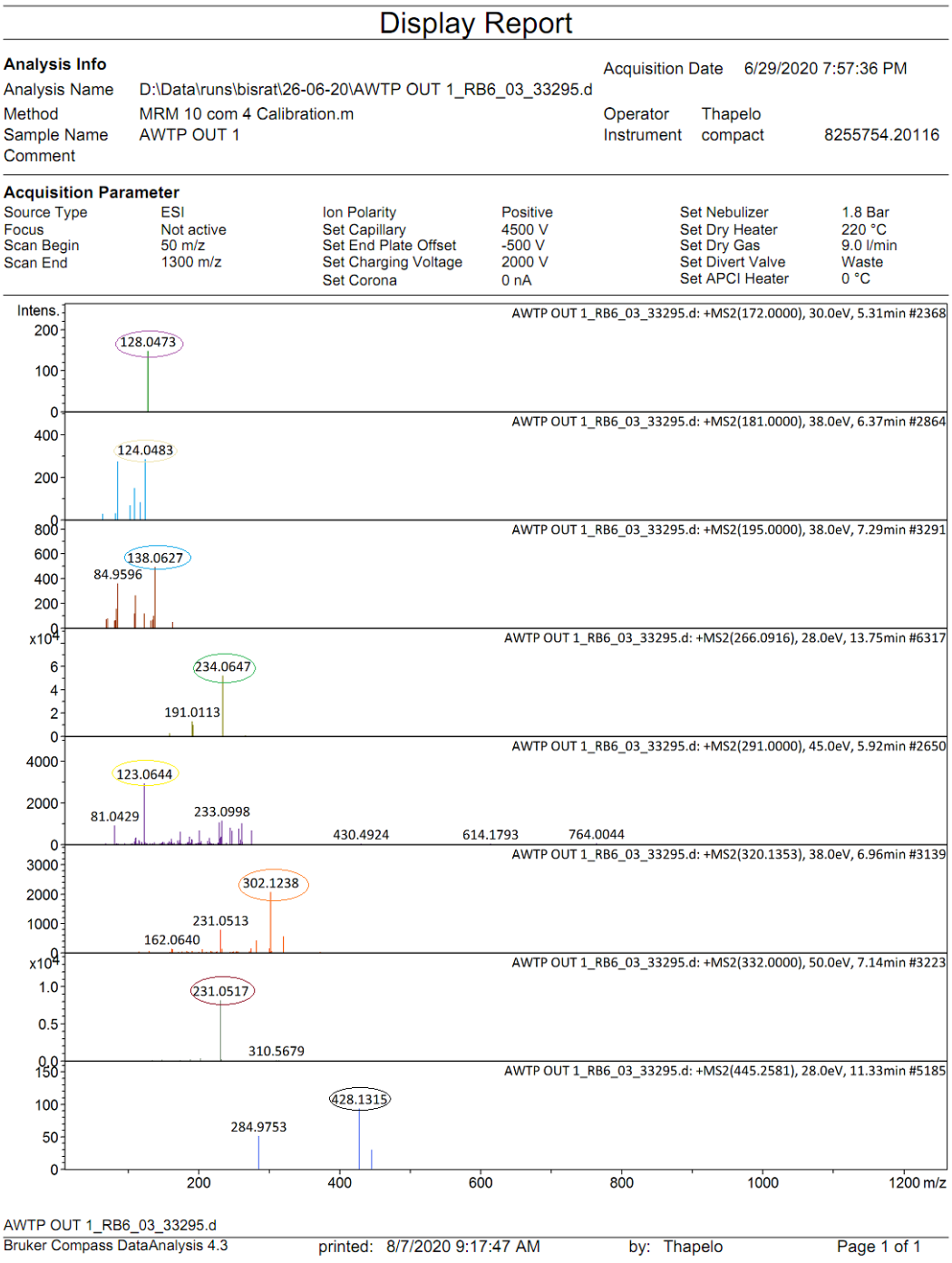
Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	220 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	9.0 l/min
Scan End	1300 m/z	Set Charging Voltage	2000 V	Set Divert Valve	Waste
		Set Corona	0 nA	Set APCI Heater	0 °C



AWTP IN 2_RB4_03_33371.d

Bruker Compass DataAnalysis 4.3 printed: 8/6/2020 9:45:16 AM by: Thapelo Page 1 of 1

Appendix D6. The fragment ions in MRM mode for water sample collected from sewerage treatment plant influent.



