EFFECT DIRECTED ANALYSIS OF EMERGING ORGANIC CONTAMINANTS (ESTRADIOL AND PROGESTERONE) IN MUKUVISI RIVER SEDIMENTS

BY

DOREEN SABONDO B0823403

SUPERVISOR MR MUSEKIWA

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ABSTRACT

Effect Directed Analysis; a method which combines biological analysis, physicochemical manipulation, concentration steps together with chemical analysis was used to separate and quantitate trace concentration of two steroids in Mukuvisi River sediments. Steroidal hormones are a class of emerging organic contaminants and the analysis was in particular focused on estradiol and progesterone. During the pre-treatment stage the target analytes were extracted from the matrix using solid liquid extraction with a buffer solution. Clean up of the extracts was achieved using two steps of solid phase extraction. Sep pak C18 cartridges were the sorbent of choice in the first clean up step and further clean up was achieved using silica based cartridges. An ELISA (enzyme-linked immunoassay) was performed before pre-concentration and after concentration with a limit of detection (LOD) of 10pg/ml for both progesterone and estradiol kits, in order to determine the fraction showing toxic effects for further analysis. The concentrated extracts were analysed using an HPLC-DAD system.

The extracts that were not purified and concentrated resulted in matrix interferences of the two steroids and the result was that the analytes were completely suppressed in the sediment. Therefore when the ELISA test was performed on these samples, the concentrations were below the limit of detection. After simultaneous purification and concentration by SPE the estradiol concentration was determined to be between 0.072 and 0.013 ng/ml in two different samples. Progesterone was not detected in any of the samples. HPLC was used to quantify and confirm these results. One sample from Matapi gave a concentration of 0.156 ng/ml estradiol on a DAD chromatogram. In the three other samples the concentration was below the LOD. Progesterone was not detected in any of the samples.

The estradiol concentration in Mukuvisi River sediments indicate that there is a risk of endocrine disrupting effects from exposure to estradiol as it is able to cause reproductive
malfunctions at ng/g levels. The maximum residue limit for estradiol in the environment is 0.25 ng/L. The results of progesterone analysis indicate that the concentrations in the environment are below the levels that can cause toxic effects to the people and the environment.
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAD</td>
<td>Diode Array Detector</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunoassay</td>
</tr>
<tr>
<td>E1</td>
<td>Estriol</td>
</tr>
<tr>
<td>E2</td>
<td>Estradiol</td>
</tr>
<tr>
<td>EE2</td>
<td>Ethynylestradiol</td>
</tr>
<tr>
<td>LC MS/MS</td>
<td>High Performance Liquid Chromatography- tandem Mass Spectrometry</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of Detection</td>
</tr>
<tr>
<td>MASE</td>
<td>Microwave Assisted Solid Phase Extraction</td>
</tr>
<tr>
<td>ND</td>
<td>Not Detected</td>
</tr>
<tr>
<td>Pg</td>
<td>Progesterone</td>
</tr>
<tr>
<td>T</td>
<td>Testosterone</td>
</tr>
<tr>
<td>WWTP</td>
<td>Wastewater Treatment Plants</td>
</tr>
</tbody>
</table>
CHAPTER ONE

INTRODUCTION

1.0 Background

Contamination of the environment by toxic substances has been associated with society since the beginning of industrialisation (Blasco and Pico, 2009). Recently there has been a growing concern over the rise in the contamination of the environment by emerging organic contaminants. Contaminants are substances that have not been intentionally added to the environment in instances as a consequence of industrial development, pollution or climate change. The term “emerging contaminants” is used to describe toxicants of concern based on their environmental persistence and the potential for harmful effects rather than “newly introduced” substances (Shareef, Kookana and Kumar, 2008). For example; a substance
present in the environment for many decades may become an “emerging contaminant” due to advances in analytical methodologies and ecotoxicological studies. Included are chemicals such as detergents, pharmaceuticals as well as natural and synthetic hormones. Steroid hormones (C18 chemical messengers derived from the triterpenoid lanosterol) are a group of emerging toxicants. Two steroid hormones under study are estradiol and progesterone.

In line with the increase in the contamination of the environment by these emerging organic toxicants, several methods have been developed with the aim of minimising their negative effects. Effect directed analysis is a method developed for the analysis of emerging organic contaminants in the environment. This method combines biological and chemical analysis with physicochemical manipulation and concentration techniques (Brack et al, 2008) to identify toxicants in environmental samples. Bio analytical tools and concentration techniques are used in the identification process of the fraction which shows toxic effects. Repetition of this process reduces the sample complexity and chemical analysis is then carried out followed by quantification and or confirmation.

1.1 Statement of the problem

Research on the pharmacokinetic activity of steroidal hormones has shown that they cannot be totally metabolised by physiological processes in humans. The table below indicates the amounts of active free hormones eliminated from the systems of different species per day.

Table 1a: Daily Excretion of hormones by humans and animals (Blasco and Pico, 2009)

<table>
<thead>
<tr>
<th>Species</th>
<th>Category</th>
<th>Urine excretion (µg day⁻¹)</th>
<th>Hormones  excreted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Humans</td>
<td>• Cycling Female</td>
<td>16-35</td>
<td>E1,E2,E3,EE2</td>
</tr>
<tr>
<td></td>
<td>• Pregnant Female</td>
<td>5270-7030</td>
<td>E1,E2,E3,Pg</td>
</tr>
</tbody>
</table>
The main route of excretion of hormones from human and pigs is via urine. Excretion via faeces forms another route of excretion largely in cattle and sheep and a small fraction in humans. The hormones are conjugated to glucuronide or sulphate moieties. After excretion they undergo deconjugation resulting in free active hormones.

![Fig 1a: Pathways through which synthetic and natural estrogenic hormones can reach the environment (adapted from Kozlick et al, 2010)](image-url)
Once these steroid hormones have reached the environment they are transferred back to humans, aquatic life and animal (both wild and domesticated) through drinking water and the food chain. It becomes impossible to control this intake of hormones or the negative effects to the target species.

