Method development and application: Solid phase extraction (SPE), Ultrasonic extraction (UE) and Soxhlet extraction (SE) for the determination of antiretroviral drugs in river water, wastewater, sludge, soil and sediment

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By

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Declaration

I hereby certify that this thesis is a result of my own investigation except where stated and has not been submitted elsewhere for any other degree.

(Signature of candidate)

(Supervisor)

7 January 2022
(Date)

Abstract

Antiretroviral drugs (ARVs) are a group of pharmaceuticals that have been recognized to be present in the environment and most of them have been reported to be environmentally persistent. However, most studies have documented their concentration levels in the aqueous matrices and not solid matrices.

This project involved the optimization of Soxhlet extraction (SE), ultrasonic extraction (UE) and solid-phase extraction (SPE) methods followed by liquid chromatography-photodiode array (LC-PDA) method for the analysis of abacavir, nevirapine and efavirenz in wastewater, river water, sludge, sediment and soil samples. The methods validation was based on linearity, limits of detection (LOD), limit of quantification (LOQ) and percentage recoveries. Good linearity was obtained for all the studied ARV drugs with R^2 values ranging from 0.9979 - 0. 9984. The recoveries, LOD and LOQ ranged from 71 - 112%, 0.7 - 0.8 µg/L and 2.07 - 2.36 µg/L, respectively for SPE. For SE they were 79 - 108%, 0.8 - 0.9 µg/kg and 2.4 - 2.8 µg/kg, respectively, while for UE they were 61 - 104%, 1.6 - 2.3 µg/kg and 4.9 - 7.0 µg/kg, respectively. These findings revealed that the methods are accurate and applicable for the monitoring of the selected ARV drugs in environmental samples. Also, they revealed that SE has high accuracy and sensitivity compared to UE due to its lower LOD, LOQ and recoveries.

The concentrations detected in real samples were 8.39 - 102 µg/L in river water, 2.47 - 814 µg/L in wastewater, and 19.8 - 6759 µg/L in sludge. The seasonal variation affect the detected ARV concentrations as lower levels were observed in spring season compared to winter. The concentrations of ARV found in sediments and soil were 22.8 - 98.9 µg/kg and 15.4 - 138 µg/kg and. The detection of ARVs in the environmental samples raise great concerns, for example, drug resistance due to build of drugs in water, soil, etc. This shows the significance of continuously monitoring these compounds that find their way to rivers including rivers (surrounding surfaces) that are used as vessels for waste including untreated and partially treated sewage from the municipalities (Hinrichsen and Tacio, 2002), that leach into surface water during various activities. Hence, large quantities of various pollutants including pharmaceuticals and antibiotics have been found in sediments, soils and surface water worldwide in which the major sources have been reported to be industries (Archer *et al.*, 2017b) and wastewater treatment plants (WWTPs) final effluents (Paiga *et al.*, 2016).

Dedication

This dissertation is dedicated to:

My late mother, Loveness Mbuyazi. I know you would be proud of this achievement.

My Uncle (Mbongeni Mbuyazi) and Aunt (Happiness Mbuyazi), for the father and mother roles you have played in my life.

Acknowledgements

I would like to express my gratitude to my supervisor Dr PN Mahlambi for presenting me to this study topic. I thank her for the support given, words of encouragement, motivation when needed and useful comments and remarks. I will forever appreciate working under her supervision.

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I am also grateful for the financial support from the South African National Research Foundation (NRF), (Thuthuka grant, no: 107091) and Freestanding, innovation and scarce skills Masters and doctoral scholarship (SFH 190115408286).

To God, the Almighty be the Glory.

Jeremiah 29:11 "For I know the Plans I have for you' Declares the Lord, 'Plans to Prosper You and Not to Harm You, Plans to Give you Hope and a Future."

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Abbreviations

ACN	Acetonitrile
ARV	Antiretroviral drugs
DWAF	Department of Water Affairs and Forestry
ECD	Electron Capture Detector
ECs	Endocrine disruptors
EPA	Environmental Protection Agency
FID	Flame Ionization Detector
GC	Gas Chromatography
HIV	Human Immune Virus
HLB	Hydrophilic-Lipophilic Balance
HPLC	Higher Performance Liquid Chromatography
LC	Liquid Chromatography
LLE	Liquid-Liquid Extraction
LOD	Limit of Detection
LOQ	Limit of Quantification
MAE	Microwave Assisted Extraction
MS	Mass Spectrometry
NRTIs	Nucleoside/ nucleotide Reverse Transcript Inhibitors
NNRTIs	Non-Nucleoside Reverse Transcript Inhibitors
PIs	Protease Inhibitors
PDA	Photodiode Arrays
PPCPs	Pharmaceuticals and Personal Care Products

RSD	Percentage Relative Standard Deviation
SE	Soxhlet Extraction
SPE	Solid Phase Extraction
UE	Ultrasonic Extraction
UV	Ultraviolet
WHO	World Health Organization
WWTPs	Wastewater Treatment Plants

CHAPTER ONE

1. Introduction

1.1. Background

Clean water is a very important resource on earth because it supports and maintains human health and a sustainable ecosystem (Madikizela, 2016). The rapid increase in population over the last century is a major contributing factor in increasing global water usage. Also, economic development and better living standards have increased the demand for freshwater resources worldwide (Hinrichsen and Tacio, 2002). Therefore, it is vital that the quality status of water be continuously monitored to ensure the good well-being of the aquatic environment and humans. In several countries, rivers are used as vessels for a variety of wastes which includes untreated and partially treated sewage from the municipalities (Hinrichsen and Tacio, 2002) that leach into surface water during various activities. Hence, large quantities of various pollutants including pharmaceuticals and antibiotics have been found in sediments, soils and surface water worldwide in which the major sources have been reported to be industries (Archer *et al.*, 2017b) and wastewater treatment plants (WWTPs) final effluents (Paiga *et al.*, 2016).

Pharmaceuticals are natural or synthetic compounds used in prescription medicines, over-the-counter veterinary and therapeutic drugs (Schoeman *et al.*, 2015). They comprise active ingredients that are beneficial to human beings. However, they can eventually end up in water and soils at trace amounts (Schoeman *et al.*, 2015). As a result of possible health effects related to exposure of human, aquatic and terrestrial life that pharmaceuticals have when they reach the environment, they have received a lot of attention (Deblonde *et al.*, 2011; Matongo *et al.*, 2015). The ARVs, like other antimicrobials, have side effects, which range from invisible to unpleasant forms. Their short-term effects include nausea, diarrhoea, occasional dizziness, fatigue, headaches, skin rash, vomiting, pain, and nerve problems. Antiretroviral drugs have also been revealed to have direct negative effects on cultured adipocytes; some (nelfinavir, indinavir, and ritonavir) encourage insulin resistance and drug resistance due to presence and build-up of trace amounts of drugs in water, alter lipid metabolism and cause lipodystrophy (Caron *et al.* (2001); Lenhard *et al.* (2000); Murata *et al.* (2002).

The evaluation and detection of pharmaceuticals (including ARV drugs) in high matrix environmental samples requires analytical techniques that have been carefully validated and which can extract low concentration compounds that mostly exist as a mixture with other compounds (Ncube *et al.*, 2018). As a result of the complexity of environmental samples and their existence in trace levels (ng/L to μg/L), it is always necessary to isolate and preconcentrate prior chromatographic analysis. The recent methods for the extraction of ARV drugs in water samples are mainly through solid-phase extraction (SPE). Sonication and QuEChERS (Schoeman *et al.*, 2017), microwave-assisted extraction (MAE) followed by SPE clean up (Aminot *et al.*, 2015), have been used for solid samples. The SPE methods reported mostly employed the oasis hydrophilic-lipophilic balance (Oasis HLB) cartridges. These cartridges have shown to be most effective for the simultaneous extraction of pharmaceuticals when compared to other sorbents (Ngumba *et al.*, 2016). This may be as a result of Oasis HLB being able to extract a mixture of compounds from environmental samples that have different chemical properties.

Instrumentation with rapid separation and sensitive detectors is required for the separation and detection of pharmaceuticals (including ARV drugs) in complex environmental samples. Liquid chromatography with a photodiode array detector (LC-PDA) has been used for the analysis of environmental samples due to its ability to fully integrate the high separation capacity of the complex samples (Pang *et al.*, 2016). The strong ability of the PDA to detect the entire wavelength spectrum which allows simultaneous determination of components in a complex mixture at their optimum wavelengths (Chan and Carr, 1990).

1.2. Problem Statement

South Africa is among countries that are lagging far behind in the assessment of pharmaceuticals drugs in various matrices such as rivers, wastewater treatment plants (WWTPs), drinking water, sludge, sediments, and soil. Also, the extent of pollution that these compounds cause in the environment is not well understood in South Africa (Madikizela, 2016). Nevertheless, recent studies in South Africa have documented environmentally significant concentrations in wastewater treatment plants (WWTP) influent and effluents (Archer *et al.* (2017a).

Moreover, South Africa is amongst the countries that have the highest number of human immunodeficiency virus (HIV) positive people with 7.52 million HIV infected people estimated in July 2018. Therefore, the treatment of ARVs in South Africa is of paramount importance, and the increase in the number of people on treatment indicates that more of these ARV drugs will inevitably reach the wastewater treatment plants (WWTPs) via domestic sewage. From the WWTPs they eventually reach the environment and waterways since they are not completely removed during water purification due to insufficient water treatment in the WWTPs. Also, research findings have shown that the existence of ARV drugs in the environment might create problems for aquatic organisms, which raises concerns (Swanepoel *et al.*, 2015). It has been reported that some ARV drugs undergo biotransformation upon consumption, whereas others are released as parent compounds from the body (Boucher and Galasso, 2002). It is therefore important to monitor and remove ARV drugs from the environment. Also, these substances exist at trace levels in the environment, hence, very sensitive methods need to be developed for their successful determination and monitoring in the environment. This study was therefore based on the optimization and validation of an SPE (for extraction of ARV drugs in liquid samples), SE and UE (for extraction of ARV drugs in solid samples), and liquid chromatography-photodiode-array (LC-PDA) for detection and quantification of antiretroviral drugs (abacavir, nevirapine and efavirenz) in wastewater, river water, sediments, sludge and soil.

1.3. Research aim and objectives

1.3.1. Aim

To develop Soxhlet extraction (SE), ultrasonic extraction (UE) and solid-phase extraction (SPE) and LC-PDA methods for the analysis of antiretroviral (ARV) drugs levels in water, sludge sediment and soil.

1.3.2. Research Objectives

- To develop an LC-PDA method using standard solutions of the analytes of interest in order to achieve acceptable separation and retention times for the selected ARV drugs.
- To optimize SPE, UE and SE methods to achieve extraction conditions that permit higher extraction efficiencies for the selected ARV drugs are obtained.
- To apply the optimum extraction conditions to extract ARV drugs in wastewater, river water, sludge, sediment and soil samples for quantitative analysis.

- To compare the extraction efficiency of SE and UE on the extraction of ARV drugs.

1.4. Research questions

- Which parameters can be optimized to improve the extraction efficiency of the targeted ARV drugs at lower concentrations?
- Can the selected ARV drugs be detected in the areas under investigation and at what concentrations?
- Are WWTPs able to effectively remove the ARV drugs in water during the water treatment processes, and what are the removal percentages?
- Are the concentrations of ARV drugs higher in the influent streams than the effluent streams of WWTPs?
- Does the effluent discharged by wastewater treatment plants contribute to ARVs concentrations present in river water?

1.5. Research Justification

The pollution of water by pharmaceuticals has been a main environmental concern since the 1990s (Doerr-MacEwen and Haight, 2006). Hence, more attention has been paid to the ecological and physiological risks that result from environmental pollution by pharmaceuticals (including ARV drugs). Powerful analytical methods are therefore required for successful detection and accurate quantification of pharmaceuticals in the environment. Liquid chromatography (LC) is the analytical method ideal for the separation of pharmaceuticals from raw water samples owing their high polarity. However, there is a need for more recent advanced methods to extract, differentiate between multiple pharmaceuticals and interfering compounds in order to achieve sensitivities that are satisfactory while saving time and cost-effective.

Liquid chromatography-mass spectrometry (LC-MS) is the main method for the analysis of drugs and their major metabolites and can provide high sensitivity and molecular structure information for the qualitative essay of drugs. However, it is expensive and hence not available in many laboratories. Also, the photodiode array (PDA) is a multichannel detector with high sensitivity however when it is employed, a range of wavelengths can be programmed and all the compounds that absorb within the programmed range can be identified in a single analysis (de Rosso and Mercadante, 2007), thus saving time. To successfully analyze many organic compounds, present at trace concentration in sample matrices, sample preparation is essential.

Sample preparation techniques should be precise, accurate, fast, and must keep sample integrity (Lucci *et al.*, 2012). Hence, there is a need for the development of sample preparation procedures that allow the analysis time to be reduced while the integrity of the extraction process is not compromised.

In this project, a reliable analytical technique based on ultrasonic extraction (UE), SPE, SE, and LC-PDA was optimized and validated for the analysis of ARV drugs in water and solid samples. The SPE was used for water samples extraction as well as for UE and SE extract clean up since it is a fascinating and mostly used technique for matrix clean up, preparation and selective extraction of analytes in complex matrices. The UE was chosen as it is simple, cheap and efficient compared to other conventional extraction techniques (Mandal *et al.*, 2015). In addition, its operating temperature allows for the extraction of thermolabile analytes that are thermolabile. The SE as a standard method with improved extraction efficiencies as a result of the sample's repeated contact with a new portion of the solvent was used to compare its extraction efficacy to UE (Halfadji *et al.*, 2013).

CHAPTER 2

2. Literature Review

2.1. The uses and effects of ARV drugs on humans

ARVs are a class of pharmaceuticals that are regarded as emerging contaminants in the environment. ARV drugs are antimicrobials used for the treatment of infection by retroviruses, primarily human immunodeficiency virus (HIV). Their function is to inhibit the damage caused by HIV and are classified into various classes. These include nucleoside and nucleotide reverse transcript inhibitors (NRTIs) e.g. abacavir, non-nucleoside reverse transcript inhibitors (NNRTIs) e.g. nevirapine and efavirenz, protease inhibitors (PIs) e.g. kaletra, lopinavir and agenerase (Jain *et al.*, 2001), fusion inhibitors and integrase inhibitors. Both the NRTIs and NNRTIs prevent HIV reverse transcriptase (RT) and suppress the duplication of the viral genome (Beach, 1998). Protease Inhibitors (PIs) inhibit virus maturation, eventually limiting the virus particles infectivity (Beach, 1998).

It has been reported that NRTIs promote lipoatrophy (Carr *et al.*, 2000) and they are also associated with neuropathy, pancreatitis, myopathy, toxicity, hepatic effects probably as a result of mitochondrial toxicity ((1999); Brinkman *et al.* (1998); Lenhard *et al.* (2000)). It has been reported that all NNRTIs (nevirapine, delavirdine, and efavirenz) are common antiretroviral drugs that cause hypertension (Carr and Cooper, 2000). Efavirenz has been linked with early neuropsychiatric side effects with up to 68% of the patients and its usage could result in damaged neurons which can lead to impaired neurocognitive performance (Decloedt and Maartens, 2013). Severe rash, including the Stevens-Johnson syndrome has been reported to be nevirapine's major toxicity effect (Metry *et al.*, 2001). This was proved in a study conducted in the United States, where nevirapine was found to be linked with effects such as rash, eosinophilia, liver function abnormality, and renal failure in women who are in their third-trimester (Joy *et al.*, 2005).