Appendix D7. The fragment ions in MRM mode for water sample collected from Akaki sewerage treatment plant effluent.

Appendix D8. Table(1 and 2) concentration of pharmaceuticals in contamination sources and therapeutic classes.

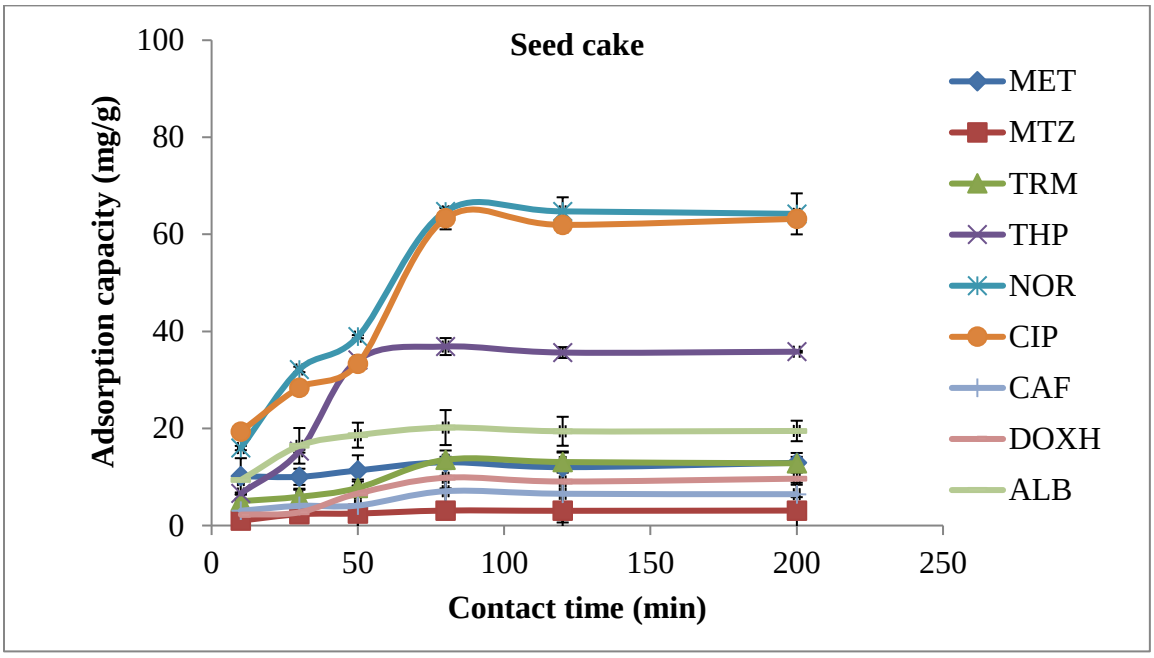
Table 1. Concentration of pharmaceuticals in contamination sources (Mean)

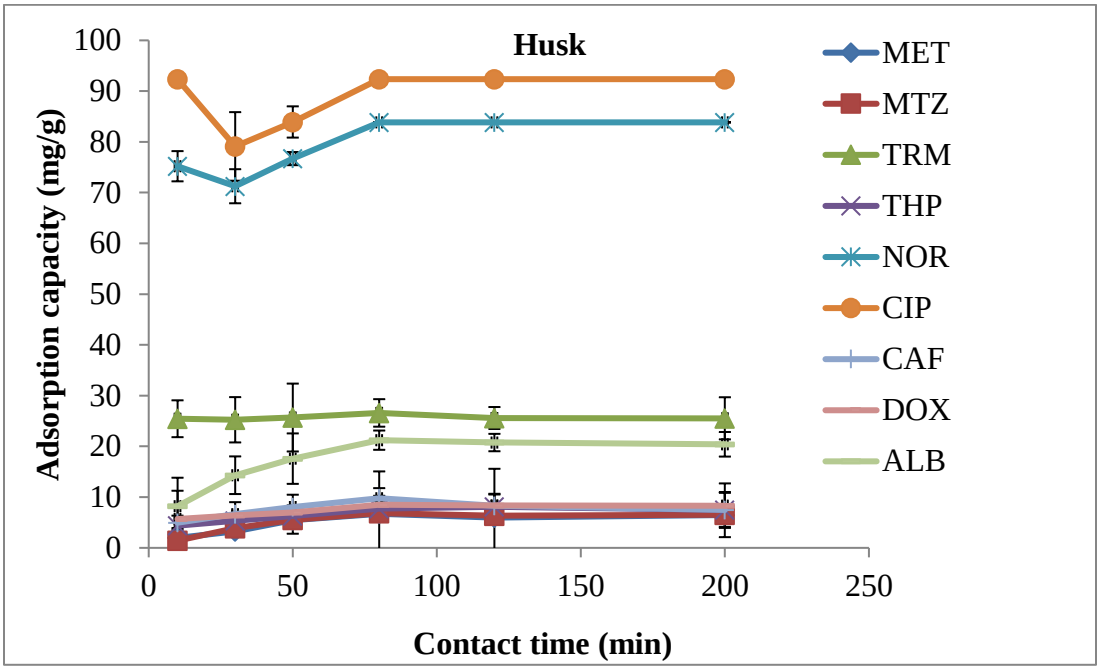
Sources	ALB	CAF	CIP	DOXH	MET	MTZ	NOR	THP	TRM
Rivers	0.551	11.62	2.966	1.869	0.333	1.860	7.628	5.616	2.034
Hospitals	2.834	23.73	3.910	1.108	0.088	4.702	6.281	5.908	2.566
ASTP	18.80	31.801	18.50	1.975	2.385	1.117	13.95	17.55	23.01
GWTP	<LOQ	1.341	1.013	0.980	ND	0.303	1.122	1.191	0.876