Hormones are the most potent endocrine disruptors even at nanogram per litre levels. The presence of the estrogenic hormone, estradiol (E2), and the progestin, progesterone in aquatic environments even at low levels (0, 1-10 ng/L) has been linked with different steroidal effects in aquatic species (Eljarrat, Cruz and Farre, 2009). This is because they are able to interact with the endocrine system. As such they interfere with reproductive, growth and development systems in both humans and animals. Some associated changes that have been slowly creeping into the wild fish populations include reduction in fertility, changes in sex ratio and imposex (alteration of sexual development) incidence and inducing feminisation.

The increase in the incidence of breast and testicular cancer as well as the decrease in the quality and quantity of sperm in humans has also been attributed to exposure to estrogens from the environment (Brack et al, 2008). As much as estrogenic hormones are linked to escalating incidences of prostate cancer they are also attributed to causing girls to reach puberty earlier than they used to (Blasco and Pico, 2009). The effects in humans are however difficult to assess due to the presence of a wide range of contaminants in the environment.

Experiments conducted have reported positive results on the effects of estrogenic hormones on other phyla. When the mollusc Potamopyrgus antipodarum was exposed to as little as 1 ng/L of estradiol, it showed an increase in embryo production (Racquet, 2012). DNA damage was also reported in such species. Evidence also exists on the negative effects of steroids in plants irrigated with waste water containing between 10-300 ng/L of estradiol.

1.2 Significance of Study
The accurate measurement of steroidal hormone concentrations in sediments is fundamental to a number of investigations, e.g. for estimating their role in the removal of steroidal estrogens from the water column (Chen and Wu, 2012), for determining their persistence and risk of permeation to ground waters and to determine the risk to benthic organisms from exposure to estrogens (Eljarrat et al, 2009). Furthermore, in case of sediment resuspension and hormone remobilization, sediments may act as a secondary source of exposure to aquatic organisms living in the water column (Chen and Wu, 2012).

1.3 Aim
To analyse sediment samples for the presence of estradiol and progesterone using Effect Directed Analysis.

1.4 Objectives

- To identify the toxic hotspots for natural hormones (steroids)
- To extract steroids from sediments
- To characterise hormones according to their biological effects.
- To quantify the steroids by chemical analysis
- To identify the ecological risk of the identified species.

1.5 Limitations
As discussed by Brack et al, the final step of toxicant identification and confirmation is far from straight forward. This step is one of the major challenges in undertaking a successful Effect Directed Analysis study. A tiered approach is employed where information from each tier contributes to accumulating evidence producing the overall result instead of a simple yes/no.

Due to the very low concentrations of oestrogenic hormones, the analytical information is restricted only to the LC analysis. Additional analysis e.g. NMR cannot be achieved due to
low sample concentrations and the high purity required for this analysis. Therefore as much information as possible is extracted from the chromatogram.

CHAPTER TWO

LITERATURE REVIEW

2.0 Effect Directed Analysis

Classical chemical analysis of environmental samples has been indicated as not sufficiently covering unintentionally produced chemicals (Blasco and Pico 2009), otherwise known as by products or metabolites which have the potential to cause negative effects even at low concentrations. In addition, in cases where sampling is covering a rather wide area the method becomes unfeasible. This is when you take into consideration the whole cost of
chemical analysis including labour and transport. As a result the method called effect directed analysis is used.

Effect directed analysis is a method where biological and chemical analyses are combined together with physicochemical manipulation and fractionation techniques with the aim of identifying toxic components in a complex environmental sample (Schymanski et al., 2008). In such scenarios, the concentration of key toxicants will be much lower than other components in the sample, usually with high matrix sample. As a result of this complexity, a combination of biological tests and physicochemical concentration procedures are used to identify those fractions showing toxic effects for further investigation (Schymanski et al., 2008). In order to sufficiently reduce sample complexity, this process is repeated. This allows for chemical analysis, quantification and confirmation that the identified compound is actually responsible for the toxic effects observed to be carried out. The detailed stages of this process are as follows:

**Toxicity Characterization**

- Characterisation of the toxicity of bulk samples with biological tests.

**Toxicity Identification**

- Identification of possible toxicants suspected for measured effects by combining chemical extraction, concentration, bioassays and chemical analysis.

**Toxicity Confirmation**

- Entails qualitative and quantitative confirmation of the toxin.

This is diagrammatically represented below:
2.1 Emerging Organic Contaminants

At the 4th Norman workshop, emerging organic contaminants were defined as substances that are not included in routine monitoring programs at continental level but are subject to regulation in the future in line with research findings on eco-toxicity, potential health effects and public perception (Brack et al., 2008). Among the large variety of compounds the following are included: pharmaceutical compounds, natural and synthetic hormones, illicit drugs, UV filters as well as detergents. These toxicants are of anthropogenic origin. They are consumer by products and are used daily in the home, agricultural lands and industry. There is however little information on the point sources (Tilghman et al., 2008) of these chemicals as well as their fate processes at a local level.

2.1.1 Hormones

Steroid sex hormones such as estrogens and progestin constitute a group of environmental emerging toxicants because of their endocrine disrupting properties (Eljarrat et al., 2009). They are chemical messengers naturally produced by the endocrine system of humans and animals. They are also known as C18 steroids as they are made up of 18 C atoms which are
distributed in the 3 hexagonal (A-C) and 1 pentagonal (D) rings (Racquet, 2012). It is the
different substitution in the cyclopentane-phenanthrene nucleus which differentiates these
molecules. These hormones find their application in human medicine as well as in animal
farming. Two hormones under study include progesterone and estradiol.

2.1.1.1 Estradiol

Estradiol (E2) is a C18 steroid hormone with a phenolic A ring. This steroid hormone has a
molecular weight of 272.4. Synthesized in the ovaries from testosterone, estradiol is
responsible for the development of female secondary sex characteristics and for regulation of
the menstrual cycle.

It is the most potent natural estrogen, produced mainly by the ovary, placenta, and in smaller
amounts by the adrenal cortex, and the male testes. The main metabolites of estradiol are
estriol and estrone. Estradiol is known to exhibit very potent estrogenic activity at a very low
concentration (~10⁻⁹ M, in vitro) (Racquet, 2012). The diagram below is an illustration of the
chemical structure of the natural estrogen estradiol. Estradiol or its metabolites play an
important role in human carcinogenesis, such as breast, prostate, and ovarian cancer (Zhao,
Guan and Liu, 2012). In addition, for the purpose of fattening animals, large amounts of
estrogens are illegally used hence the recent trend in the development of interest in estrogens.