2.2. Sources of antiretroviral drugs in the environment

Concerns regarding the presence of endocrine disruptors (ECs), such as pharmaceuticals (including ARV drugs), in water sources has increased. Researchers have reported that multiples of pharmaceuticals are being continuously discharged into the environment due to inefficient wastewater treatment (Ferrer and Thurman (2012); Wood *et al.* (2015) in wastewater treatment plants (WWTPs). In addition to discharge from WWTPs sewage effluents, there are

countless sources of contamination. These includes excretion and incorrect dumping of expired pharmaceuticals, for example leachates from landfilling (Peng *et al.*, 2014) and manufacturing effluents (Daughton, 2013). The WWTPs sewage effluents have been stated as the major source of various pharmaceuticals and their metabolites, entering water bodies. A significant pathway in sewage effluent is by consumption of medication after therapeutic use, followed by excretion and discharge into the sewage stream (Ebele *et al.*, 2017). The excretion of antiretroviral drugs varies depending on the compound. For example, efavirenz, abacavir, and nevirapine are excreted via urine at 62% (Rakhmanina and van den Anker, 2010), 18% (Yuen *et al.*, 2008) and 2.7% (Riska *et al.*, 1999), respectively. Therefore, if a mean of 30% excretion to sewage via urine and faeces is assumed to be 374 imperial tons of ARV drugs could end up in South Africa WWTPs each year. After sewage treatment, the wastewater is usually used for irrigation with biosolids (treated sludge) in which are applied as fertilizer to arable land. This can transfer traces of ARV drugs to the soil and these maybe absorbed by plants and thus can be ingested by human beings.

Another source of ARV drugs into the environment is *via* their manufacture. Wastewater from the pharmaceutical manufacturing industries goes directly into sewage treatment plants. The sludge is then used as soil fertilizer and the liquid sewage is thrown back into the freshwater environment. Sewage groundwater may also be the source of pharmaceuticals entering freshwater sources (Gaw *et al.*, 2014). It has been reported that pharmaceuticals can make their way into groundwater through leaching from the soil, hence causing threats to drinking water (Ebele *et al.*, 2017). In Spain (Mallorca), recycling of treated inland wastewater for irrigation was identified to contribute to groundwater pollution by pharmaceutical (Rodriguez-Navas *et al.*, 2013). Once these pharmaceuticals are released into the environment, range transport is possible (depending on their physicochemical properties). These compounds are highly polar, hydrophilic and have low volatility, therefore the primary source of environmental distribution is via aqueous medium transportation and dispersal of the food chain.

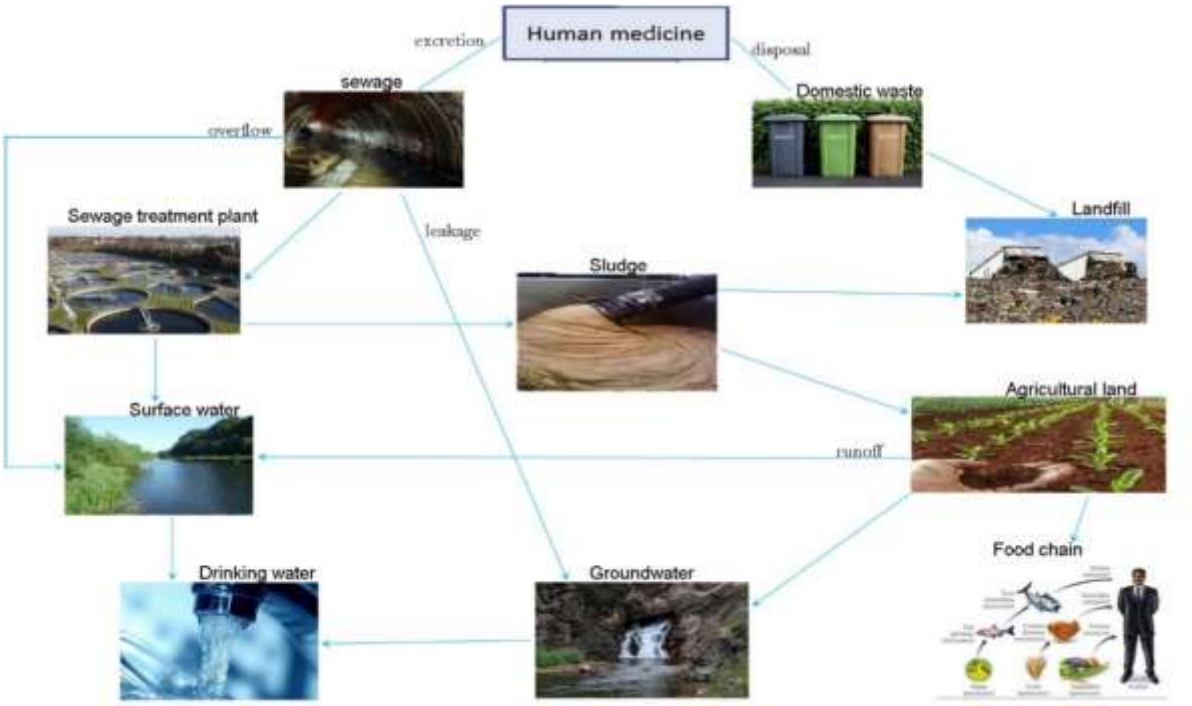


Figure 2.1: Illustration of environmental contamination by antiretroviral drugs.

2.3. Occurrence of antiretroviral drugs in aquatic and soil environment

Scientific research attention is currently devoted to the occurrence of ARV drugs and other endocrine disruptors in the environment. This has resulted in an increasing number of published reports of pharmaceuticals (including ARV drugs) being detected in low concentrations in different environmental matrices, e.g. wastewater, river water, sediments and soils. In the following sections, a general overview will be given on the existence of pharmaceuticals in various environmental matrices.

2.3.1. Aquatic environment

The existence of pharmaceuticals in the environment was reported for the first time in 1976 by Garrison *et al*., in Kansas City, U.S.A where clofibric acid (0.8 – 2 µg/L) was detected in treated wastewater (Fent *et al.*, 2006) (Ebele *et al.*, 2017). In 1981, Richardson *et al.* documented the existence of 25 pharmaceuticals in river water with concentrations up to 1 µg/L (Richardson and Bowron, 1985). The reported concentrations may be low but a lot of pharmaceuticals are environmental persistent for many years. Their discovery in the environment differs between countries and regions of the same country that is pharmaceuticals detected in one country/region may not be detected in countries/regions where they are mostly

unprescribed (Ebele *et al.*, 2017). In previous years, knowledge and research about pharmaceuticals presence in the environment has risen significantly as a result of new analytical techniques that are capable of determining trace levels of polar compounds.

In Germany, Ternes *et al.* (1998), detected 32 pharmaceuticals from different medicinal classes in sewage treatment plant effluents and river water. Also, carboxylated transformation products of different ARV drugs (including abacavir) have been found in drinking water in Germany (Funke *et al.*, 2016). In South Africa, a maximum concentration of efavirenz (34 µg/L) has been recorded (Abafe *et al.*, 2018). In Kenya, zidovudine, nevirapine and lamivudine have been reported in rivers and dams at levels up to 17.4, 5.62 and 167 µg/L, respectively (K'Oreje *et al.* (2016); Ncube *et al.* (2018). Moreover, maximum concentrations of 3.5 and 0.3 µg/L were, respectively reported for ibuprofen in influent and effluent samples from German WWTPs. In South Africa, KwaZulu Natal, Madikizela *et al.* (2014) detected triclosan and ketoprofen in wastewater and river water with concentrations ranging from 1.2 µg/L to 9 µg/L. Wood *et al.* (2015), reported the contamination of various surface water sources in South Africa by 12 ARV drugs with average concentrations ranging between 0.027 µg/L – 0.43 µg/L. The study indicated that the effectiveness of the WWTP and population density of the specific area have affect the detection/non-detection of these compounds.

2.3.2. Terrestrial (Soil and Sediments)

Sources of contamination in the terrestrial environment include irrigation using wastewater effluent, the use of sludge in agriculture as manure to reclaim inorganic nutrients, application of livestock sewage on farmland and improper disposal of out-of-date medicines in landfills (Bottoni *et al.*, 2010). In such cases, an impact on terrestrials is expected, but currently only a small number of published papers (especially on ARV drugs) on this matrix is available. The environmental persistence of the ARV drug tenofovir has been evaluated in agricultural soils, where no extractable transformation products were detected (Al-Rajab *et al.*, 2010). Berger *et al.* (1986) reported that multiple drug resistance had developed in livestock and intestinal flora of untreated pigs due to the manure that was applied to agricultural soils. Hence, 366 strains were able to find their way to the food chain. Also, in soil amended with poultry manure, Chlortetracycline have been found in soil (Warman and Thomas, 1981).

The presence of pharmaceuticals in sediments has been reported by several authors. In Minnesota (United State), a high concentration (0.82 µg/g) of triclocarban in freshwater sediments was reported (Venkatesan *et al.* (2012). In Australia, 21 out of 46 target

pharmaceuticals in estuarine sediments were quantified at levels from 0.002 – 0.008 μg/g (Ebele *et al.,* 2017). In South Africa (KwaZulu Natal), residues of ibuprofen were detected from the Msunduzi river sediments with concentrations as high as 0.66 μg/g (Matongo *et al.*, 2015). Also, in sediment samples collected from Msunduzi River, KwaZulu-Natal, South Africa, ibuprofen, diclofenac and aspirin were quantified with concentrations ranging from 0.005 – 0.011 μg/g, 0.057 – 0.31 μg/g and 0.21- 0.43 μg/g, respectively (Agunbiade and Moodley, 2016).

2.4. The concerns of antiretroviral drugs in the environment

It has been reported that the WWTPs that meet the requirements for wastewater treatment are only moderately effective in removing the pharmaceuticals. The treatment plants with tertiary treatment (Li, 2014) are more effective in removing pharmaceuticals. Also, it has been reported that due to the lack of published data, the ecotoxicological risks associated with pharmaceuticals in the environment have not been evaluated (Prasse *et al.*, 2010). However, when biologically active compounds (such as antibiotics) are released into the environment, they can directly impact on organisms and indirectly affect humans (Prasse, 2012). Unlike other pharmaceuticals, ARV drugs are different in that their therapeutic effects works in contrast to a virus that simply changes into resistant strains if the medication prescription is taken accordingly (Ncube *et al.*, 2018). Hence, any traces of ARV drugs in food and drinking water sources may have a more negative effect on the health of humans compared to other pharmaceuticals.

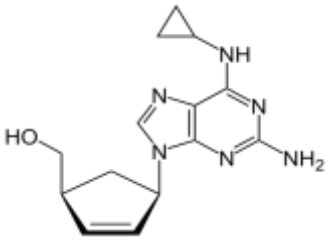
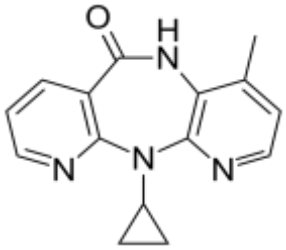
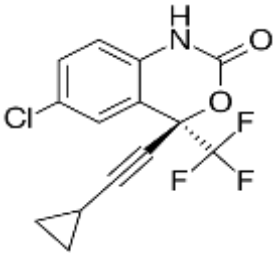
2.5. Physico-chemical properties and ARVs

The interaction i.e. sorption of ARV drugs into the solid or liquid media depends of their physical and chemical properties. The properties include octanol (organic)/water partition coefficient (Log K_{ow}), water solubility and polarity (Piwoni and Keeley, 1990). Log K_{ow} is an essential parameter for predicting the interaction of a substance with various environmental compartments (water, soil, sediments, etc). Contaminants with high log K_{ow} values mostly adsorb to organic matter found in soils or sediments due to their low affinity for water. Hence, ARV drugs with low octanol/water coefficient (Log K_{ow}) will be expected to be dominant in the aqueous phase Schoeman *et al.* (2017) and vice versa. The water solubility of ARV differs from drug to drug and those that have high solubility are expected to be dominant in water than solid phase. Abacavir is highly soluble in water (77000 mg/L) with the lowest Log K_{ow}

followed by nevirapine and efavirenz, hence abacavir is likely to be found in higher levels in water than soil compared to other ARVs.

The total sum of all polar regions in the molecule's surface is called the Topological polar surface area (TPSA) (Fernandes and Gattass, 2009). As water is a highly polar molecule, it is expected that the ARV drug with the highest TPSA will be dominant in the water environment.

Table 2.1: Abacavir, nevirapine and efavirenz structures, molecular masses, Log K_{ow} and pKa values. (Source: PubChem (2004))

NAME (Molecular Mass, g/mol)	Structure	Log K_{ow} (@25°C)	Solubility in Water @25°C (mg/L)	Topological Polar Surface Area (Å^2)
Abacavir (286.33)		1.2	77 000	102
Nevirapine (266.30)		3.89	100	58.1
Efavirenz (315.68)		4.7	0.093	38.3

2.6. Sample preparation techniques

Sample preparation techniques (mainly called extraction techniques) are surface dependent processes because the kinetics of the analyte transference between the phases is directly reliant on the extracting phase. Extraction techniques are commonly used for the preliminary purification and fractionation of analytes from matrices (Nnane *et al.*, 2000). They are a prerequisite for the analytical determination of organic and inorganic compounds in samples (Bendicho and Lavilla, 2000). The extraction techniques usually employed for the extraction of ARV drugs and pharmaceuticals in water and solid matrices include microwave assisted

extraction (MAE), solid phase extraction (SPE), Soxhlet extraction (SE), liquid-liquid extraction (LLE).

2.6.1. Solid-phase extraction

Solid-phase extraction is a method that is mostly employed for the extraction of desired analytes from a sample matrix. The SPE includes the analytes retention mechanisms such as ion exchange, normal phase, and reversed-phase. The reversed-phase consists of non-polar functional groups (e.g., C_{18}, C_8, phenyl and cyclohexyl) and contact amongst the analyte of interest and the sorbent is *via* Van der Waals forces. The sorbents are used for the extraction of compounds of interest with non-polar functional groups from matrices that are polar and the contact between the analyte and the sorbent surface group is eased by the use polar of solvents. The normal phase is used for polar analytes extraction from a non-polar media. This is because the sorbent has polar active sites (e.g., diols, aminopropyl, unbonded silica and alumina). The analytes interact and are retained at the sorbent surface via hydrogen or dipole-dipole interactions (Lucci *et al.*, 2012). To maximize analyte-sorbent interaction, non-polar solvents are used. To interrupt these interactions and to elute the analyte, a solvent with some polar character is used. In ion-exchange, both the compound of interest and sorbent functional groups need to be in their ionized form as the mechanism of retention is via electrostatic interaction (Lucci *et al.*, 2012). This is conducted via the sample pH adjustment.

SPE can be employed to extract compounds of interest from diverse matrices such as blood, urine, beverages, water, soil and animal tissue. It is designed for sample preparation and purification of compounds in a solution by adsorption onto a sorbent. This is followed by elution with a suitable solvent (Thurman and Mills, 1998). A range of commercially available sorbents used in SPE includes Oasis HLB Sep-Pak C_{18}, Oasis MAX or MCX, (Su *et al.*, 2017), silica, C_8, C_5, Phenyl, diol, amino bonded silica, ion exchange phases and polymer phases (Stevenson, 2000). The C_{18} sorbent (non-polar) is normally employed for the extraction of analytes that have non-polar functional groups from predominately polar mediums (e.g. water). Advantages of SPE includes the usage of small solvent amounts, short extraction times and simpler processing procedures (Poole and Poole, 2012). A solid-phase extraction contains four manual steps, i.e. conditioning, sample loading, washing, and elution (Figure 2.2).