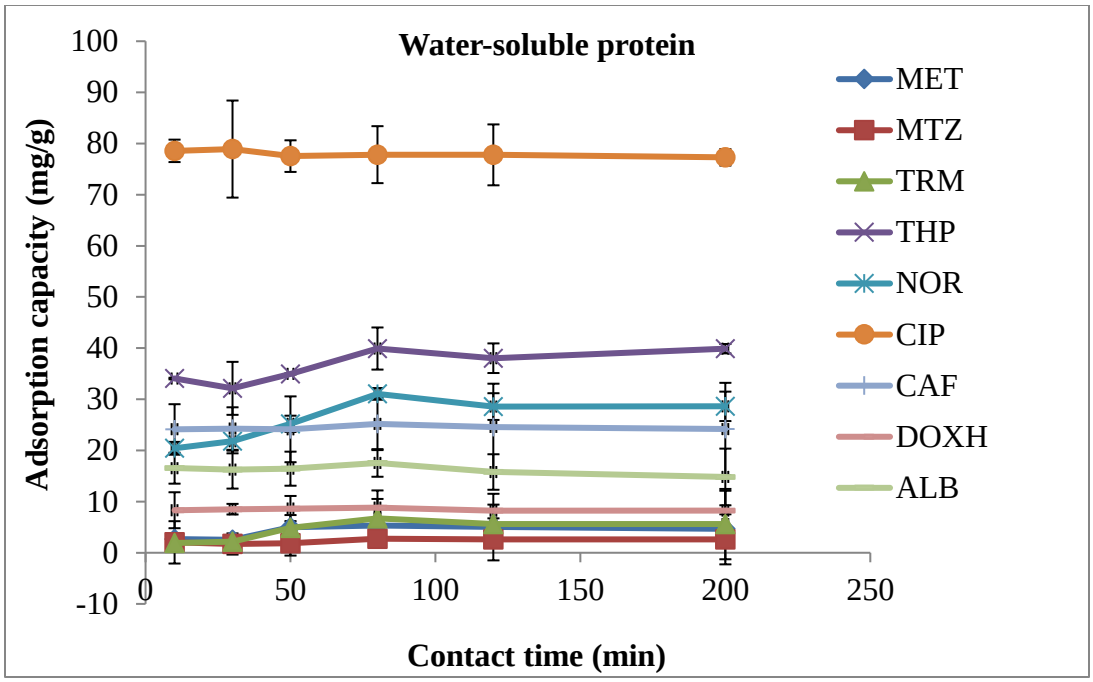
Table 2. Concentration of pharmaceuticals in therapeutic classes in contamination sources (mean)

Classes	Rivers	Hospitals	ASTP	GWTP
Antibiotics	3.271	3.713	11.71	0.859
Antidiabetic	0.333	0.088	2.385	ND
Anthelmintic	0.551	2.834	18.80	ND
Stimulant	11.62	23.73	31.80	1.341
Xanthine	5.616	5.908	17.55	1.191