![Estradiol structure](image)

Fig 2.1 (a) Estradiol (Adapted from Zhao et al., 2012)

2.1.1.2 Progesterone
Progesterone is one of the most important progestin. It is essential for preparing the uterus for implantation of a fertilized ovum during pregnancy.

**Fig 2.1(b) Progesterone (Barceló et al., 2006)**

Progesterone is excreted in the urine of mammals and also in faeces. It enters waterways through the effluent of sewage treatment plants or through run off of sewage sludge which is mainly used for agriculture. This sequence of events may lead to reproductive and developmental alterations in aquatic organisms such as feminisation and hermaphroditism (Barceló et al., 2006)

### 2.1.1.3 Pharmacokinetics

In humans and animals, hormones undergo many transformations and this usually occurs in the liver. The processes include oxidation, hydroxylation, deoxygenation and methylation. Thereafter they become conjugated to glucuronide and/or sulfate moieties, forming more polar conjugated forms (Eljarrat et al., 2009). Estradiol is rapidly oxidized to estrone, which can be further converted into estriol, the major excretion product. Many other polar metabolites like 16-hydroxy-estrone, 16-ketoestrone or 16-epiestriol are formed and can be present in urine and faeces. Therefore, estrogens are excreted mainly as inactive conjugates of sulphuric and glucuronic acids. Although steroid conjugates do not possess a direct biological activity, they can act as precursor hormone reservoirs able to be reconverted to free
steroids by bacteria in the environment. Progesterone is metabolised into allopregnanolone and pregnanolone (Andreen et al., 2006) and can be converted to the free form after excretion

2.1.2 Occurrence of steroids in the environment

When they complete their action in humans/animals the hormones are either excreted in conjugated form through urine or in free form via faeces. Hence they consequently enter the environment in active forms. In addition hormones show moderate affinity with solids as shown by their high hydrophobic properties (log octanol-water partition coefficient 3-5) (Eljarrat et al., 2009). This means that these chemicals will be sorbed to sludge, sediments and the particulate matter in sewage.

The major point of collection of human excretion is the sewers. Animal hormones however are generally found in manure. Eventually these hormones will find their way into the environment through wastewater treatment plants, direct excretion into the fields or spreading of manure. The fate of these hormones is dependent on the physicochemical properties of the hormones as well as the conditions prevailing in the land as well as microbial activity.

2.1.3 Fate of steroids in the environment

2.1.3.1 Sorption

Sorption is the process by which chemicals become associated with the solid phases. Included is the process of adsorption and absorption. By definition, absorption occurs when components penetrate into a three dimensional surface whereas in adsorption, the molecules only associate themselves with a two dimensional matrix.

The major factors determining the presence and distribution of steroids in sediments is a combination of their physicochemical properties and site-specific environmental conditions.
Studies have shown that sorption of estrogens in the soil and sediment of aquatic environments is moderate to high with typical sorption coefficients ($K_d$) ranging from 30 to 123 L/Kg for E2 (Eljarrat et al., 2009). The behaviour of steroids has been shown to be non-linear in soil and sediment and is modelled using the Freundlich isotherm with $\log K_f = 1.71$ (Chowdhury, 2010).

The sorption of steroids correlates with the presence of organic carbon content and increase with salinity in water (Lai et al., 2000).

Because both progesterone and estradiol are hydrophobic, they exhibit an affinity towards humic substances. In aquatic environments, higher ionic strength solutions lead to greater sorption and higher salt concentrations enhance particle aggregation and flocculation. All these factors combined, favour the sedimentation of steroids out of the water column.

2.1.3.2 Degradation

Degradation of steroids occurs through biotic and abiotic pathways. The degradation of E2 to E1 occurs under anaerobic conditions (Holthaus et al., 2002). The degradation is very slow under anaerobic conditions in marine and water samples.

Degradation of progesterone also occurs through direct photolysis. Progesterone is a chromophore due to the presence of carbonyl groups. As a result direct photolysis occurs by photon absorption by the carbonyl groups, resulting in Pg being excited to its singlet state, and undergoing chemical transformation to generate one or more product species. Since Pg absorbs in the UV visible range (290-750 nm) it is prone to direct photolysis (Eljarrat et al., 2009). However photodegradation is affected by sediment loading and high algal content in aquatic systems and presence of humic substances which can absorb or attenuate light. These factors reduce the rate of photodegradation of progesterone in aquatic systems.

In urban wastewater treatment plants, there is efficient removal of steroids from the liquid phase. However due to their lipophilic properties, the steroids tend to be adsorbed onto the
solid phase which is the sludge. Previous work on anaerobic reactors treating sludge concluded that hormones were poorly eliminated or not at all in methanogenic conditions. E2 is transformed into E1 which is further partially removed (17%). Similarly, in experiments using methanogenic reactors with sludge spiked with hormones, E1 was reduced to E2 which was not further degraded (Eljarrat et al., 2009). Therefore the sorbed steroids are not degraded and are persistent in the environment. In Zimbabwe these sewage sludge which contain hormone steroids are often applied to agricultural land. In Harare there are five farms that are used for the deposition of sewage sludge and include the Ingwe farm, Pension Farm and Mufakose Pasteurised land with a total 15000 ha of land. Manure from animals also contain steroids and are applied directly on to land for agricultural use.

### 2.1.4 Endocrine Disrupting Effects

Anthropogenic sources of natural hormones result in concentrations that can cause endocrine disrupting effects even at ng L\(^{-1}\) levels. Natural hormones present the greatest potency, and exposure to certain concentrations can cause disruption to endocrine systems that control development in aquatic organisms and wildlife (Damstra et al., 2002).

For example, the exposure of fish to 2 ng L\(^{-1}\) concentrations of E2 under laboratory conditions has been shown to induce measurable reproductive effects (Snyder et al., 2003). In addition the effects of these hormones has been shown to be additive. Exposure to such trace levels can harm early life stages of fish populations, including embryonic or larval development and these effects can only be detected in adulthood or in subsequent offspring. Other effects investigated include increased sex reversal, reduced hatch/birth rate, and disrupted breeding behaviour in wildlife.

In humans however it has been difficult to assess the effects of these ECDs in the reproductive systems. However the exposure to natural hormones in the environment has
been linked to decrease in sperm count and ejaculate volume, increase in testicular and breast cancer, and increase in deformities of the genital organs in humans (Sharpe, 2001).