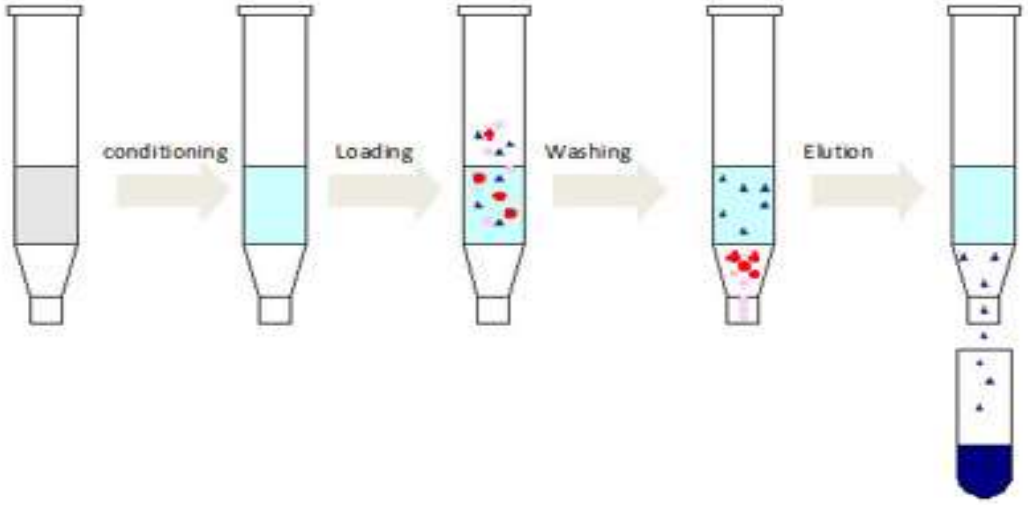


Figure 2.2: Process of Solid-phase Extraction (Thurman and Mills, 1998)

Conditioning step: The solvent is distributed in the sorbent to wet the packing material, thus activating the sorbent's functional groups to allow active interaction with the analytes (Thurman and Mills, 1998). Typically, the conditioning solvent is accompanied by water or an aqueous buffer.

Sample loading: The sample comprising the analyte is inserted into the column to be adsorbed by the sorbent. This step includes hydrogen bonding, Van der Waals interaction, ion exchange and dipole-dipole forces as the analyte is concentrated on the sorbent.

Washing or rinsing of impurities: This includes removing the compounds that may have been retained on the sorbent together with the target analytes during the loading step. This step is significant as it minimizes interferences of undesirable compounds during the analysis stage (Simpson, 2000). The solvent used in this step should be sufficient to remove all the impurities but not too strong to remove the analytes of interest.

Elution of the analyte: In the sorbent, the analyte is eluted with a suitable solvent precisely selected to break the bond between the analyte and the sorbent. The solvent used in this step should be strong enough to remove all the adsorbed analytes with as small a volume as possible.

The SPE technique has been employed in the analysis of different ARV drugs in human blood plasma due to better resolution of the C_{18} silica column (Rezk *et al.*, 2003). The percentage recovery ranged from 75.2 to 98.1%. The limit of quantification (LOQ) range of 10 – 10 000 ng/mL (except zalcitabine (10 – 5000 ng/mL)) was reported. The SPE has also been used for

the extraction of antibiotics and ARVs in wastewater and surface water. Recoveries of pure standards ranged from 41 to 116%. The low recovery was reported to be associated with matrix effects leading to signal suppression. The LOQs and LODs ranged from 5 -63 ng/L and 10 – 570 ng/L, respectively (Ngumba *et al.*, 2016).

2.6.2. Ultrasonic extraction

Ultrasonic extraction is an extraction technique employed for the preparation of solid samples. It is mostly used for pre-treatment of environmental samples for the extraction of semi/non-volatile organic compounds (Bendicho and Lavilla, 2018). The method involves the use of ultrasonic energy, which when imparted to solutions, it causes sound cavitation resulting in the creation of bubbles and subsequent disintegration of a solid sample (Ashley *et al.*, 2001). The collapse which is formed by sonication of solutions results in enormously high temperature and pressure gradients being created. This generates (under sonochemical conditions) local pressures and energies of approximately 10^5 atm and 1 eV, respectively on a timescale of about 10^{-10} seconds. These high-energy environments formed are then used to target analytes extraction from solid matrices (Ashley *et al.*, 2001). Compared to other conventional extraction techniques, UE is inexpensive, simple and efficient (Mandal *et al.*, 2015). The UE can subsequently decrease the operating temperature allowing the extraction of thermolabile compounds. Parameters such as extraction solvent and polarity, mass of sample, extraction time and temperature, and the ultrasonic source (intensity and frequency) need to be optimized as they can have an effect on the extraction efficiency of UE (Kataoka, 2019).

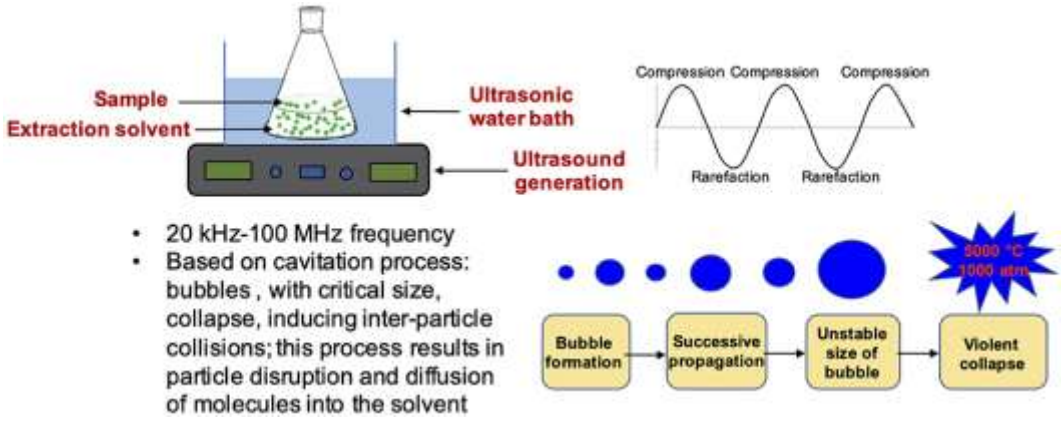


Figure 2.3: Schematic illustration of Ultrasonic extraction (UE) equipment and its characteristics (Panzella et al., 2020)

2.6.3. Soxhlet extraction

Soxhlet extraction is a method used to transfer partially soluble components from a solid to the liquid phase using Soxhlet extractor. The Soxhlet extractor was developed by van Soxhlet in 1879 and has been the most widely used leaching technique (Luque de Castro and Priego-Capote, 2010). The method includes placing a solid sample in a permeable thimble above a flask of solvent but below a cold-water condenser. The extraction solvent boils, condenses and runs back down to the apparatus containing the sample which is then extracted with the solvent as the condenser fills. The process is repeated several times continuously and the end result is a large volume of a volatile liquid (Flanagan, 1996). The advantages of SE are that no filtration of sample is needed after leaching. In addition, SE has a very simple procedure, and can be used to extract large sample mass compared to most of the latest techniques (e.g. microwave-assisted extraction) (Luque de Castro and Priego-Capote, 2010). Its limitations include long extraction time and, a large volume of solvent is employed which is not environmentally friendly.

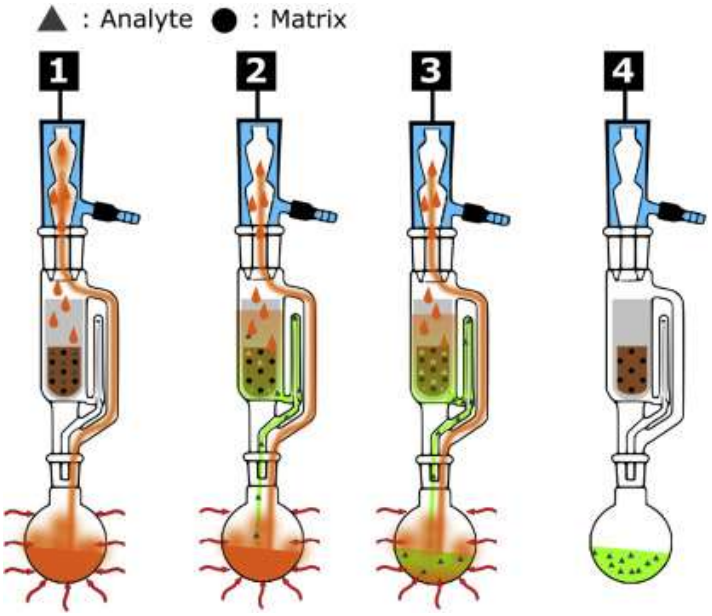


Figure 2.4: Soxhlet extraction (SE) schematic illustration. **1**) Solid sample/matrix is placed in a SE thimble and solvent is heated under reflux. **2**) Condensation and reflux with "fresh" solvent, solutes are transferred from the extraction chamber into the reservoir. **3**) Continuous repetition of the extraction process. **4**) Extraction is complete with solutes in the chamber. (Weggler et al., 2020)

2.7. Chromatographic techniques

Chromatographic techniques are techniques used to separate the target analytes from sample matrix. A separation method is a methodology to achieve any mass transfer phenomenon that converts a mixture of constituents into one or more different products. In chromatography, a mixture of compounds is distributed between a mobile phase and a adjacent stationary phase. The mobile phase is either a liquid or a gas; contrary the stationary phase can be a solid or liquid (Giddings, 2016). Chromatographic methods such as gas chromatography and high-performance liquid chromatography have been employed for the ARV drugs and pharmaceuticals separation in water and solid matrices.

2.7.1. High-performance liquid chromatography

The principle of High-performance liquid chromatography (HPLC) involves the separation of a complex mixture into its components based on their different interaction with the mobile phase and stationary phase. The analyte with a high affinity for the mobile phase spends more time in this phase than the analyte which has a greater attraction for the stationary phase. There are several applications in HPLC. The most common is the reverse phase and normal-phase chromatography. Normal-phase chromatography is used for the separation of neutral species based on their polarity while reversed-phased is used for the separation of species based on their hydrophobicity (Weston and Brown, 1997). In the normal phase chromatography, the higher the polarity of the solute, the greater it is retained in the column. This is because the mobile phase is less polar than the stationary phase, hence increasing the mobile phase polarity results in decreased retention time of the analyte. The normal phase is usually used for the analysis of samples that are soluble in non-polar solvents (Weston and Brown, 1997). The most used, reversed-phase chromatography involves the use of a polar mobile phase. Therefore, a decrease in the mobile phase polarity results in a decrease in the retention time of a solute. The reversed-phase is used in the analysis of samples with polar analytes. High Performance Liquid Chromatography assures fast separation times, improved resolution, high recoveries (Ian Smith, 1993), and high sensitivity (Seneca, 2007).

When choosing a detector, the type of sample matrix must be considered. The detectors that are usually used in HPLC are the fluorescence detector, ultraviolet absorbance detectors (photodiode array (PDA) and UV/Vis), refractive index detector (RI) (Lindon *et al.*, 2003) and Mass Spectrometry (MS) detector.

Fluorescence detector

The fluorescence detector is specifically used for analytes that can fluoresce. It is mostly used in environmental, food and pharmaceutical analysis. The fluorescence detector provides high sensitivity, high selectivity and repeatability. It measures the optical radiation of light by molecules in a solute that has been excited at a higher wavelength (Swartz, 2010). The deuterium or xenon flash lamp is used as a light source. Although the fluorescence detector has a relatively large dynamic range (~ 1000-fold), it is often smaller for many analytes. Also, it can be used when the sample has high levels of impurities because they are not detected as they do not absorb light at the specifically chosen analyte wavelength.

Fluorescence detection has been used simultaneously with UV detector for detection of pharmaceuticals in water. Average recoveries in the range of 65 – 104% and RSD ≤ 16% were obtained. The LOQs reported ranged from 10 – 1100 ng/L (Patrolecco *et al.*, 2013).

Ultraviolet absorbance detectors

The photodiode array (PDA) and ultraviolet/visible light (UV/Vis) detectors are all referred to as ultraviolet absorbance detectors. They have excellent sensitivity for compounds that adsorb light at a picogram level. They are easy to use and provide good stability. The UV-visible detector is the most used and uses light to analyse samples. The sample passes the separation column and goes through a flow cell (colourless glass cell) on which the UV light is illuminated. The sample then absorbs some of the illuminated UV light. Hence, the intensity of the UV light that is observed for the eluent containing the sample and the mobile phase (without the analyte) differ. The difference measured is equal to the analyte concentration present in a sample at a chosen wavelength. A standard UV/Vis detector has wider selection wavelength ranges (195-700 nm) but 254 nm is mostly used as many compounds containing benzene rings can absorb light at this wavelength. Contrarily, the PDA detector detects an entire spectrum (multichannel detector). This allows for simultaneous determination of components contained in the mixture and each at its optimum wavelength (Chan and Carr, 1990). PDA also provides low noise spectral analysis. An HPLC-PDA is coupled to eluates of separation devices by molecular weight and reverse phase (hydrophobicity) making it significant for HPLC.

The ultraviolet detection at 246 nm has been employed for the quantitative determination of efavirenz in human plasma. In this study, Veldkamp *et al.* (1999), reported recovery and LOQ

of 106.4% and 10 ng/mL, respectively. da Silva *et al.* (2018) reported the LOQ and LOD in the range of 0.058 – 0.752 and 0.019 – 0.247 mg/L, respectively, in their study analysis of the concentration of pharmaceuticals in river water samples.

2.7.2. Gas Chromatography

Gas Chromatography (GC) is an analytical method of separating the component mixtures of gases and vapours. GC is a powerful technique for the analyses of organic materials and is also beneficial for the detection of low levels of various contaminants in the environment (Sudhakar *et al.*, 2016). There are two types of methods, namely, gas-liquid chromatography and gas-solid chromatography.

The principle includes the partition of the sample between the gaseous mobile phase and a non-volatile liquid layer coated on the inert solid particles. The samples are introduced as liquid or gas through special sample introduction valves, eluted through a column with an adsorbent (solid/liquid phase) by a carrier gas. Each analyte that is volatile in the column is partitioned between the solid/liquid and the carrier gas (Sudhakar *et al.*, 2016) depending on their retention time. The analytes come through from the column at different times (retention time) and are detected by an appropriate detector.

The choice of carrier gas depends on the nature of the detector to be used (Fothergill, 1968). The most widely used carrier gases are hydrogen and nitrogen. Helium is less reactive therefore ideal to use; however, its cost limits its use. Some of the detectors used in gas chromatography include the mass spectrometry detector (MS), electron capture detector (ECD) flame ionization detector (FID).

Flame ionization detector

Flame ionization detector (FID) is based on the measurement of the electrical conductivity of gases. FID is mostly considered a universal response to organic volatile compounds. FID has a low limit of detection and a wide linear response range (10^7) (Ojanperä and Rasanen, 2008). The FID response originates from the organic compounds that are combusted in a small hydrogen- air diffusion flame. The compounds burned produce ions and electrons that can conduct electricity (Milne and Morrow, 2009). Therefore, the produced ions are proportional to the concentration of analytes (in the form of ions) present in the detector (Stauffer *et al.*, 2008). Its limitations are that it is not sensitive to amine, alcohols and carbonyl functional

groups as well as non-combustible gases (e.g. H_2O and CO_2) and halogens (Milne and Morrow, 2009).

Electron capture detector

Electron capture detector (ECD) is used mostly for the sensitive study of compounds that have high electron affinities (Ojanperä and Rasanen, 2008). A source (3-ray) is used to attain slow electrons in the sample. This is achieved through ionization of the carrier gas (preferably nitrogen) passing through the detector (Sudhakar *et al.*, 2016). As the electrons flow in the direction of the anode under a stationary potential, they give rise to a fixed current. After the analytes of the sample being analysed pass through the column and goes through the detector where the electrons are trapped, they are then substituted by ions that are negatively charged with much greater mass which reduces the current measured. The compensation for this reduction is noted as a positive peak in the chromatogram. The limitations of ECD is that its linear response is limited (10^4), which may result in a need to dilute the sample (Ojanperä and Rasanen, 2008).