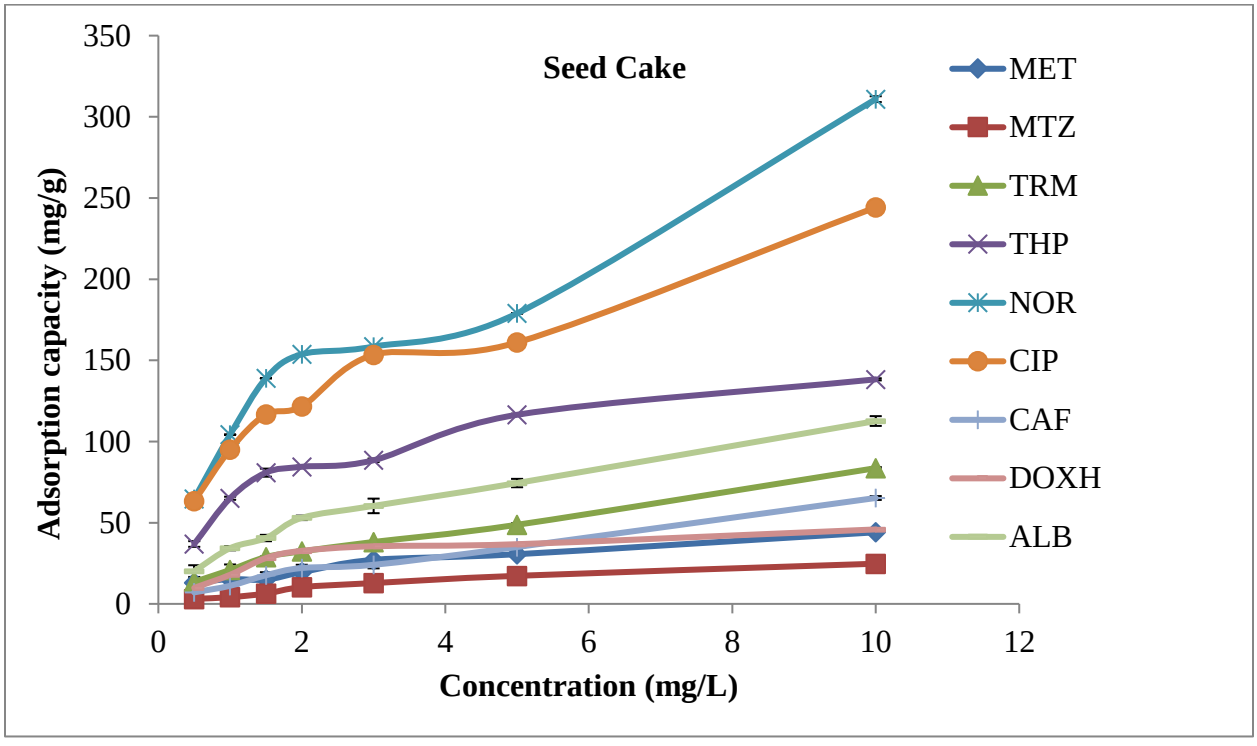
Appendix E: Removal using *Moringa Oleifera*