2.2 Methods for analysis hormones in sediments

Chemical analysis of sediment samples generally requires both extraction and clean up steps. The methods used are usually the same regardless of whether the analytical quantification and identification of the steroidal compounds is based on liquid or gas chromatography (Streck, 2009). For solid matrices extraction is usually carried out by solvent extraction assisted with ultrasonication.

2.2.1 Sample preparation

The objective of sample preparation is to convert a matrix into a sample suitable for analysis. Applicability of different sample preparation techniques and analytical methodologies is dependent upon the chemical and physical properties of both the analyte and the matrix. This extends to the efficacy and reproducibility of the methods. Therefore physicochemical characterisation of a sample should be a prior step to further sample preparation. Sample preparation should also be designed with respect to the instrumentation to be used, degree of accuracy desired and whether qualitative or quantitative analysis is to be achieved. Different methods can be used in relation to the above factors and below are some that are applicable to the analysis of steroids in sediments.

2.2.1.1 Solid Phase Extraction

Analytes to be extracted are distributed between the solid phase and a liquid phase. The analyte should have a higher affinity for the solid phase than for the sample matrix. A critical point is the selection of the sorbent. The interaction of the sorbent and the functional groups of the analyte and the kind of sample matrix have an effect on selectivity, affinity and
capacity of the sorbent. Examples of sorbets include chemically-bonded silica with the C8 or C18 organic group among others, carbon or ion-exchange materials, polymeric materials and immunosorbents.

Hajkova et al., 2010, tested three different SPE adsorbents: ENVI-Carb, Supelclean LC-18, and Oasis HLB and different elution solvents for cleanup of sediment extracts in 10% acetonitrile-water. While elution of the target compounds failed with the ENVI-Carb cartridge under the chosen conditions, Oasis HLB, in combination with an acetonitrile-1% ammonium hydroxide mixture (95:5, v/v), achieved the best recoveries as well as good repeatability.

2.2.1.2 Solid Phase Micro-extraction

SPME is a modern sample-preparation method used for isolating and pre-concentrating organic molecules from gaseous, liquid and solid samples. It is highly sensitive and can be used for polar and non-polar analytes with different types of matrix. The mechanism of SPME is similar to that of SPE because SPME is a miniature version of SPE, the only difference being the volume of sorbent (Pavlovic et al., 2007). This technique of sample preparation can be used for samples to be analysed with gas chromatography or HPLC. The sorbent used is a short piece of a fused silica fiber coated with a polymeric stationary phase placed on a syringe.

2.2.1.3 Ultrasonic Extraction

This method can be used for the extraction of hormones and other pharmaceuticals. It uses mechanical energy in the form of a shearing action. The sample with added solvent is immersed in an ultrasonic bath and subjected to ultrasonic radiation for few minutes. Extracted analytes are separated from the matrix by vacuum filtration or centrifugation. The process is repeated two or three times to achieve higher extraction efficiency and the extracts are combined for analysis (Pavlovic et al., 2007).
2.2.1.4 Microwave Assisted Solvent Extraction (MASE)

In MASE a solid sample-solvent mixture is heated in closed vessel with microwave energy under temperature-controlled and pressure-controlled conditions. This closed extraction system enables analyte extraction with elevated temperatures and pressure accelerating the extraction process (Pavlovic et al., 2007). Polar solvents are used as they are the only type of solvents that can absorb microwave energy.

After the heating, the samples are cooled and filtered in order to separate the extract for analysis. Labadie and Hill, 2006, employed this method of sample preparation for the analysis of steroids in river sediments by liquid chromatography–electrospray ionisation mass spectrometry and determined levels of E2 of 0.40 ng/g.

2.2.2 Analytical Methodologies

2.2.2.1 GC-MS

GC-MS achieves high separation and has a greater identification capacity of the analyte. Derivatization of hydroxyl or carbonyl groups is advisable in order to achieve good chromatographic separation of the analytes, since that increases thermal stability and volatility of the compounds, while reducing their polarity due to decreased dipole-dipole interactions. Silylation techniques have been applied most often, and that generally results in more favourable mass-fragmentation patterns (Streck, 2009).

2.2.2.2 LC-MS/MS

This method has the lowest limit of detection and is also superior in terms of selectivity. It has the advantage of avoiding false positives when analysing complex matrices. Estrogenic hormones are often analysed in negative ionisation mode and androgens and progestagens in positive ionic mode.
Compared with GC-based methods, LC methods have the advantage of avoiding derivatization step before analysis, thus eliminating a potential source of analytical errors (Streck, 2009). With respect to LODs, GC-MS methods are generally somewhat less sensitive than LC-MS/MS techniques.

CHAPTER THREE

RESEARCH METHODOLOGY

Materials
Estrogen Standard (purity >99%)

Ethynylestradiol (purity>98%)

Estrogen solutions (stock solution:1mg/ml, working solution 0.1µg/L) prepared in methanol and stored at -20°C.

Ultrapure water

Butylated hydroxytoulene (BHT>99%)
Sodium iodide
Sodium acetate >99%
Sulfamethoxine >98%
Acetonitrile
Methanol
Formic Acid

**Instruments**

HPLC Agilent Technologies 1200 Infinity Series, Quaternary Pump with UV/VIS Diode Array Detector
pH meter - Cyber Scan
Centrifuge tube filters (nylon membrane pore size 0.22µm)
Analytical Balance - Sartorius
Estradiol ELISA test kit - Ridascreen 17β Estradiol Assay
Progesterone ELISA test kit - Ridascreen progesterone assay

**3.0 Sampling Site**

Collection of samples was done at three locations in the Mukuvisi River catchment. From each location four samples were collected. The first site is located in Harare CBD along Seke Road. The second site, a further 5km downstream, is located along Magaba industrial area. The 3rd site is along Mbare residential area adjacent to Matapi Flats where urban wastewater represents a significant input to the river water.

The last site is located downstream of the Mufakose pasteurised farm where sewage sludge from wastewater treatment plants is abandoned.

**3.1 Sample Collection**
Surface samples (0-10 cm) were collected using a grab sampler into clean methanol rinsed plastic containers (Streck et al., 2009). The collected sediment samples were be packed in cool boxes with ice (< 4°C) and transported to the laboratory to freeze at -20°C.