Mass spectrometry

Mass spectrometry (MS) measures the mass-to-charge ratio (m/z) of charged particles from a compound (Osweiler and Imerman, 2012). In gas chromatography-mass spectrometry (GC-MS), a gas is used to fragment the compound giving a pattern specific to the compound while liquid chromatography-mass spectrometry (LC-MS) uses a liquid as a mobile phase and detects the whole mass of the compound. The MS can be used for quantification, small organics, inorganics, macromolecules and it can identify trace impurities and target analytes that are present in complex matrices (Thatcher and Caputo, 2008). The high mass accuracy, high sensitivity, and structural information (Vandell and Limbach, 2010) are major advantages of the MS detector. Its limitations include operational complexity and the need for highly qualified personnel.

The MS detector has been used for the determination of ARV drugs in human blood plasma. The recoveries were between 77.3 and 90.1%. The LOD reported fell below, 3 – 8 mg/L which is the clinically relevant therapeutic range (Mwando *et al.*, 2017). Also, recoveries (27 – 139%) were reported for the study of ARV drugs in WWTPs (both in influents and effluents) from KwaZulu Natal using the MS as the detector. The LOQs and LODs ranged from 12 – 65 and 2-20 ng/L, respectively (Abafe *et al.*, 2018).

CHAPTER 3

3. Research Methodology

3.1. Chemicals and reagents

The ARV drug standards (abacavir, nevirapine, and efavirenz), HPLC grade solvents (acetone methanol, ethyl acetate and acetonitrile), were bought from Sigma Aldrich (Steinheim, Germany). Formic acid (98%) was bought from Fluka (Steinheim, Germany). Thimbles and Oasis hydrophilic-lipophilic balance (HLB) cartridges were obtained from Separations (Johannesburg, South Africa) and Biotage (Uppsala, Sweden), respectively.

3.2. Preparation of stock solution

The stock solution of target ARV drugs was prepared by dissolving 1.0 mg of each analyte (abacavir, nevirapine and efavirenz) in acetonitrile (10 mL) to make the final concentration (100 mg/L). Working standard mixtures of different concentrations (0.1-1.0 mg/L) were made in acetonitrile to calibrate the LC-PDA. All solutions were stored in the fridge at 4ºC.

3.3. Instrumentation

The analysis of the selected ARV drugs was performed using LC Shimadzu 2020 series purchased from Shimadzu (Tokyo, Japan), coupled with a photodiode array detector (PDA) purchased from Europe (Germany). The separation of the target analytes was performed using Shim-Pack GIST $C_{18\text{-HP}}$ (150 × 4.6 mm i.d, 3 µm particle size) column purchased from Shimadzu (Tokyo, Japan). The column temperature was kept at 40ºC. A lab developed gradient method with a mobile phase composition of 0.1 % formic acid in water and acetonitrile (30:70) between 0 - 5min and (50:50) between 6 - 12 min was employed for the separation of the analytes. The SPE vacuum manifold employed for the extraction of the analytes from water samples as well as clean-up of Soxhlet and ultrasonic extracts was bought from Sigma Aldrich (Steinheim, Germany). The vacuum pump connected to the SPE manifold was bought from Edwards (Munich, Germany). Oasis HLB cartridges purchased from Biotage (Uppsala, Sweden) were employed as SPE sorbents (60 mg, 3 mL). The ARV drugs were extracted from the soil and sediment samples using ultrasonic purchased from Science Tech (Durban, South Africa) and Soxhlet extractor bought from the University of KwaZulu Natal Glassblower (Pietermaritzburg, South Africa). The centrifuge used for the separation of the solids and the

supernatant liquid was purchased from Shalom laboratory (Durban, South Africa). The rotary evaporator bought from Labotec (Pty) LTD (Durban, South Africa) was employed to concentrate the extracts.

3.4. The study area

The study sites were in the city of Pietermaritzburg and Durban located in KwaZulu-Natal Province in South Africa. Both cities are highly populated and are used for business and recreational activities. Hence, substantial residential and industrial areas are found along the banks of the rivers. This kind of exposure to urbanization and population growth have negatively affected the aquatic environment of the surrounding rivers and sediment bed which in turn affects the aquatic species and human beings

The wastewater samples were collected from five WWTPs of which four (Northern, Umbilo, Umhlathuzana and Amanzimtoti WWTPs) are situated in the Durban area and one (Darvill WWTP) is situated in the Pietermaritzburg area. The river water samples were collected in five sampling sites (YMCA, Camps Drift, College Road, Bishopstowe and Woodhouse) along the Msunduzi river and also in the rivers where the studied WWTPs discharges their treated effluents (Umbilo, Umhlathuzana, Umngeni and Mbokodweni river). The river and wastewater samples were collected in amber glass bottles using a grab sampling technique. The liquid sludge samples were collected at Amanzimtoti and Northern WWTPs. Sediment samples were collected at Bishopstowe, Woodhouse, Camps Drift, Umngeni River and Mbokodweni River. Soil samples were collected in agricultural sites, namely; Curry post, Umgeni Valley, Donny Brook, Richmond and Gilboa Farm. The sediment and soil samples were collected in aluminium foil using an auger. The water samples were transported to the laboratory in a cooler box. All the samples were collected during winter and spring seasons to assess the effect of seasonal variation in the concentrations of ARV drugs with seasonal changes.

3.4.1. Description of sampling sites

3.4.1.1. Msunduzi River and Darvill wastewater treatment plant

The Msunduzi River situated in the Pietermaritzburg area runs through the Midlands of the KwaZulu Natal province. The river has a length of 21.55 km from the source to the mouth. The river passes through highly industrial areas in the city centre of Pietermaritzburg. It meets the Umngeni River between Nagle and Inanda dams and flows out into the Indian ocean in Durban. The river receives runoff from rural communities as well as agricultural areas of Msunduzi

Municipality which contributes to the pollution in the river by organic compounds (Agunbiade and Moodley, 2016). The discharge of inefficiently treated wastewater effluent into rivers has been reported to be the main path accountable for river water pollution with pharmaceuticals. This is because pharmaceuticals are not removed completely by water treatment processes and thus end up in drinking water (Matongo *et al.*, 2015). The five points of sampling along the Msunduzi River and the Darville WWTP were chosen to purposively represent domestic, industrial, municipal and agricultural activities happening nearby the Msunduzi watershed. The map of sampling points along Msunduzi River is shown in Figure 3.1.

Camps Drift is mainly an industrial area located upstream of Msunduzi River and has a length of 5.09 Km. The college road and YMCA are residential areas, and next to the sampling site, is a Mediclinic hospital and a gymnasium. Woodhouse is just after the YMCA. Bishopstowe is a populated area located downstream of the Msunduzi river and it is mainly a residential area. All these places (hospitals and industrial areas) and illegal dumping of garbage (including pharmaceuticals) by residences can contribute to the overall concentration of the target analytes along the Msunduzi River. Figure 3.2 shows pictures of the sampling points along the Msunduzi River.

Darvill WWTP is within the Msunduzi local municipality (Pietermaritzburg), serving over 300 000 people. The plant also treats raw municipal wastewater and treated industrial wastewater with a design capacity of ± 75megalitre/day. It receives industrial and domestic effluent from the Msunduzi area and discharges its treated effluent into the Msunduzi River just before the Bishopstowe area (Mhlanga *et al.*, 2009).

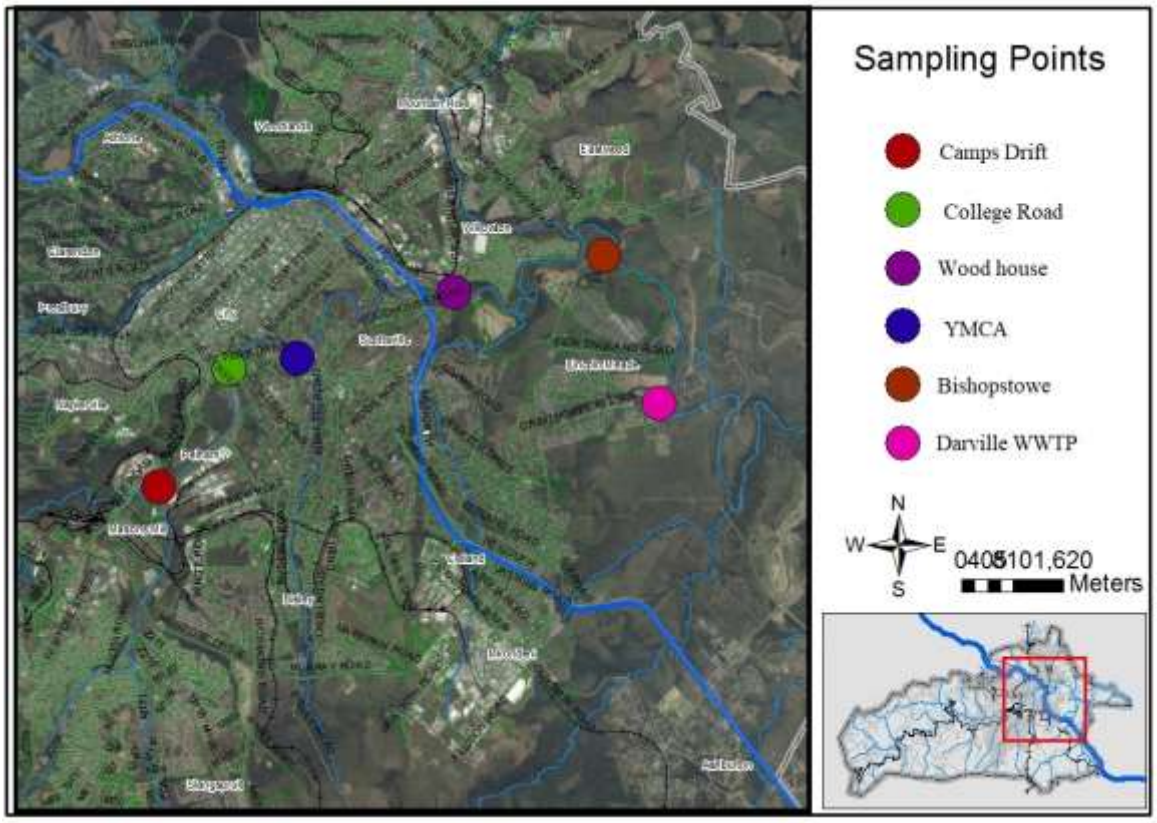


Figure 3.1: Map of sampling points along the Msunduzi River

Figure 3.2: Picture of sampling points along Msunduzi River

3.4.1.2.Umhlathuzana WWTP

Umhlathuzana WWTP is located about 25 km from Durban. The plant receives wastewater from two sources which are the Marianridge and Shallcross (Mhlanga *et al.*, 2009). Marianridge receives an average inflow of 8 megalitre (ML)/day in which 30% is industrial wastewater and 70% is domestic wastewater, whereas Shallcross receives about 2 ML/day of pure domestic water. The final effluent from the two sources is mixed, chorine treated, and then released into Umhlathuzana River (Brouckaert and Mhlanga, 2013) (Figure 3.3). The river (with a length of 50 km) then carries the effluent treated from its upper reaches (Naidoo, 2013). Further downstream, there is the Marianhill Industrial areas as well as residential areas in close range to the river.

Figure 3.3: Umhlathuzana WWTP sampling points

3.4.1.3.Amanzimtoti WWTP

The Amanzimtoti WWTPP is situated in Isipingo (Durban) between the Mbokodweni and the Southern N2 national road (Figure 3.4). The area is mainly residential with some industries. The plant has a capacity of 30 000 ML/day. 2000 kg/day of thin sludge is back washed and disinfected with chlorine gas. The plant receives raw water and potable water from the Nungwane Dam (yield of 9.04 ML/day) and Wiggins WWTP, respectively. The Amanzimtoti

WWTP provides wastewater treatment for effluent from the Amanzimtoti, Isipingo, Prospecton (which is flanked by vast industrial areas) and Kwamakhutha areas. Figure 3.4 shows the surrounding areas and sampling points.

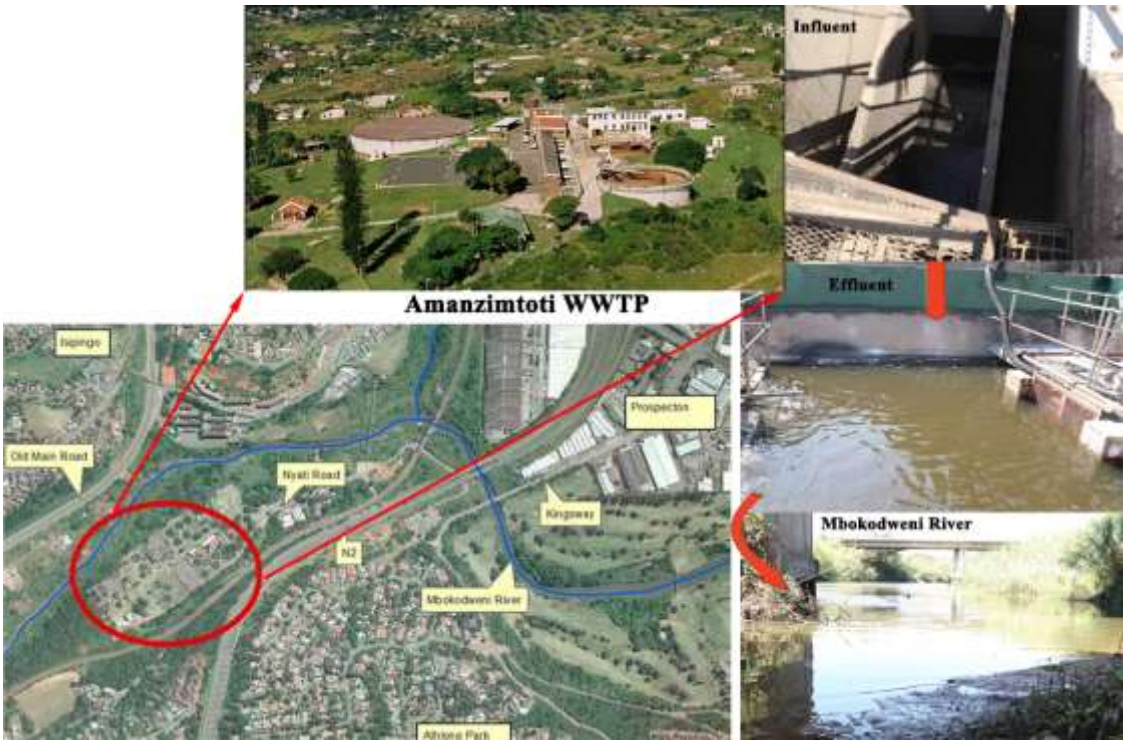


Figure 3.4: Amanzimtoti WWTP, surrounding areas and sampling points

3.4.1.4.Northern WWTP

The Northern WWTP is a conventional biological WWTP, containing primary settling tanks, activated sludge, secondary settling tanks. The plant uses the chlorination process and its effluent is discharged into the Umngeni River. It has a design capacity, treatment capacity and ultimate flow capacity of 58 ML/day, 66 ML/day and 99 ML/day, respectively. The Umngeni River has a length of 255 km and catchment of 4416 km^2 (WRC, 2002) and runs through the Valley of a Thousand Hills (starting from Inanda dam) before it discharges out to the sea. The river flows through the valley and it is surrounded by residential, and industrial areas that has been modified to accommodate human activities (WRC, 2002). The sampling points for the Northern WWTP are shown in Figure 3.5.

Figure 3.5: Northern WWTP sampling points

3.4.1.5.Umbilo WWTP

Umbilo WWTP purifies sewage and is located in Pinetown (at the bottom of Paradise Valley) in Durban Metropolitan. The area is 10 km^2 and mostly consist of residential areas with few large factories. The plant is subdivided into two plants (old and new), and each consist of mechanical and biological treatment (Figure 3.6). The whole plant has an inlet flow of 23 m^3/day. The biological treatment at the new plant contains trickling filters and the new plant have an activated sludge unit that is operated as a reactor that is continuously stirred. The effluent is discharged to Umbilo River with the sludge dumped on landfills located in Durban Metropolitan. The effluent makes up approximately 90% of the river flow during the winter season (Naidoo, 2013).