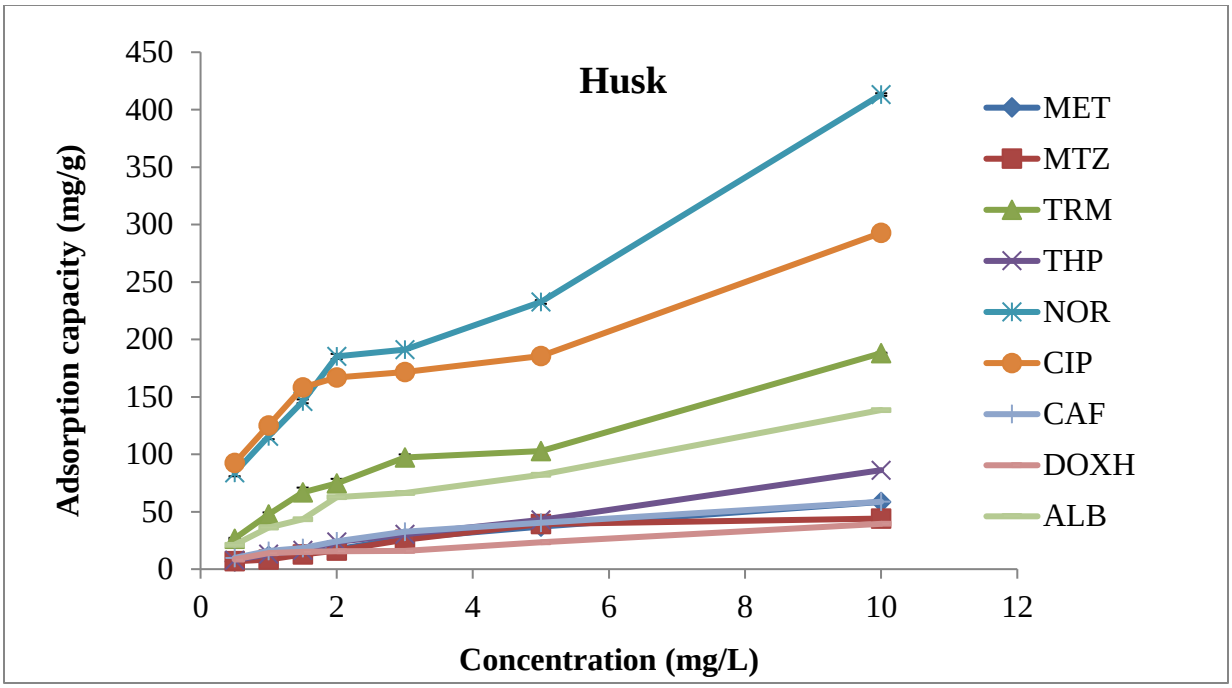


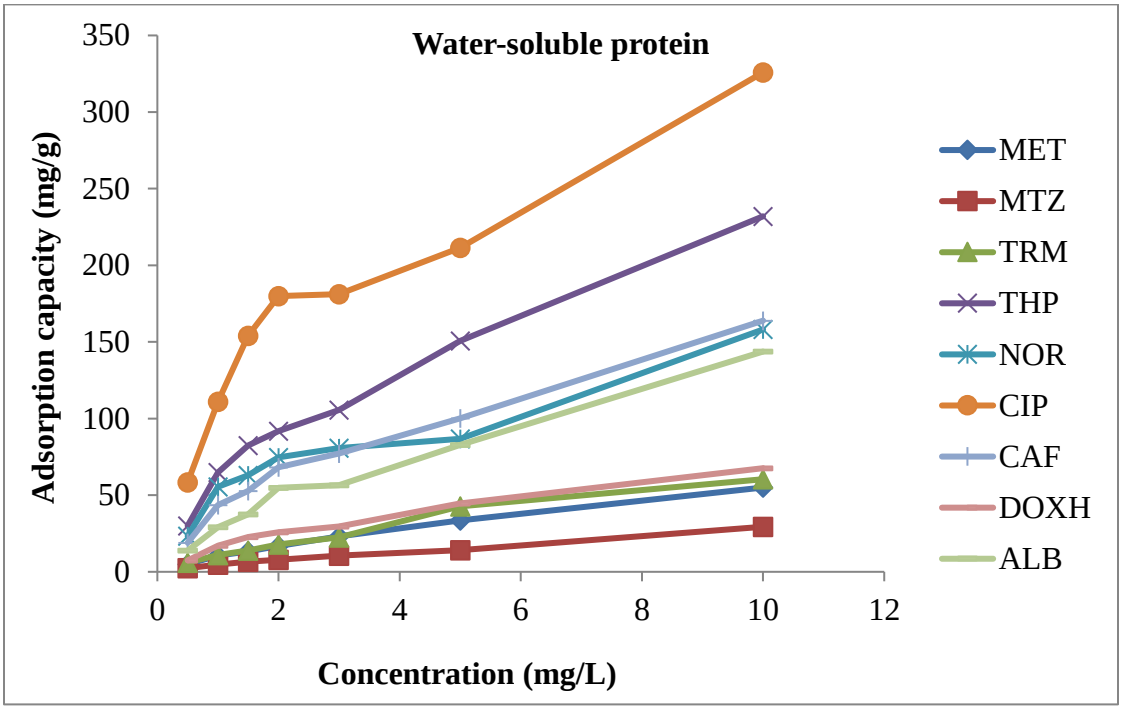




Appendix E1. Adsorption capacity versus contact time







Appendix E2. Adsorption capacity versus initial concentration