3.2 Preparation of Standards

3.2.1 Stock Standards (400 µg/ml)

0.25 g of estradiol/ progesterone was dissolved in 100 ml methanol in a volumetric flask. The stock solution was stored in a refrigerator at 4°C.

3.2.2 Intermediate Mixed Standard (1 µg/ml)

0.25 ml of the stock standard was taken into a 100 ml volumetric flask and diluted to the mark with methanol. This solution was stored at 4°C.

3.3 Sample Preparation

3.3.1 Extraction

10 g of the sample was weighed into a Petri dish. 10 ml of acetonitrile was added into the sample. The mixture was vortexed for 30 seconds. The combined salts (4 g MgSO4 and 1 g NaCl) were added and shaken for a minute. The mixture was centrifuged at 3500 rpm for 12 minutes at 4 °C. The supernatant was decanted and evaporated to 20 mL under nitrogen.

3.3.2 Sample Purification

- Sediment samples were purified on silica Sep-Pak cartridges. The cartridges were prepared by conditioning them with 5 mL each of ethyl acetate, methanol and water in sequence. A syringe of air was pushed through it to dry afterwards. The sediment extract was transferred to the silica Sep- Pak and allowed to dry. The cartridge was
washed with 4 mL of methanol/pH 7.0 acetate buffer (4:6, v/v) and dried under vacuum for 30 minutes. The natural hormones were eluted with 7 mL of ethyl acetate. The eluates were evaporated under vacuum and reconstituted in 500 µL of cyclohexane/ethyl acetate (9:1, v/v) (Labadie and Hill, 2006)

3.4 Biological Analysis

3.4.1 ELISA test

3.4.1.1 PREPARATION OF SAMPLES

1mL sample was extracted with 5 mL tert butyl methyl ether in plastic universal containers by shaking thoroughly at room temperature for 20 minutes.

<table>
<thead>
<tr>
<th>Spike ID</th>
<th>Spike Level Expected</th>
<th>Amount of 3200 ppt of Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spike 1</td>
<td>80 ppt</td>
<td>25 µL</td>
</tr>
<tr>
<td>Spike 2</td>
<td>40 ppt</td>
<td>12.5 µL</td>
</tr>
<tr>
<td>Spike 3</td>
<td>20.5 ppt</td>
<td>6.5 µL</td>
</tr>
</tbody>
</table>

The sample was left to settle for 20 min. 3 mL of the ether supernatant was pippeted into a bijou bottle and evaporated at 60 °C in a water bath. The dried residue was redissolved in 400
µL sample dilution buffer and mix thoroughly by swirling. 20 µL each was used per well in the assay

**3.2.1.2 TEST PROCEDURE**

The standard and sample positions were recorded as follows (Ridascreen, 2013)

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>S1</td>
<td>S1</td>
<td>S1</td>
<td>1</td>
<td>1</td>
<td>9</td>
<td>9</td>
<td>17</td>
<td>17</td>
<td>25</td>
<td>25</td>
<td>33</td>
</tr>
<tr>
<td>B</td>
<td>S2</td>
<td>S2</td>
<td>S2</td>
<td>2</td>
<td>2</td>
<td>10</td>
<td>10</td>
<td>18</td>
<td>18</td>
<td>26</td>
<td>26</td>
<td>33</td>
</tr>
<tr>
<td>C</td>
<td>S3</td>
<td>S3</td>
<td>S3</td>
<td>3</td>
<td>3</td>
<td>11</td>
<td>11</td>
<td>19</td>
<td>19</td>
<td>27</td>
<td>27</td>
<td>34</td>
</tr>
<tr>
<td>D</td>
<td>S4</td>
<td>S4</td>
<td>S4</td>
<td>4</td>
<td>4</td>
<td>12</td>
<td>12</td>
<td>20</td>
<td>20</td>
<td>28</td>
<td>28</td>
<td>34</td>
</tr>
<tr>
<td>E</td>
<td>S5</td>
<td>S5</td>
<td>S5</td>
<td>5</td>
<td>5</td>
<td>13</td>
<td>13</td>
<td>21</td>
<td>21</td>
<td>29</td>
<td>29</td>
<td>35</td>
</tr>
<tr>
<td>F</td>
<td>S6</td>
<td>S6</td>
<td>S6</td>
<td>6</td>
<td>6</td>
<td>14</td>
<td>14</td>
<td>22</td>
<td>22</td>
<td>30</td>
<td>30</td>
<td>35</td>
</tr>
<tr>
<td>G</td>
<td>37</td>
<td>38</td>
<td>39</td>
<td>7</td>
<td>7</td>
<td>15</td>
<td>15</td>
<td>23</td>
<td>23</td>
<td>31</td>
<td>31</td>
<td>36</td>
</tr>
</tbody>
</table>

50 µL of diluted enzyme conjugate was added to the bottom of each well. 20 µL of standard was added to triplicate wells or prepared sample to duplicate wells respectively. 50 µL of the diluted 17B-estradiol antibody was added to each well. It was mixed thoroughly and incubated for 2 hours at room temperature. The liquid was poured out of the wells and the micro well holder tapped three times against absorbent paper to ensure complete removal of liquid from the wells. All the wells were filled with 200 µL distilled water and the liquid poured out as in step 5. The step was repeated 2 more times. 50 µL of substrate and 50 µL of chromogen were added to each well and mixed thoroughly and incubated for 30 min at room
temperature in the dark. 100 µL of stop solution was added to each well and mixed well and the absorbance measured at 450 nm against an air blank. It was read within 60 minutes.

3.4 Solid phase extraction

Further cleanup of these extracts was achieved using silica gel cartridges (500 mg, Sigma–Aldrich). 0.5g of anhydrous sodium sulphate was packed on top of the silica cartridge. The cartridge was preconditioned in sequence with 4 mL of cyclohexane/ethyl acetate (6:4, v/v) and 4 mL of cyclohexane. The cartridge was not allowed to dry at this point. The sample was loaded on to the cartridges and washed with 4mL of cyclohexane. The estradiol and progesterone were eluted with 6 mL of cyclohexane/ethyl acetate (6:4, v/v). These extracts were dried under vacuum and reconstituted in 60 µL water/acetonitrile (7:3, v/v). The extracts were passed through 0.22 µm centrifuge filters. Filters were further rinsed with 20 µL of water/acetonitrile (7:3, v/v). The extracts were stored at -20°C until further analysis (Chen et al., 2012).