Figure 3.6: Umbilo WWTP overview and sampling points

The plant has difficulties with organic matter that is present in large amount in the effluent due to the industrial content in the raw sewage. Umbilo River has streams around the Richmond Farm located west of Durban. The streams meet in the suburban area of Ashely. The river meander through Pinetown, Queensburgh and Durban before being channelled in the suburb of Umbilo (Naidoo, 2013). Therefore, the influent and effluent water must be analysed for the presence of ARV drugs and observe the treatment plants contribution to the nearby rivers.

3.5. Optimization of liquid chromatography – photodiode array detector

The LC-PDA method reported by Mtolo *et al.* (2019) was used with further modifications to improve separation and retention times due to additional compounds used in this work. The mobile phase composition using gradient and isocratic elution as well as flow rate are the conditions optimized for LC-PDA.

3.6. Sample pre-treatment

The samples were filtered using a vacuum frit filter with 45 µm Whatman® filter paper for the removal of suspended materials inorder to prevent SPE sorbent blockage during extraction. Collected sediment and soil samples were air-dried (to remove moisture) in a fume hood. Thereafter they were ground by pestle and mortar, followed by sieving through a 60 mm sieve to remove macro substances to allow homogeneity prior to extraction.

3.7. Sample extraction

3.7.1 Solid-phase extraction (SPE) procedure

Samples were extracted using the modified procedure reported by Mtolo *et al.* (2019). Oasis HLB (60 mg, 3 mL) were employed as SPE sorbents for the extraction of the targeted analytes due to its enhanced retention for polar compounds and ability to extract neutral, basic and acid analytes. The conditioning of the cartridge was done with 1 mL acetonitrile and 1 mL methanol followed by sample loading (50 mL) to permit analytes adsorption by the sorbent. The sorbent was rinsed using 2 mL of 10% methanol in water to remove any co-adsorbed impurities and subsequently dried under vacuum for 10 minutes. The 2 mL acetonitrile was then used to elute the adsorbed analytes.

3.7.2 Ultrasonic extraction (UE) procedure

The extraction of ARV drugs was conducted using a modified method reported by Al-Khazrajy *et al.* (2017). Under optimum conditions, a 5 g of dried soil was weighed into a 20 mL centrifuge tube, then 10 mL of 1:1(v/v) acetone/methanol was added into the centrifuge tube and the mixture was vortexed for 1 minute. The mixture was shaken using the ultrasonic bath sonicator for 15 minutes and then centrifuged at 4500 rpm for 10 minutes. The supernatant was filtered through 0.45 µm Whatman® filter paper a then reduced to approximately 1 mL using a rotary vacuum evaporator at 55ºC. This was followed by dilution to 50 mL using deionized water and clean-up using SPE and then analysed by LC-PDA.

3.7.3 Soxhlet Extraction (SE) procedure

The ARV drugs of interest were extracted in soil samples using Soxhlet extraction procedure reported by Sabourmoghaddam *et al.* (2012). Under optimum conditions, a 20 g sample of dried soil was placed in a thimble and the apparatus was fitted in a 250 mL round bottom flask containing 100 mL of 1:1 acetone/methanol. Samples were extracted for 8 hours, after which,

the solution was filtered through 45 µm Whatman® filter paper. It was then reduced to approximately 1 mL using a rotary vacuum evaporator at 55ºC and diluted to 50 mL using deionized water. The SPE was then used for sample clean up prior to analysis with LC-PDA.

Optimization of SPE, UE and SE

The sample loading volume was the optimized SPE condition. Each of the studied sample loading volumes (50, 100 and 200 mL) were spiked with the analytes of interest to make a final concentration of 10 µg/L. The UE conditions optimized were the extraction time and the extraction solvent. The extraction times investigated were 15, 30 and 45 minutes and the extraction solvents were acetone, methanol and acetone: methanol (1:1). The SE optimized condition was the extraction solvent where methanol acetone and acetone: methanol (1:1) were assessed. The samples used for UE and SE were fortified with the target compounds to make a final concentration of 1 mg/L for the assessment of the ARVs recoveries.

3.8. Analytical method validation

The method was validated in terms of the limit of detection (LOD), the limit of quantification (LOQ) that were calculated using a signal to noise ratio (S/N) of 3 and 10, respectively. Linearity was evaluated by means of six-point calibration curves for all the target ARV drugs with concentrations ranging from 0.1-1 mg/L (Figure A2). Each method's precision was assessed as reproducibility and repeatability which was expressed as a percent of relative standard deviation (%RSD). The accuracy of an analytical method is related to the amount of a target compound that is determined as a percentage of the theoretical amount present in the matrix. Accuracy is mostly given as the percentage recovery of the spiked analyte amount. Recovery was assessed using spiked wastewater, river water, deionized water, sediment and soil samples.

CHAPTER 4

4. Results and Discussion

4.1. Optimization of LC-PDA

Initially an isocratic elution with a mobile phase of 60:40% (acetonitrile: water) was employed, with 10 µL and 0.5 mL/min as the injection volume and flow rate, respectively. Under these conditions efavirenz eluted at 14 minutes. The elution mode was then changed to gradient to reduce the retention time for efavirenz. The mobile phase composition composed of 0.1% formic acid in water: acetonitrile was used with the gradient elution held at 70:30% (acetonitrile: water) between 0-5 minutes then 50:50% (acetonitrile: water) between 6-12 minutes. Formic acid is added in the mobile phase to facilitate ionization (by controlling the pH), ensuring the analyte is more basic than the solvent, hence, improving separation by reproducible retention times. A flow rate of 0.4 mL/min was used. Under these conditions, all target ARV compounds were well separated, and the run time was reduced to 10 minutes (Figure A1), hence these were taken as optimal conditions.

4.2. Optimization of SPE

4.2.1. The Effect of sample volume on SPE

The sample volumes examined were 50, 100 and 200 mL. For abacavir, the recoveries displayed a decrease as the sample volume increases, while for nevirapine and efavirenz, recoveries slightly increased from 50 to 100 mL and then decreased with further increase in volume to 200 mL (Figure 4.1). This decrease may be attributed to the shift in adsorption/desorption equilibrium favouring increased desorption from the sorbent packings hence causing a net loss of adsorbate from the SPE cartridge (Sibiya *et al.*, 2012). The t-test analysis done in the recovery mean of sample volume indicated that they are insignificantly different with the $p>1$ for 50 versus 100 mL, $p>0.42$ for 50 versus 200 mL, $p>0.49$ for 100 versus 200 mL which are all above 0.05 (Table A1). Hence, a 50 mL volume of sample was chosen as the ideal volume, taking into consideration the time required for the extraction process.

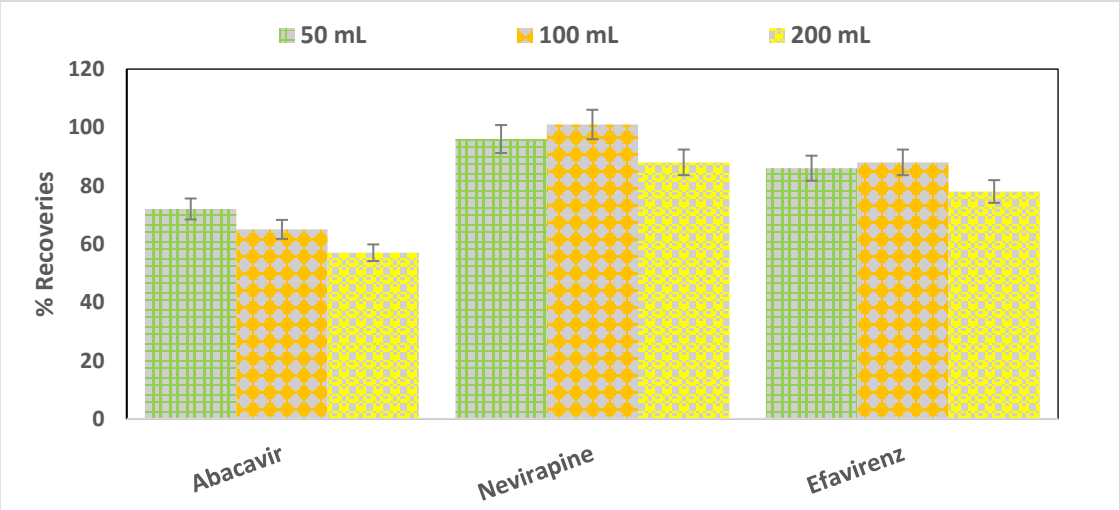


Figure 4.1: Effect of sample volume on the extraction efficiency of ARV drugs by SPE

4.3. SPE-LC-PDA method quality assurance

The method was assessed in terms of linearity, recovery, LOD, LOQ, reproducibility and repeatability. The calibration curves of all compounds gave a good coefficient of determination (R^2) which is above 0.997 (Figure A2). This indicated that the relationship between the method response and the concentration of the analytes in the matrix is directly proportional. The method's accuracy was determined by spiking the river water, wastewater and deionized water with the concentration of each compound ranging from 10 to 100 µg/L. The recoveries after SPE and LC-PDA determination were calculated and good recoveries (70 – 112%) were obtained which indicated the robustness of the optimised SPE-LC-PDA method. This was within an acceptable recovery range as a function of the analyte concentration (10 µg/L/ 10ppb) (Nash and Wachter, 2003). The recoveries obtained were comparable regardless of the spike concentration used which showed that they are independent of the concentration in the sample. This insignificant difference was also statistically confirmed where $p>0.6$ for 10 versus 50 µg/L, $p>0.42$ for 10 versus 100 µg/L, $p>0.69$ for 50 versus 100 µg/L which are all above 0.05 (Table A2). The LOD and LOQ were found to be 0.7 - 0.8 µg/L and 2.1 - 2.4 µg/L, respectively. The lower LOD and LOQ obtained signifies good sensitivity of the method and indicates that it can be able to detect these compounds at trace levels in real samples. The repeatability and reproducibility of the instrument ranged from 0.12 - 1.5% and 12 - 16%, respectively. All RSD values reported were less than 20% (Table 4.1) which indicated good precision (SW-846, 2003) and accuracy of the instrument and the method.

Table 4.1: Correlation efficient, LOQs, LODs, % recovery and % RSD, (n = 3)

Compound	LOD (µg/L)	LOQ (µg/L)	R^2	% Recovery for Deionized water			River water	Wastewater
				10 µg/L	50 µg/L	100 µg/L	10 µg/L	10 µg/L
Abacavir	0.77	2.4	0.9984	72 ± 11	81 ± 10	71 ± 5	107 ± 16	80 ± 13
Nevirapine	0.70	2.1	0.9983	96 ± 1	89 ± 7	74 ± 1	112 ± 2	88 ±11
Efavirenz	0.68	2.1	0.9979	86 ± 2	70 ± 6	86 ± 10	101 ± 12	73 ± 6

4.4. Application of SPE-LC-PDA method to water and sludge samples

4.4.1. Physico-chemical parameters of the collected samples

Before the analysis, the physical parameters for all the samples collected were measured. The parameters measured include dissolved oxygen, chemical oxygen demand, pH, dissolved solids, conductivity and salinity (Table A3 and Table A4).

The pH of the water was found to be 6.5 – 8.2 and 6.9 – 8.3 for river water and wastewater samples, respectively. These pH values are within World Health Organization (WHO (2003a) acceptable pH range (6.5 – 8.5) for raw water. At a pH below pKa, pharmaceuticals exist in a neutral form (hydrophobic), and in an anionic form (hydrophilic) at pH above pKa (Bui and Choi, 2009). Therefore, at a basic pH, higher concentrations of ARV drugs are expected as they will mostly dominate in the aqueous phase.

The measurement of salinity showed that all the samples had slightly higher amounts of inorganic soluble salts in wastewater (0.25 - 0.76 psu) as compared to river water (0.07 – 0.61 psu), except Amanzimtoti river (1.48 psu). These salinity results suggest that higher concentrations of the ARV drugs are expected in Amanzimtoti soil/sediment samples. This is due to the fact that the interaction of pharmaceuticals with water is affected by high salinity resulting in their adsorption to the soil/ sediments. The total dissolved solids (TDS) in wastewater samples (225 – 1551 ppm) were higher compared to river water samples (75.7 – 1503 ppm). These TDS results are higher than WHO (2003b) acceptable range (300 – 900 ppm) in some samples and hence can cause a negative effect on water bodies. Also, the concentration of TDS was very high in samples from rivers where the WWTP discharge their effluent (225 -1503 ppm) compared to the river water samples obtained along the Msunduzi River (75.7 – 200 ppm). This is caused by discharging waste or saline industrial effluents into

the rivers (Naidoo, 2013). An increase of TDS in rivers may also be caused by the discharge of high amounts of sewage effluent into inland waters (Dallas and Day, 2004). The conductivity measured ranged between 3.0 - 1276 µS in river water and 3.10 – 1657 µS in wastewater.

In general, the dissolved oxygen (DO) in water is wide-ranging. However, it has been reported that a minimum of 5 mg/L DO is required in the water for fish not to be affected (Ahn *et al.*, 2019). The DO measured ranged between 7.8 - 18.26 mg/L in river water and 12.6 - 19.02 mg/L in wastewater. This indicated that the water does not pose threat to fish in rivers as the measured DO concentration is above the minimum required. There was an increase in DO in all wastewater samples between the influent and the final effluent, which is due to the aeration process (Madikizela and Chimuka, 2017).

4.4.2. Concentrations of ARV drugs obtained in wastewater

The wastewater effluent and influent samples were collected in Umhlathuzana, Darvill, Umbilo, Amanzimtoti, Northern WWTPs, while sludge was collected in Northern and Amanzimtoti WWTPs. The highest detected concentration of ARV drugs was for nevirapine (6759 µg/L) in the activated sludge of the Amanzimtoti WWTP (Table 4.2). This could be attributed to nevirapine's medium sorption potential ($2.5 < \log K_{ow} < 4.0$), hence it binds mostly to sludge/sediments. It could also be attributed to its photostability and low biodegradability in a closed bottle system (Vankova, 2010). Moreover, at low pH levels, organic substances do not decompose (Naidoo, 2013), hence large concentrations of abacavir and nevirapine were found in the sludge of Amanzimtoti and Northern WWTPs.

Nevirapine was also found at higher levels in the effluents of Northern and Umbilo WWTP although it was not detected in the influents. The reason could be that the residues of ARVs that are bound to a bile acid before being released from the patient's body may undergo deconjugation in the WWTP, therefore can yield high levels of ARVs in the effluent compared to its influent (Schoeman *et al.*, 2015). It has been stated that the treatment efficiency of WWTPs is reduced with high concentrations of bioactive pharmaceuticals such as ARV's (Slater *et al.*, 2011) which could also result in higher concentrations in the effluent. These observations agree with those reported by Schoeman *et al.* (2015) and Prasse *et al.* (2010) whereby increased levels of nevirapine were observed in the effluents of WWTPs. This indicates the persistence of nevirapine in the wastewater stream (Abafe *et al.*, 2018). The prevalence is most likely to be attributed to its environmental persistence as well as frequent use for HIV treatment and to prevent mother-to-child transmission (Schoeman *et al.*, 2015).

Data on the presence of efavirenz in WWTP effluents is not commonly available (Schoeman *et al.*, 2017), however, this study showed significant concentrations of efavirenz in the influent and effluent. This drug was detected in all WWTP effluents ranging from 2.47 – 22.0 µg/L, while the concentrations that entered the WWTPs ranged between 4.32 µg/L to 23.3 µg/L. These results agreed with those reported in Gauteng, South Africa whereby efavirenz concentrations in the influents ranged from around 5.50 µg/L to 14.0 µg/L (Schoeman *et al.*, 2017). The use of chlorination as a wastewater treatment mechanism has been shown to result in the transformation of ARV's which can lead to the formation of many undescribed disinfection transformation products (Wood *et al.*, 2016) and this can result in lower concentrations of ARVs detected in the effluent. The concentrations of efavirenz measured in influents and effluents of this work are lower than those observed in another study in KwaZulu Natal, South Africa where influents and effluents ranged from 24.0 – 34.0 µg/L and 20.0 – 34.0 µg/L, respectively (Abafe *et al.*, 2018).