3.6 HPLC Analysis

The samples were analysed using an LC-DAD with the conditions below (Agilent Technologies).

**HPLC Conditions**

- **Column:** C-18 reversed-phase HPLC column, 25 cm×4.6 mm, 5 µm
- **Injection Volume:** 20 µL
- **Flow rate:** 0.3 mL/min
- **Column temperature:** 30 °C
- **Mobile Phase:** 30% H₂O, 40% CH₃OH and 30% ACN & 0.1%formic acid in each of the solvents.
- **Detector:** DAD
CHAPTER FOUR

RESULTS

4.0 Bioanalysis

4.0.1 Estradiol Calibration Curve

ELISA was used for the qualitative determination of estradiol in sediments.
The following absorbance readings were observed for the respective estradiol concentrations and the mean absorbance was used to draw up the calibration curve.

Table 3.0: Absorbance of estradiol standards

<table>
<thead>
<tr>
<th>Concentration (pg/ml)</th>
<th>Absorbance</th>
<th>Mean Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1. 2.401</td>
<td>2.450 ±0.076</td>
</tr>
<tr>
<td></td>
<td>2. 2.411</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3. 2.539</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>1. 1.883</td>
<td>1.886 ±0.029</td>
</tr>
<tr>
<td></td>
<td>1.</td>
<td>2.</td>
</tr>
<tr>
<td>-----</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>30</td>
<td>1.868</td>
<td>1.793</td>
</tr>
<tr>
<td>100</td>
<td>1.437</td>
<td>1.522</td>
</tr>
<tr>
<td>300</td>
<td>0.822</td>
<td>0.819</td>
</tr>
<tr>
<td>1000</td>
<td>0.436</td>
<td>0.411</td>
</tr>
</tbody>
</table>

As shown in Fig 4.0.1 below the standard curve for estradiol was presented in the ranges 10 to 1000 pg/ml.
Fig 4.0.1(a): Standard Curve for Estradiol ELISA test

The logarithm of concentration gave a well-fitted linear regression: \(Y = -0.888x \times 3.099\), \(R^2 = 0.991\), \(n=4\).
Fig 4.0.1(b): Logarithm of standard curve

4.0.2 Progesterone Calibration Curve

Concentration of standards ranged from 0.4-40 pg/ml as shown in the calibration curve below:
<table>
<thead>
<tr>
<th>Concentration (pg/ml)</th>
<th>Absorbance Readings</th>
<th>Mean Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4</td>
<td>1.055 1.055 1.109</td>
<td>1.073 ±0.031</td>
</tr>
<tr>
<td>1.0</td>
<td>0.834 0.911 0.822</td>
<td>0.855 ±0.048</td>
</tr>
<tr>
<td>2.0</td>
<td>0.668 0.639 0.655</td>
<td>0.654 ±0.014</td>
</tr>
<tr>
<td>4.0</td>
<td>0.538 0.491 0.536</td>
<td>0.522 ±0.026</td>
</tr>
<tr>
<td>10.0</td>
<td>0.378 0.400 0.393</td>
<td>0.390 ±0.011</td>
</tr>
<tr>
<td>20.0</td>
<td>0.314 0.322 0.401</td>
<td>0.345 ±0.048</td>
</tr>
<tr>
<td>40.0</td>
<td>0.344 0.393 0.341</td>
<td>0.359 ±0.029</td>
</tr>
</tbody>
</table>
Fig 4.0.2(a) Standard Curve for Progesterone ELISA

The logarithm of concentration gave a well fitted linear regression as shown in fig 4.0.2(b) below:
Fig 4.0.2(b) Logarithm of standard curve

4.0.3 Estradiol ELISA Test for Samples

The limit of detection for the determination of estradiol using the RIDASCREEN 17β Estradiol assay was 10 pg/ml. The table below shows the assay results for the analysis of the samples before concentration.
Table 4.0(a) Sample results for estradiol ELISA

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Sampling Site</th>
<th>Observation</th>
<th>Deduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Matapi flats, Mbare</td>
<td>The sample changed from colourless to light blue colour.</td>
<td>Estradiol activity in assay</td>
</tr>
<tr>
<td>2.</td>
<td>Magaba light industrial area</td>
<td>Colourless Solution.</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>3.</td>
<td>Seke Road flyover</td>
<td>Colourless Solution.</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>4.</td>
<td>Mufakose pasteurised land</td>
<td>Colourless Solution</td>
<td>&lt;LOD</td>
</tr>
</tbody>
</table>

4.0.4 Progesterone ELISA test for samples

Table 4.0(b) Sample results for progesterone ELISA

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Sampling Site</th>
<th>Observation</th>
<th>Deduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Matapi flats, Mbare</td>
<td>Colourless solution.</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>2.</td>
<td>Magaba light industrial area</td>
<td>Colourless solution.</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>3.</td>
<td>Seke Road flyover</td>
<td>Colourless solution.</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>4.</td>
<td>Mufakose pasteurised land</td>
<td>Colourless solution.</td>
<td>&lt;LOD</td>
</tr>
</tbody>
</table>

4.1 Solid Phase Extraction

Table 4.1: Pre-concentration by solid phase extraction
### Table 4.2(a): Estradiol ELISA for concentrated sample

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Sampling Site</th>
<th>Observation</th>
<th>Deduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Matapi Flats, Mbare</td>
<td>The sample changed from colourless to light blue colour.</td>
<td>Estradiol activity in assay</td>
</tr>
<tr>
<td>2.</td>
<td>Magaba light industrial area</td>
<td>Change of colour from colourless to blue solution</td>
<td>Estradiol activity in assay</td>
</tr>
<tr>
<td>3.</td>
<td>Seke Road flyover</td>
<td>Colourless Solution</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>4.</td>
<td>Mufakose Pasteurised Area</td>
<td>Colourless Solution</td>
<td>&lt;LOD</td>
</tr>
</tbody>
</table>

**4.2 ELISA Test after Concentration**

The results below show the colour changes that were observed when the concentrated samples were exposed to an ELISA.

*Absorbance Readings for estradiol ELISA for concentrated sample*
After pre-concentration the following absorbance readings were taken for the two samples above that showed estradiol activity in the assay.

**Table 4.2(b): absorbance readings for concentrated Matapi sample**

<table>
<thead>
<tr>
<th>Absorbance 1 (450nm)</th>
<th>Absorbance 2 (450nm)</th>
<th>Absorbance 3 (450nm)</th>
<th>Mean Absorbance (450nm)</th>
<th>Concentration(ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.731</td>
<td>1.649</td>
<td>1.688</td>
<td>1.689±0.041</td>
<td>0.072</td>
</tr>
</tbody>
</table>

**Table 4.2(c): absorbance readings for concentrated Magaba sample**

<table>
<thead>
<tr>
<th>Absorbance 1 (450 nm)</th>
<th>Absorbance 2 (450 nm)</th>
<th>Absorbance 3 (450 nm)</th>
<th>Mean Absorbance (450 nm)</th>
<th>Concentration(ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.688</td>
<td>2.135</td>
<td>2.460</td>
<td>2.428±0.278</td>
<td>0.0126</td>
</tr>
</tbody>
</table>

**Table 4.2 (d): progesterone ELISA for concentrated sample**

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Sampling Site</th>
<th>Observation</th>
<th>Deduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Matapi flats, Mbare</td>
<td>Colourless solution.</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>2.</td>
<td>Magaba light industrial area</td>
<td>Colourless solution.</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>3.</td>
<td>Seke Road flyover</td>
<td>Colourless solution.</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>4.</td>
<td>Mufakose pasteurised land</td>
<td>Colourless solution.</td>
<td>&lt;LOD</td>
</tr>
</tbody>
</table>
4.3 HPLC Analysis

4.3.1 Estradiol Calibration Curve

A total of thirteen standards were used to draw the calibration curve.

Table 4.3.1(a): HPLC-DAD Results for analysis of estradiol standards

<table>
<thead>
<tr>
<th>Concentration of estradiol standard (µl/ml)</th>
<th>Retention times</th>
<th>Peak area</th>
<th>Mean Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>5.877</td>
<td>0.189</td>
<td>0.189±2.220E-5</td>
</tr>
<tr>
<td></td>
<td>5.881</td>
<td>0.188</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.877</td>
<td>0.179</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.186</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>5.903</td>
<td>0.345</td>
<td>0.342±0.066</td>
</tr>
<tr>
<td></td>
<td>5.899</td>
<td>0.346</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.889</td>
<td>0.481</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.353</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>5.882</td>
<td>0.485</td>
<td>0.483±0.54</td>
</tr>
<tr>
<td></td>
<td>5.881</td>
<td>0.483</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.882</td>
<td>0.492</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>5.877</td>
<td>0.590</td>
<td>0.593±1.15E2</td>
</tr>
<tr>
<td></td>
<td>5.876</td>
<td>0.593</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.881</td>
<td>0.597</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>5.885</td>
<td>0.773</td>
<td>0.774±0.0001</td>
</tr>
<tr>
<td>Temperature</td>
<td>Value 1</td>
<td>Value 2</td>
<td>Value 3</td>
</tr>
<tr>
<td>-------------</td>
<td>----------</td>
<td>----------</td>
<td>----------</td>
</tr>
<tr>
<td>150</td>
<td>5.901</td>
<td>0.995</td>
<td>0.993±0.002</td>
</tr>
<tr>
<td>200</td>
<td>5.892</td>
<td>1.601</td>
<td>1.604±0.004</td>
</tr>
<tr>
<td>300</td>
<td>5.881</td>
<td>2.135</td>
<td>2.133±0.002</td>
</tr>
<tr>
<td>350</td>
<td>5.889</td>
<td>2.622</td>
<td>2.528±0.230</td>
</tr>
<tr>
<td>500</td>
<td>5.870</td>
<td>3.444</td>
<td>3.349±0.136</td>
</tr>
</tbody>
</table>
Fig 4.3.1(a): Calibration curve for estradiol standards

4.3.2 Progesterone Calibration Curve

Table 4.3.2: HPLC-DAD analysis of progesterone standards

<table>
<thead>
<tr>
<th>Concentration of estradiol standard (µl/ml)</th>
<th>Retention time</th>
<th>Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>14.597</td>
<td>0.653</td>
</tr>
<tr>
<td>40</td>
<td>14.602</td>
<td>1.457</td>
</tr>
<tr>
<td>60</td>
<td>14.523</td>
<td>2.694</td>
</tr>
<tr>
<td>80</td>
<td>14.583</td>
<td>3.229</td>
</tr>
<tr>
<td>100</td>
<td>14.591</td>
<td>4.166</td>
</tr>
<tr>
<td>150</td>
<td>14.577</td>
<td>6.418</td>
</tr>
</tbody>
</table>
Concentration of estradiol standard (µl/ml) | Retention time | Peak Area
---|---|---
200 | 14.587 | 8.366
300 | 14.584 | 13.506
350 | 14.575 | 16.032
500 | 14.553 | 23.327

**Fig 4.3.2 (b): Calibration curve for progesterone standards**

\[
y = 0.0475x - 0.5329 \\
R^2 = 0.9987
\]

**4.2.3 HPLC-DAD Analysis Results for Samples**

**Table 4.2.3(a) Estradiol Analysis**
Table 4.3.3(b) Progesterone Analysis

<table>
<thead>
<tr>
<th>Sample description</th>
<th>Peak Area</th>
<th>Retention Time</th>
<th>Concentration(ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Matapi</td>
<td>0.896086</td>
<td>5.927</td>
<td>0.12±0.034</td>
</tr>
<tr>
<td>2. Magaba light</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>industrial area</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Seke rd Flyover</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>4. Mufakose pasteurised land</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND- Not Detected

4.4 Statistical Analysis

From the analysis carried out in the methodology, progesterone was not detected in any of the samples. Only one sample from Matapi was detected and managed to give four different readings of the concentration as shown on the chromatograms attached (appendix 1). Therefore the statistical analysis used was descriptive and based on the four concentrations of estradiol read from the Matapi sample.
**Table 4.3: Descriptive Statistics**