Abacavir was found to be the most dominant and higher in concentrations as compared to nevirapine and efavirenz. This is due to its low log K_{ow} (0.22) thus it is found mostly in the aqueous phase. High concentrations of abacavir ranging from 278 - 814 µg/L were observed in influents of WWTPs whilst lower concentrations were observed in the effluents, which resulted in better removal efficiency.

Abacavir had the highest removal efficiency in all WWTP (75-91%), while nevirapine had 44-87% and efavirenz had 6-53%. The poor removal of ARVs could lead to their frequent detection in WWTPs effluents and surface water. Also, the amounts removed by the WWTPs varied which could mostly be attributed to both the nature of the influents and the operation of the plants (Schoeman *et al.*, 2015). Amanzimtoti WWTP was the most polluted plant which could be because it serves residential, and the vast majority of industrial waste and it also has a larger capacity. Although Darvill WWTP had a different distribution of ARV drugs due to its more localised nature (serves over 300 000 people) has a lower capacity and is still under upgrade), it removed most of the ARVs compared to the other WWTPs.

Table 4.2: Concentration of ARVs detected in wastewater samples

WWTP	Sampling Site	Concentration/ µg L^{-1}			Removal Efficiency/%		
		Abacavir	**Nevirapine**	**Efavirenz**	**Abacavir**	**Nevirapine**	**Efavirenz**
Darvill (WWTP)	Influent	278	261	4.32	85	87	42
	Effluent	40.8	34.2	2.47			
Northern (WWTP)	Influent	621	nd	6.39	86	-	-
	River	19.0	28.5	18.7			
	Effluent	85.2	18.8	22.0			
	Liquid sludge	474	nd	19.6			
Amanzimtoti (WWTP)	Influent	502	106	9.39	75	44	53
	River	102	35.6	5.67			
	Effluent	124	59.1	4.45			
	Liquid sludge	134	6759	<LOQ			
Umhlathuzana (WWTP)	Influent (Marianridge)	661	68.4	9.60	91	58	6
	Influent (Shallcross)	605	nd	<LOQ			
	River	57.4	36.0	8.52			
	Effluent	116	28.6	9.01			
Umbilo (WWTP)	Influent	814	nd	23.3	85	63	47
	River	80.7	52.3	21.7			
	Effluent	103	74.7	3.13			

*nd = not detected; LOQ = not quantified

4.4.3. The concentration of ARV drugs obtained in river water

The river water samples were collected at the YMCA, Bishopstowe, College Road, Wood house, Camps Drift, Northern, Amanzimtoti, Umbilo. Abacavir and nevirapine showed the highest concentrations in the rivers (102 µg/L in Amanzimtoti and 52.3 µg/L in Umbilo River, respectively) where WWTPs discharge their treated effluent (Table 4.2). This agrees with the finding reported in another study on the occurrence of ARVs in surface water where nevirapine

was frequently detected with the highest concentration in the samples analysed (Ferrer and Thurman, 2012).

More ARV compounds were observed during the winter season compared to spring. The highest concentration of efavirenz (87.1 µg/L) was found in College Road in the winter season (Table 4.3). Efavirenz is excreted at 62% via faeces and/or urine (Rakhmanina and van den Anker, 2010), hence, this suggests possible contamination of the Msunduzi River by faeces and/or urine as the area is highly populated. Efavirenz was not detected in the sample collected in the spring season at the College Road area. This is likely to be because of the heavy rains during the spring season which could result in dilutions beyond quantification limits. A similar trend was observed where pharmaceuticals were found in rivers at higher concentrations during the cold season (Vieno *et al*., 2005). In surface water, the main elimination processes are sorption, photodegradation and biodegradation, hence, the higher levels of ARVs in the cold season could be attributed to inhibited degradation due to lower temperature and sunlight. This could be indication that ARV drugs are temperature sensitive. It has been reported that extreme/high temperatures cause degradation of various drugs through chemical reactions such as hydrolysis, decarboxylation and oxygenation (Küpper *et al.*, 2006).

The highest concentration found in river water was that of abacavir (102 µg/L) in Amanzimtoti River which corresponds to the high concentration (124 µg/L) that was found in the effluent of Amanzimtoti WWTP (Table 4.2). A similar trend was observed for both efavirenz and nevirapine in which the concentrations obtained in the river corresponds to the effluents of the relevant WWTPs. This indicated that the WWTPs indeed contributes to river water contamination. Higher concentrations in river water compared to the corresponding effluent could be due to direct disposal of waste in or near the rivers, while low concentrations could be due to river flow dilutions. Abacavir (pKa = 5.04) and nevirapine (pKa = 2.8) both have pKa below the pH of water and thus attracted more to water compared to efavirenz (pKa = 12.52) which has a pKa above pH. Hence, high concentrations of abacavir and nevirapine were obtained in water as they existed in the anionic form compared to efavirenz which was in neutral form.

Umbilo River was found to be most polluted compared to other rivers. The river has many incoming streams around the Richmond Farm which meet in the suburban area of Ashely. The river also passes through Pinetown, Queensburgh and Durban (highly populated) before being channelled in the suburb of Umbilo (Poulsen and Lauridsen, 2005). Therefore, all these streams can contribute to high concentrations detected in the river. Bishopstowe sampling site was

found to be polluted with all ARVs although efavirenz was below the limit of quantification. This may be due to the release of the Darvill WWTP effluents just before this site as the discharge of insufficiently treated effluent into rivers can result in surface water pollution with pharmaceuticals (Matongo *et al.*, 2015).

Table 4.3: Concentration of ARVs detected in river water collected along Msunduzi River

		Concentration (µg/L)		
Season	**Sampling Site**	**Abacavir**	**Nevirapine**	**Efavirenz**
Winter	YMCA	nd	nd	Nd
	Bishopstowe	9.76	8.45	<LOQ
	College Road	nd	nd	87.1
	Woodhouse	nd	nd	Nd
	Camps Drift	nd	nd	Nd
Spring	YMCA	nd	nd	Nd
	Bishopstowe	nd	nd	<LOQ
	College Road	8.39	nd	Nd
	Woodhouse	nd	nd	Nd
	Camps Drift	<LOQ	nd	Nd

*nd = not detected; LOQ = not quantified

4.5. Ultrasonic extraction method optimization

4.5.1. The effect of extraction solvent on UE

The optimization of extraction solvent is critical as the solvents have different polarities and affinity towards different chemical compounds and thus affects the recovery percentage Annegowda *et al.* (2012). The influence of extraction solvent was investigated using methanol: acetone (1:1 v/v), methanol: acetonitrile (1:1 v/v) and methanol alone and the extraction was done for 15 minutes. The results obtained revealed that the extraction solvent mixture of methanol: acetone (1:1 v/v) was the best extraction solvent as it gave higher recoveries (61 -

104%) for all the ARV drugs (Figure 4.2). This may be due to a mixture of different polarities of solvents used (i.e. methanol is polar while acetone is least polar of the selected solvents) which accounted for different polarities of the target analytes and thus improved affinity of the analytes to the solvents (Kunene and Mahlambi (2020). The t-test analysis indicated that these recoveries are not significantly different as they gave p-values that are greater than 0.05. The values obtained are $p>0.72$ for methanol:acetonitrile versus methanol, $p>0.34$ for methanol:acetonitrile versus methanol:acetone, $p>0.50$ for methanol versus methanol:acetone (Table A5). Hence, methanol:acetone was chosen as the best optimum extraction solvent.

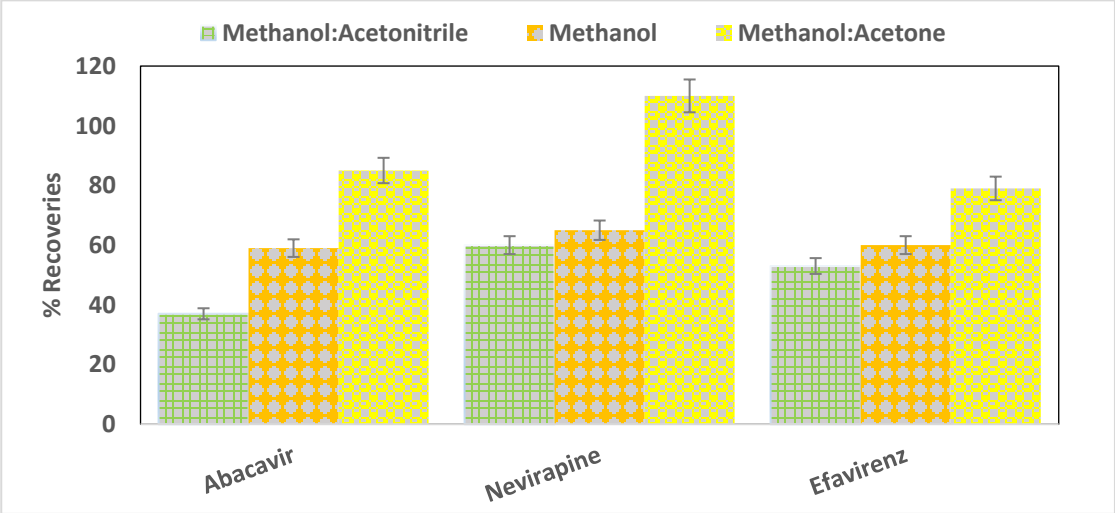


Figure 4.2: The effect of solvent system on analytes recoveries by UE

4.5.2. The Effect of extraction time on UE

One of the aims of the extraction process is to obtain higher percentage recoveries in a short period of extraction time. The influence of extraction time on the analytes recoveries was evaluated using 15, 30and 45 minutes. The methanol:acetone mixture was used as the extraction solvent. The recoveries showed a decrease in recoveries with extraction time the highest recoveries (61-104%) were obtained at 15 minutes extraction time (Figure 4.3). This indicates that 15 minutes was enough to allow proper analytes transfer from the matrix to the solvent. A possible reason for recovery decrease with increased extraction time could be that the analytes degraded as they spent more time in contact with the solvent. The t-test carried out on the mean recovery showed that they are not significantly different with the $p>0.70$ for 15 versus 30 minutes, $p>0.16$ for 15 versus 45 minutes, $p>0.27$ for 30 versus 45 minutes which

are all above 0.05 (Table A6). Fifteen minutes was therefore selected as the suitable extraction time as shorter extraction process that gives acceptable recoveries is desired.

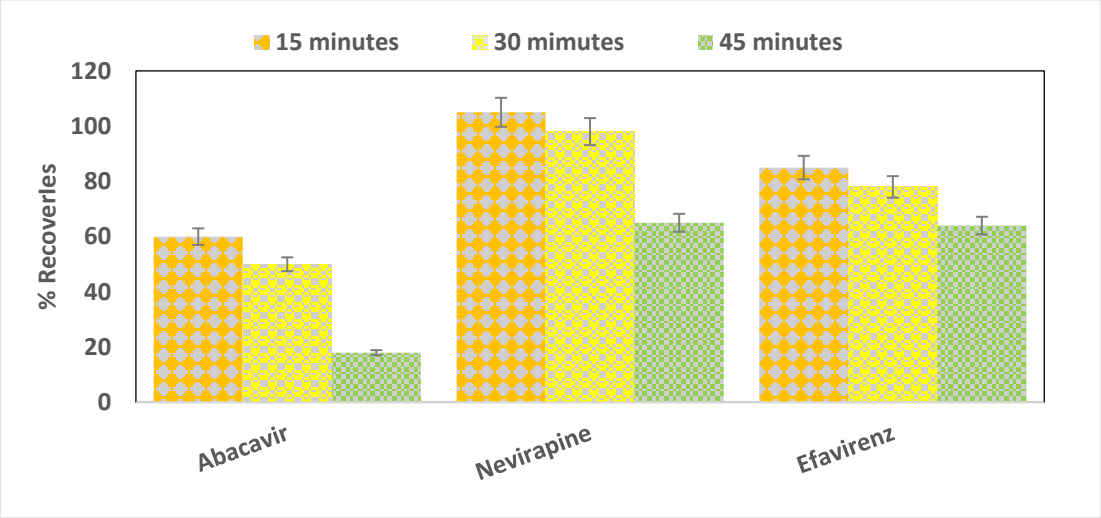


Figure 4.3: The effect of extraction time on analytes recoveries by UE

4.6. Soxhlet extraction method development

4.6.1. The Effect of extraction solvent on SE

The extraction solvent effect was studied using methanol:acetonitrile (1:1 v/v) and methanol:acetone (1:1 v/v). Similar to ultrasonic extraction, methanol:acetone mixture was more effective in penetrating the soil and sediment pores to efficiently interact with the compounds leading to improved analytes transfer to the solvent (79 - 108%), (Figure 4.4). Moreover, acetone has low viscosity, hence adding it improved the penetration of the solvents into the pores of the soil particles and thus enhanced analytes transfer to the solvent leading to higher recoveries. The performed t-test revealed that only methanol:acetonitrile versus methanol:acetone mean recoveries are significantly different as they gave $p>0.024$. The $p>0.25$ was obtained for methanol:acetonitrile versus methanol, $p>0.09$ for methanol versus methanol:acetone which is more than 0.05 and thus not significantly different (Table A7). Methanol:acetone was chosen as the idea extraction solvent.

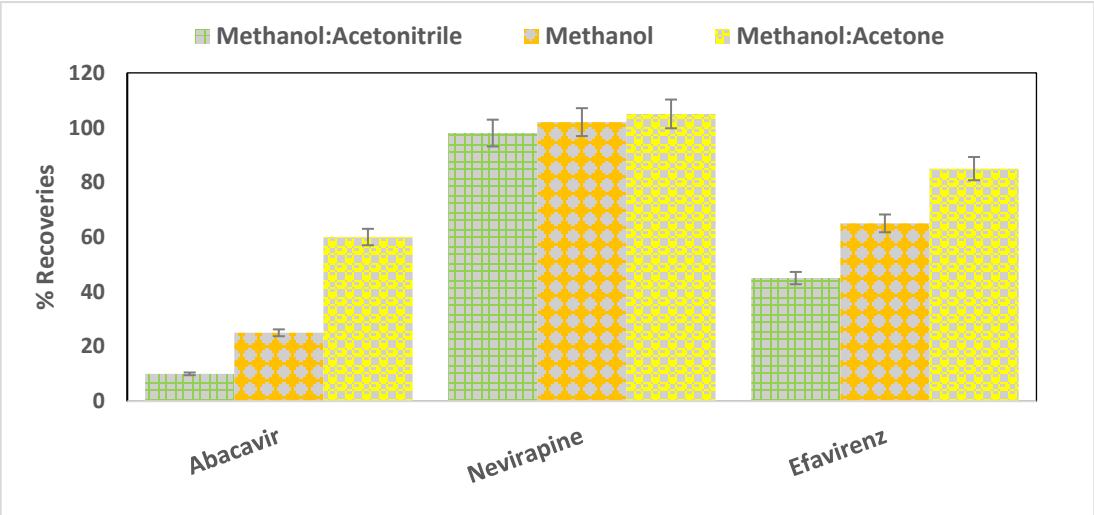


Figure 4.4: The effect of solvent system on analytes recoveries by SE

4.7. UE and SE-LC-PDA methods quality assurance

The methods quality assurance was assessed based on measuring linearity, recovery, LOD, LOQ. Good correlation of determination (R^2) greater than 0.99 were obtained for all compounds (Table 4.4). This indicated a relationship between the method response and the concentration of the analytes in the matrix is directly proportional.

The recoveries obtained were 79 – 108% for SE and 61 – 104% for the UE method. The calculated LOD and LOQ were found to be 1.6 – 2.3 µg/kg and 4.9 – 7.0 µg/kg for UE while for and SE they were 0.8 - 0.9 µg/kg and 2.3 – 2.8 µg/kg, respectively (Table 4.4). These lower LOD and LOQ signifies good sensitivity of the methods and indicates that it can be able to extract these analytes at low concentrations in real samples. Comparing UE and SE, low LOD and LOQ were obtained for SE showing its high sensitivity than UE. Also, SE gave higher recoveries which revealed that it is more accurate than UE. However, UE showed to be more accurate for efavirenz. The significant difference of the mean LOD, LOQ and recovery results was evaluated by conducting a t-test analysis which proved that LOD and LOQ results are statistically different with the $p>0.043$ and 0.041, respectively. However, the mean recoveries were found to be insignificantly different with the $p>0.61$which is more than 0.05 (Table A8).

Table 4.4: The correlation efficient, %recoveries (%R), limit of detection (LOD) and quantification (LOQ) (µg/kg) of the analytical methods

Analyte	***Equation***	***R^2***	***Soxhlet Extraction***			***Ultrasonic Extraction***		
			%R	***LOD***	***LOQ***	***%R***	***LOD***	***LOQ***
Abacavir	y= 65549x	0.9984	88	0.8	2.4	61	2.3	7.0
Nevirapine	y= 67255x	0.9983	108	0.8	2.3	104	1.6	4.9
Efavirenz	y= 112887x	0.9979	79	0.9	2.8	85	1.7	5.2

4.8. UE and SE method application

4.8.1. Concentrations of ARV drugs in soil and sediment using UE

None of the target ARV drugs was detected in soil samples collected from Donny brook, Cedara and Gilboa Farm (Table 4.5). The possible reason is that these areas are agricultural lands and mostly populated by the farming community and hence there may be very little or no usage of the ARV drugs in these areas. Nevirapine was detected in Richmond, UMngeni and College Road soils as well as Northern sediment samples, with the highest concentration in Richmond soil (31.3 µg/kg) (Table 4.5). The presence of nevirapine could be due to its high usage. It is mostly used for the HIV treatment and the prevention of mother-to-child transmission Schoeman *et al.* (2015). Abacavir was only quantified at 35.1 µg/kg (Table 4.5) in College Road soil samples which was also found to be the most contaminated area as all of the target ARV drugs were detected. The other contributing factor towards pollution in this area could be incorrect disposal of unused medicines which has been found to largely contribute to the detection of pharmaceutical drugs in the environment (Schoeman *et al.* (2017). Efavirenz was quantified in College Road, Amanzimtoti, Curry Post soil as well as in Northern sediment samples. The highest concentration of efavirenz (43.6 µg/kg) was found in Curry Post. The high ARV concentrations in this farmland could an indication of possible usage of sludge as manure or irrigation using wastewater effluent which could contain ARV residues. It has been reported that efavirenz is used in a constituent of a cocktail prescribed to HIV patients and only replaced with nevirapine where contraindications are observed (Health (2013). The

presence of ARV drugs in sediments could be due to the effluent discharge from the wastewater treatment plants.

The absence of ARV drugs in most of the sampling areas could be due to that their hydrophilic and polar nature will dominate in the aqueous phase (Schoeman *et al.* (2017). This could be the reason studies conducted on similar ARVs and other pharmaceutical drugs have only reported their extraction in liquid media.

Table 4.5: Concentration of drugs detected in soil and sediment samples

Matrix	**Sampling Site**	**Concentration (µg/kg)**		
		Abacavir	**Nevirapine**	**Efavirenz**
Soil	Donny Brook	nd	nd	nd
	Cedara	nd	nd	nd
	Gilboa Farm	nd	nd	nd
	Richmond	nd	31.3	nd
	UMngeni	nd	24.7	nd
	Curry Post	nd	nd	43.6
	College Road	35.1	30.7	15.4
Sediment	Amanzimtoti	nd	nd	27.1
	Northern	nd	22.8	31.2

Note: nd – not detected

4.8.2. Comparison of SE and UE on the extraction of ARV drugs in soil and sediment

The sediment and soil samples obtained from Bishopstowe, Woodhouse, and Camps Drift areas were used to compare the extraction efficiency of UE and SE methods. Only efavirenz was found in Woodhouse and Camps Drift soil and sediment samples, although in the Woodhouse soil it was detected below quantification limits. The contamination in Woodhouse sediment could be because it is after Darville wastewater treatment plant; hence, it could be receiving contaminants discharged by the treatment plant. This is because treatment plants have been recognised as the source of contamination for the rivers. The quantified ARV drugs were only detected from SE extracts and not UE (Table 4.6). This could be due to the higher sensitivity and accuracy of SE compared to UE resulting from the low LOD and LOQ values as well as high recoveries obtained for the SE. Additionally, the SE uses high temperatures and thus

improves its efficiency in extracting the target analytes is enhanced (Marshal *et al.* (1967); Kunene and Mahlambi (2020). This result indicates that despite the drawbacks of SE, such as high solvent consumption and longer extraction time, it is more effective in extracting the studied ARV drugs from soil and sediments.

Table 4.6: Comparison of concentrations obtained using SE and UE

Matrix	Sampling Site	Ultrasonic Extraction			Soxhlet Extraction		
		Concentration (µg/kg)			Concentration (µg/kg)		
		Abacavir	Nevirapine	Efavirenz	Abacavir	Nevirapine	Efavirenz
Soil	Woodhouse	Nd	nd	nd	nd	nd	<LOQ
	Bishopstowe	Nd	nd	nd	nd	nd	nd
	Camps Drift	Nd	nd	nd	nd	nd	138
Sediment	Woodhouse	Nd	nd	nd	nd	nd	53.6
	Bishopstowe	Nd	nd	nd	nd	nd	nd
	Camps Drift	Nd	nd	nd	nd	nd	98.9

4.8.3. Comparison of ARV drugs obtained in different environmental matrices

The levels of ARV drugs obtained in various matrices were also compared (Figure 4.5). As expected, abacavir was found to be dominant in water due to its low Log K_{ow} and high polarity (77000 mg/L) in water. Nevirapine was found to be dominant in the sludge, which could be due to the drug environmental persistence. Additionally, nevirapine has a high Log K_{ow} and lower polarity in water (100 mg/L), thus is expected to be retained more in the sediments/solid-like phase. Efavirenz was found to be highly dominant in soil and sediments which could be due to its low TPSA, high Log K_{ow} and very low solubility in water (< 1mg/L).

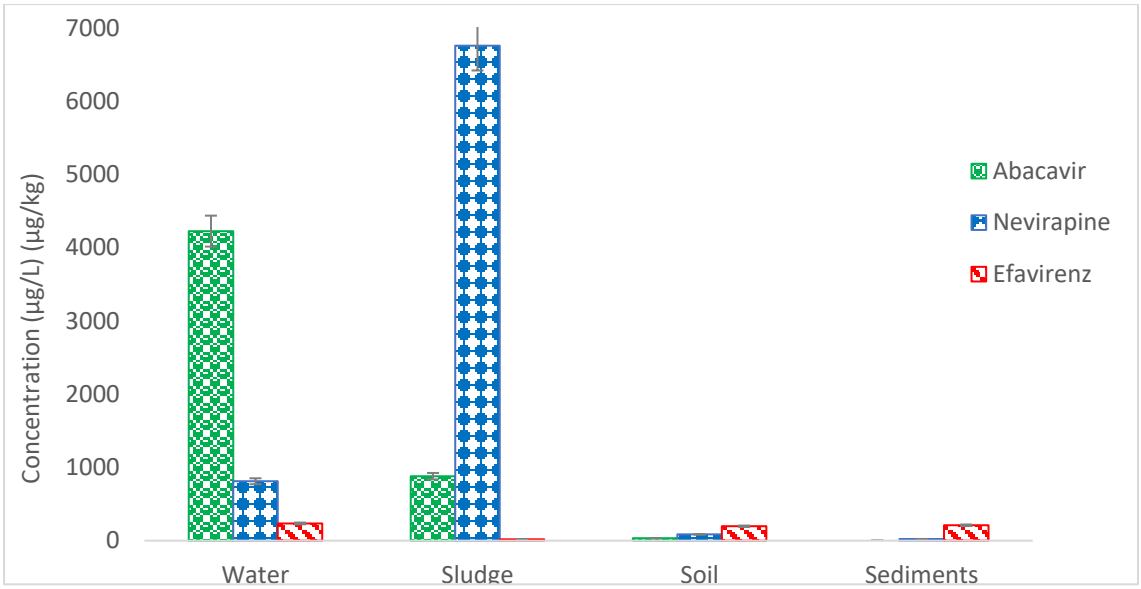


Figure 4.5: The comparison of the concentration of ARV drugs in different environmental medium.

4.8.4. Comparison of obtained concentrations to other studies

A limited number of studies have previously been reported on the presence of abacavir (especially in South Africa). It was however observed that the majority of studies have reported concentrations less than those observed in this work (Table 4.7). This could be attributed to the number of increasing HIV/AIDs cases reported every year, hence an increasing demand in the usage of these drugs. The concentrations of abacavir quantified in this study exceeded those reported in the previous studies in South Africa (Abafe *et al.*, 2018; Swanepoel *et al.*, 2015). Also, the concentrations of nevirapine reported in this study were above those reported in the previous reported in South Africa from the year 2015 – 2018 (Abafe *et al.*, 2018; Schoeman *et al.*, 2017; Schoeman *et al.*, 2015) and those found in Kenya, Nairobi (K'Oreje *et al.*, 2016). This could imply a high usage of nevirapine over the years with an increase in the number of people infected with HIV/AIDS. This leads to high consumption and discharging of ARV drugs metabolites through the sewage, which is one of the major sources of ARV's into the environment. On the contrary, high levels of efavirenz have been reported in WWTP influents and effluents from previous studies conducted in South Africa, KwaZulu Natal (Abafe *et al.*, 2018; Mtolo *et al.*, 2019) compared to those observed in this work. Schoeman *et al.* (2015) has previously reported comparable concentrations of efavirenz to this study in WWTP influents. These results indicate that African countries are more contaminated than overseas countries, however, water contamination by ARV drugs is a worldwide problem. Therefore, continuous

monitoring of these drugs in our environment is of importance. Based on the data in table 4.7, it is evident that the presence of these ARV drugs in the water bodies and surrounding surfaces is likely to increase rapidly over the years. Therefore, necessary studies must be conducted nationwide in order to assess the presence, use-pattern and removal rate of ARV drugs.

Table 4.7: Comparison of ARV drugs levels to other various studies in South Africa and other countries

Analyte	Country/Area	Maximum concentration (ng/L)			Reference
		WWTP influent	WWTP effluent	Surface water	
Abacavir	South Africa/ KwaZulu-Natal	814 000	124 000	102 000	This Study
	South Africa/ KwaZulu-Natal	14 000	-	-	(Abafe *et al.*, 2018)
	France/Talence	-	33	0.7	(Aminot *et al.*, 2015)
	South Africa/ Northwest	-	<LOQ	1.6	(Swanepoel *et al.*, 2015)
Nevirapine	South Africa/ KwaZulu-Natal	26 1000	74 700	52 300	This Study
	South Africa/ KwaZulu-Natal	2 800	1 900	-	(Abafe *et al.*, 2018)
	South Africa/ Gauteng	200	473	-	(Schoeman *et al.*, 2017)
	South Africa/ Gauteng	2 100	320	-	(Schoeman *et al.*, 2015)
	Kenya/ Nairobi	3 300	2 080	5 620	(K'Oreje *et al.*, 2016)
Efavirenz	South Africa/ KwaZulu-Natal	23 300	22 000	87 100	This Study
	South Africa/ KwaZulu-Natal	140 400	93 100	2 450	(Mtolo *et al.*, 2019)
	South Africa /KwaZulu-Natal	34 000	34 000	-	(Abafe *et al.*, 2018)
	South Africa/Gauteng	14 000	4 000	-	(Schoeman *et al.*, 2017)
	South Africa/ Gauteng	17 400	7 100	-	(Schoeman *et al.*, 2015)
	Kenya/Nairobi	1 020	110	560	(K'Oreje *et al.*, 2016)

CHAPTER 5

5. Conclusion and future recommendations

5.1. Conclusion

The proposed analytical extraction methods (SE, UE and SPE) and LC-PDA were successfully optimized, validated and applied for the extraction of abacavir nevirapine and efavirenz in wastewater, sludge, sediment, river water, and soil samples. The methods showed good accuracy with the recoveries ranging from 71 - 112% for SPE, 79 -108% for SE and 61 - 104% for UE. In comparison, SE proved to be more accurate and sensitive for the extraction of ARV drugs compared to UE, which was shown by lower LODs and LOQs, and higher recoveries. However, UE was more accurate for abacavir. Hence, SE is recommended for the monitoring of these compounds regardless of this time-consuming process.

The concentrations detected were 8.39 - 102 µg/L in river water, wastewater 2.47 - 814 µg/L and 19.6 – 6759 µg/L in sludge samples. Higher concentrations of ARV drugs were obtained in samples collected during the winter season compared to spring season. This could be due to a decrease in the photodegradation and biodegradation of the ARVs in the cold season. This can also be attributed to dilution due to rain. While the pharmacokinetics of the drugs may largely contribute to the high concentration found in WWTP, discharge of WWTP effluents contributes greatly to concentrations found in river water. The concentrations of ARV drugs detected in soil were 15.4 - 138 $\mu g.g^{-1}$ while they were 22.8 – 98.9 $\mu g.g^{-1}$ in sediments.

Abacavir was found to be the dominant compound in water. This could be due to its low log K_{ow} and therefore, retain more in water samples, while efavirenz with high log K_{ow} was found to be the most dominant in solids with the highest concentration obtained in Camps Drift soil. The Amanzimtoti WWTP was identified to be the most highly contaminated plant. This could be due to contributions received by the plant from residential and vast majority of industrial waste.. The average removal efficiency of the abacavir, nevirapine and efavirenz was 85, 63, and 47%, respectively, which revealed that the processes used in WWTPs are efficient in removing abacavir which retains in water.

Levels of ARV drugs detected in water samples in this work are high and comparable to those reported in literature. This indicates the importance of monitoring and removing these

compounds in our waters. In addition, more analysis of these ARV drugs must be done to increase their database so that their allowable limits can be regulated.

5.2. Recommendations and Future work

- This study demonstrated that ARV drugs are partially eliminated during the process of wastewater treatment and therefore high concentrations are found in receiving surface waters. Therefore, the impact of these compounds on aquatic life needs to be further assessed.
- Continuous monitoring of ARV drugs in various matrices and cities of KwaZulu Natal to have a better understanding of the ARV pollution in the province.
- Expand analysis to other SA provinces to produce more data that can be used by policymakers to set MRL values for African countries and worldwide since there are no set limits currently.
- Initiate collaborative research study with Municipal companies e.g., Umgeni Water for the production of materials that can be employed in the wastewater treatment processes for complete removal of the ARV residues.
- Development of shorter extraction methods that are sensitive enough for the extraction and detection of ARV drugs in soil and sediments.
- Further analysis of fruits and vegetables to determine how much is being absorbed by the plants as slugde and wastewater are often used as manure and for irrigation purposes, respectivel

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CHAPTER 6

6.1. Paper emanated from this work

1. Ngwenya N.P., Mahlambi P.N. Determination of antiretroviral drugs in soil and sediment: Optimization and application of ultrasonic extraction and Sohxlet followed by solid phase extraction and liquid chromatography-photodiode array (Sent to Water Science and Engineering Journal)

6.2. Presentations

1. Ngweya N.P., Mahlambi P.N. SPE-LC-PDA method development and application for the determination of antiretroviral drugs in river water, wastewater and sludge. SACI/ChromSA Research Colloquium, 6th March 2019, Durban University of Technology. *Oral presentation.*

2. Ngweya N.P., Mahlambi P.N. SPE-LC-PDA method development and application for the determination of antiretroviral drugs in river water, wastewater and sludge. University of KwaZulu-Natal. College of Agriculture, Engineering and Science, Postgraduate research day October 2018, *Oral presentation.*

Appendix

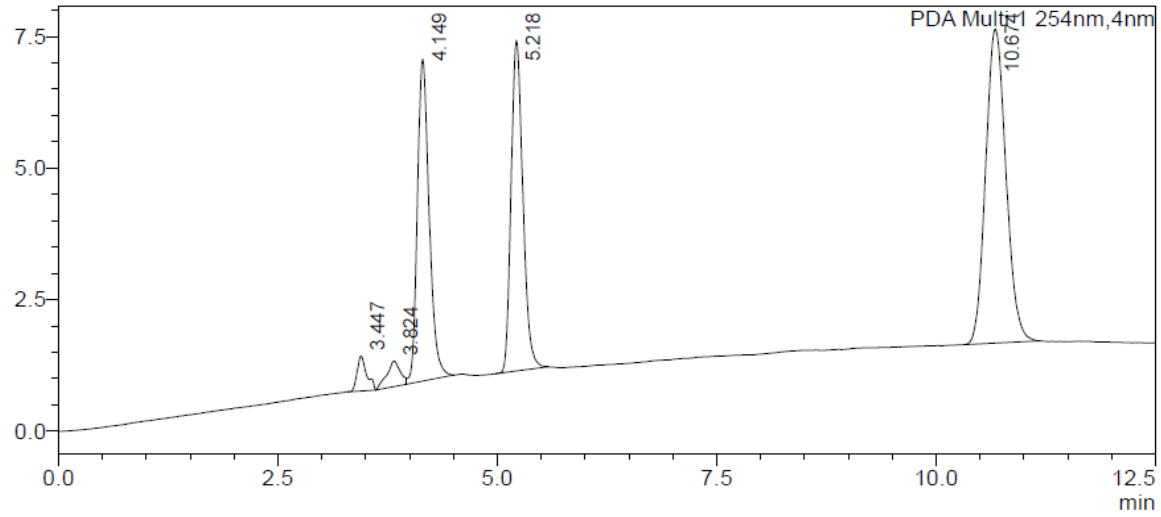

Figure A1: Typical chromatogram of 1ppm standard of ARV drugs

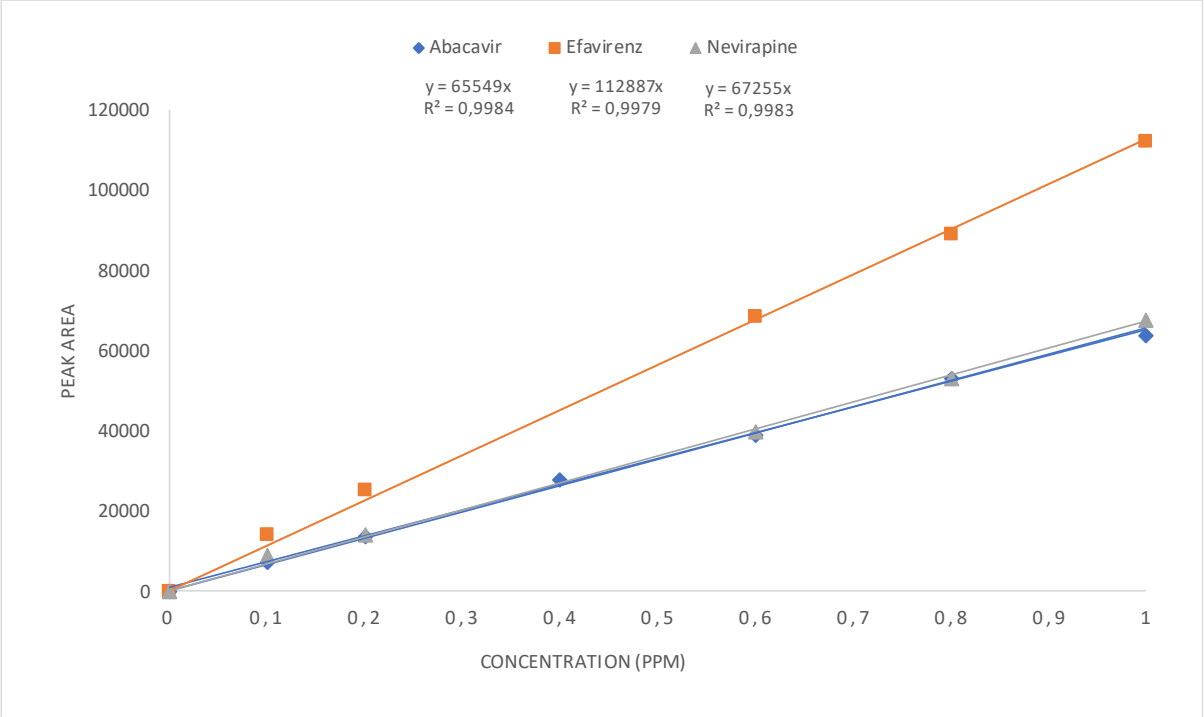

Figure A2: Calibration curve of the target ARV drugs

Table A1: Statistical analysis on the effect of extraction solvent on SPE

t-Test: Two-Sample Assuming Unequal Variances		
	50 mL	*100 mL*
Mean	84,6666667	84,6666667
Variance	145,333333	332,333333
Observations	3	3
Hypothesized Mean Difference	0	
df	3	
t Stat	0	
P(T<=t) one-tail	0,5	
t Critical one-tail	2,35336343	
P(T<=t) two-tail	1	
t Critical two-tail	3,18244631	

t-Test: Two-Sample Assuming Unequal Variances		
	50 mL	*200 mL*
Mean	84,66666667	74,33333333
Variance	145,3333333	250,3333333
Observations	3	3
Hypothesized Mean Difference	0	
df	4	
t Stat	0,899779997	
P(T<=t) one-tail	0,209554794	
t Critical one-tail	2,131846786	
P(T<=t) two-tail	0,419109588	
t Critical two-tail	2,776445105	

t-Test: Two-Sample Assuming Unequal Variances		
	100 mL	*200 mL*
Mean	84,66666667	74,33333333
Variance	332,3333333	250,3333333
Observations	3	3
Hypothesized Mean Difference	0	
df	4	
t Stat	0,741465554	
P(T<=t) one-tail	0,249791085	
t Critical one-tail	2,131846786	
P(T<=t) two-tail	0,49958217	
t Critical two-tail	2,776445105	

Table A2: Statistical analysis on the effect of extraction time on SPE

t-Test: Two-Sample Assuming Unequal Variances		
	10 microliter	*50 microliltre*
Mean	84,6666667	80
Variance	145,333333	91
Observations	3	3
Hypothesized Mean Difference	0	
df	4	
t Stat	0,52578104	
P(T<=t) one-tail	0,31342036	
t Critical one-tail	2,13184679	
P(T<=t) two-tail	0,62684072	
t Critical two-tail	2,77644511	

t-Test: Two-Sample Assuming Unequal Variances		
	10 microliter	*100 microlitre*
Mean	84,66666667	77
Variance	145,3333333	63
Observations	3	3
Hypothesized Mean Difference	0	
df	3	
t Stat	0,92	
P(T<=t) one-tail	0,212710829	
t Critical one-tail	2,353363435	
P(T<=t) two-tail	0,425421657	
t Critical two-tail	3,182446305	

t-Test: Two-Sample Assuming Unequal Variances		
	50 microlitre	*100 microlitre*
Mean	80	77
Variance	91	63
Observations	3	3
Hypothesized Mean Difference	0	
df	4	
t Stat	0,418717895	
P(T<=t) one-tail	0,348463956	
t Critical one-tail	2,131846786	
P(T<=t) two-tail	0,696927913	
t Critical two-tail	2,776445105	

Table A3: Physicochemical parameters of water samples collected along the Msunduzi River during spring and winter seasons

Season	**Site**	**Sampling Point**	**pH**	**Temp (°C)**	**Salinity (psu)**	**TDS (ppm)**	**Conductivity (µS)**	**D.O (mg/L)**
Spring Sampling	**Msunduzi River**	Camps Drift	7.4	11.8	0.11	124.5	248	17.45
		College Rd	8.0	14.6	0.11	117.9	235	16.76
		YMCA	6.5	13.3	0.11	119.8	241	15.93
		Wood House	7.7	18.6	0.10	110.4	220	12.04
		Bishopstowe	7.3	19.8	0.19	200	404	7.85
Winter Sampling	**Msunduzi River**	Camps Drift	8.1	18.2	0.18	191.3	188.1	14.82
		College Rd	8.2	18.9	0.08	81.9	194.5	15.76
		YMCA	8.1	17.9	0.09	88.6	189.8	13.48
		Wood- house	7.6	14.9	0.07	75.7	201	14.33
		Bishopstowe	7.5	18.4	0.15	159.7	351	13.62
	Darvill WWTP	Effluent	7.7	23.1	0.29	312	751	14.48
		Influent	7.3	24.5	0.37	387	773	12.63
		Digested Sludge	7.3	31.3	-	-	4.87	-

		Activated Sludge	7.4	22.3	-	-	758	-

Table A4: Physicochemical parameters of the water samples collected in Durban during spring season

Sampling point		**pH**	**Temp (°C)**	**Salinity (psu)**	**TDS (ppm)**	**Conductivity (µS)**	**DO (mg/L)**
Northern WWTP	Effluent	7.4	19.2	0.27	276	568	11.53
	Influent	7.9	18.2	0.41	450	899	14.09
	River	7.3	11.8	0.61	639	1276	18.26
	Sludge	6.0	11.8	0.44	466	932	16.48
Amanzimtoti WWTP	Effluent	8.3	11.6	0.53	566	1140	17.95
	Influent	7.4	11.4	0.76	829	1657	17.86
	River	7.7	11.2	1.48	1503	3.00	16.96
	Sludge	6.9	13.4	1.55	1551	3.10	12.23
Umhlathuzana WWTP	Effluent	7.2	10.1	0.27	298	597	19.02
	Influent (Marian ridge)	7.0	12.1	0.43	456	915	16.76
	Influent (Shallcross)	7.5	14.8	0.25	262	528	14.89
	River	7.7	11.0	0.21	225	451	18.26
Umbilo WWTP	Effluent	7.8	14.1	0.39	414	830	15.40
	Influent	7.2	14.0	0.44	468	935	15.15
	River	7.5	11.9	0.35	367	733	16.03

Table A5: Statistical analysis on the effect of extraction solvent on SE

t-Test: Two-Sample Assuming Unequal Variances

	Methanol:Acetonitrile	*Methanol*
Mean	50	61,3333333
Variance	139	10,3333333
Observations	3	3
Hypothesized Mean Difference	0	
df	2	
t Stat	-1,60634901	
P(T<=t) one-tail	0,124715832	
t Critical one-tail	2,91998558	
P(T<=t) two-tail	0,249431664	
t Critical two-tail	4,30265273	

t-Test: Two-Sample Assuming Unequal Variances

	Methanol:Acetonitrile	*Methanol:Acetone*
Mean	50	91,33333333
Variance	139	270,3333333
Observations	3	3
Hypothesized Mean Difference	0	
df	4	
t Stat	-3,538526902	
P(T<=t) one-tail	0,01202263	
t Critical one-tail	2,131846786	
P(T<=t) two-tail	0,02404526	
t Critical two-tail	2,776445105	

t-Test: Two-Sample Assuming Unequal Variances

	Methanol	*Methanol:Acetone*
Mean	61,33333333	91,33333333
Variance	10,33333333	270,3333333
Observations	3	3
Hypothesized Mean Difference	0	
df	2	
t Stat	-3,101604826	
P(T<=t) one-tail	0,045059824	
t Critical one-tail	2,91998558	
P(T<=t) two-tail	0,090119647	
t Critical two-tail	4,30265273	

Table A6: Statistical analysis on the effect of extraction solvent on UE

t-Test: Two-Sample Assuming Unequal Variances			t-Test: Two-Sample Assuming Unequal Variances			t-Test: Two-Sample Assuming Unequal Variances		
	Methanol:Acetonitrile	*Methanol*		*Methanol:Acetonitrile*	*Methanol:Acetone*		*Methanol*	*Methanol:Acetone*
Mean	51	64	Mean	51	83,33333333	Mean	64	83,33333333
Variance	1963	1483	Variance	1963	508,3333333	Variance	1483	508,3333333
Observations	3	3	Observations	3	3	Observations	3	3
Hypothesized Mean Difference	0		Hypothesized Mean Difference	0		Hypothesized Mean Difference	0	
df	4		df	3		df	3	
t Stat	-0,383571511		t Stat	-1,126536956		t Stat	-0,750404422	
P(T<=t) one-tail	0,360405753		P(T<=t) one-tail	0,170957096		P(T<=t) one-tail	0,253751896	
t Critical one-tail	2,131846786		t Critical one-tail	2,353363435		t Critical one-tail	2,353363435	
P(T<=t) two-tail	0,720811505		P(T<=t) two-tail	0,341914193		P(T<=t) two-tail	0,507503793	
t Critical two-tail	2,776445105		t Critical two-tail	3,182446305		t Critical two-tail	3,182446305	

Table A7: Statistical analysis on the effect of extraction time on UE

t-Test: Two-Sample Assuming Unequal Variances			t-Test: Two-Sample Assuming Unequal Variances			t-Test: Two-Sample Assuming Unequal Variances		
	15 minutes	*30 minutes*		*15 minutes*	*45 minutes*		*30 minutes*	*45 minutes*
Mean	83,33333333	75,33333333	Mean	83,33333333	49	Mean	75,33333333	49
Variance	508,3333333	581,333333	Variance	508,3333333	721	Variance	581,3333333	721
Observations	3	3	Observations	3	3	Observations	3	3
Hypothesized Mean Difference	0		Hypothesized Mean Difference	0		Hypothesized Mean Difference	0	
df	4		df	4		df	4	
t Stat	0,419762639		t Stat	1,696062174		t Stat	1,263878673	
P(T<=t) one-tail	0,348112111		P(T<=t) one-tail	0,082557612		P(T<=t) one-tail	0,13745535	
t Critical one-tail	2,131846786		t Critical one-tail	2,131846786		t Critical one-tail	2,131846786	
P(T<=t) two-tail	0,696224222		P(T<=t) two-tail	0,165115224		P(T<=t) two-tail	0,2749107	
t Critical two-tail	2,776445105		t Critical two-tail	2,776445105		t Critical two-tail	2,776445105	

Table A8: LOD, LOQ and recovery statistical analysis for UE and SE methods

t-Test: Two-Sample Assuming Unequal Variances			t-Test: Two-Sample Assuming Unequal Variances			t-Test: Two-Sample Assuming Unequal Variances		
	SE LOD	*UE LOD*		*SE LOQ*	*UE LOQ*		*SE RECOVERY*	*UE RECOVERY*
Mean	0,8333333	1,866667	Mean	2,5	5,7	Mean	91,66666667	83,33333333
Variance	0,0033333	0,143333	Variance	0,07	1,29	Variance	220,3333333	464,3333333
Observations	3	3	Observations	3	3	Observations	3	3
Hypothesized Mean Difference	0		Hypothesized Mean Difference	0		Hypothesized Mean Difference	0	
df	2		df	2		df	4	
t Stat	-4,673426		t Stat	-4,75271		t Stat	0,551619727	
P(T<=t) one-tail	0,0214317		P(T<=t) one-tail	0,020766		P(T<=t) one-tail	0,305287354	
t Critical one-tail	2,9199856		t Critical one-tail	2,919986		t Critical one-tail	2,131846786	
P(T<=t) two-tail	0,0428633		P(T<=t) two-tail	0,041532		P(T<=t) two-tail	0,610574708	
t Critical two-tail	4,3026527		t Critical two-tail	4,302653		t Critical two-tail	2,776445105	