<table>
<thead>
<tr>
<th>Item</th>
<th>Calculated Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observations</td>
<td>4.000</td>
</tr>
<tr>
<td>Arithmetic Mean</td>
<td>0.156</td>
</tr>
<tr>
<td>Variance</td>
<td>0.001</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>0.034</td>
</tr>
<tr>
<td>Coefficient of Variation</td>
<td>0.221</td>
</tr>
<tr>
<td>Median</td>
<td>0.154</td>
</tr>
<tr>
<td>Range</td>
<td>0.074</td>
</tr>
<tr>
<td>Max.</td>
<td>0.194</td>
</tr>
<tr>
<td>Min.</td>
<td>0.120</td>
</tr>
<tr>
<td>Interquatile</td>
<td>0.057</td>
</tr>
</tbody>
</table>

The standard deviation was used to describe the distribution of the values around the arithmetic mean which was calculated as 0.156 ng/ml from the four observations. The value of the standard deviation showed a higher degree of precision as well as accuracy of the results.
CHAPTER FIVE

DISCUSSION

An effect Directed analysis approach was taken for the analysis of natural hormones in sediments along Mukuvisi River. Effect Directed Analysis (a combination of biological and chemical analysis) was the method of choice due to the low sample concentrations of the micro-pollutants as well as the complexity of the matrix. The sample was subjected to biological analysis followed by concentration techniques and repeated biological analysis to reduce the complexity of the sample and to ascertain the fraction showing the steroidal effects. Chemical analysis was then done to quantify and confirm the presence/absence of the extracted steroids in the sediments.

5.1 Sampling Sites

Samples were collected along Mukuvisi River and four sites were chosen. The choice of sites was based on the fact that the major sources of steroidal pollutants are of anthropogenic origin. These contaminants are derived from excretion, household use and disposal. In Harare, the unavailability of a pharmaceutical industry which manufactures contraception as well as hormones for growth promotion or supplementation reduces the occurrence of steroidal contaminants from the industry. Hence the major source of contamination remains that of human handling, use and disposal.

Mukuvisi was chosen because of the densely populated areas located along the river including Mbare, Sunningdale, and Highfield etc. Mukuvisi also runs along the Harare CBD thereby giving wide representation of the effects of populations on contamination. Samples were collected from Mbare residential area which has a high population and wastewater has a significant input into the river approximately 500 m downstream. Due to the close proximity
of the river to both Matapi Flats and Magaba industrial area, direct excretion into the rivers is high given the high people-toilet ratio of the areas. Illegal panners as well as squatters contribute their share of contamination of the river waters through direct excretion into the waters as well as the river banks. Disposal of contraception into the river waters is also part of contamination resulting from the people using the river as dumping sites. The sewer pipes in the residential area are routinely bursting thereby exposing the untreated sewer to the river banks just 500 m downstream. This is a major source of contact between the contaminants and the Mukuvisi River.

Harare CBD was chosen as another sampling site in order to compare the effects of large populations on the concentrations of steroids in the sediments. The contamination of the river sediments in the city centre is limited due to absence of dumping sites and lower human activities with regards excretion. The sewer pipes in the city centre have a lower frequency of bursting thereby reducing contamination rates.

Mufakose Pasteurised land was also chosen because of the deposition of the sewage sludge for agricultural use as well as the large animal farm just approximately 1.6 km upstream. The washing away and deposition into the Mukuvisi River of this sludge by rainwater and weathering agents has a significant effect on contaminant concentrations in the sediments.

5.2 Extraction

The initial solid liquid extraction made use of the combined salts MgSO₄ and NaCl. This is a buffering solution and is important in this pre-SPE step to stabilise the base sensitive estradiol and progesterone. The MgSO₄ polarises the water and separates the organic layer into the inorganic layer thereby achieving extraction.

5.2.1 Clean Up and Solid Phase Extraction

Clean up of the extracts was achieved using Sep Pak C18 (500 mg) cartridges. This is a silica based bonded stationery phase with strong hydrophobicity and capable of adsorbing analytes
of even weak hydrophobicity from aqueous solutions (Waters) Estradiol has high hydrophobic properties as shown by its log $K_{ow}$ of 4.8. Thus this high affinity to solids resulted in estradiol adsorbing well onto the C18 cartridge. Progesterone is moderately hydrophobic with a $K_{ow}$ value of 3.87 and thus adsorbed well onto the C18 cartridge. The Sep pak cartridges use the reverse phase mode of interaction whereby the stationery phase is less polar than the mobile phase. The steroids under investigation are non-polar compounds and as a result they interacted more with the non polar C18 stationery phase resulting in efficient separation from other more polar interferences. The cartridges were conditioned with a polar mobile phase of ethyl acetate, methanol and water in sequence (Labadie and Hill, 2006). This was done in effect to improve separation efficiency and ensure complete desorption of the analytes.

However, use of Sep Pak clean up still resulted in poor method detection limits and severe ion suppression of the analytes and so a second (silica) SPE clean up was used (Labadie and Hill, 2006). In addition to further clean up SPE was also used as a pre-concentration step. Preconditioning of the silica cartridges was also done to improve separation efficiency. During preconditioning and sample loading the cartridges were not allowed to dry in order to keep the sorbent ligands active and prevent air from trapping in the cartridges. One of the challenges faced with solid phase extraction was the clogging of the sorbent material therefore the upper layer containing less suspended solids was transferred first and the bottom layer containing the heavily suspended particles transferred last. A concentration factor of 200 was achieved and could not be concentrated further as a result of the suspended particles that resulted in clogging therefore more amount of time was required to further concentrate the analyte.

5.3 Bioanalysis
The enzyme linked immunosorbent assay was used as the Bioanalysis method of choice. The concentrations of the samples were below the limit of detection before concentration. The concentration of the sample allowed the ELISA kit to determine concentrations of the estradiol. The limit of the ELISA test was low in the ranges of pg/ml. However due to the variability of compounds that can be found in sediment samples, test interferences caused by matrix effects cannot be completely excluded. As with any analytical technique, positive results requiring some actions should be confirmed by an alternative method.

5.4 HPLC Analysis

5.4.1 Stationary and Mobile Phase

A reversed phase C18 column was used. Reversed phase chromatography is a term which is used to describe the state in which the stationary phase is less polar than mobile phase. The analytes being investigated are non-polar compounds and thus their interaction with the stationary phase was high.

Because water is the weakest reversed phase mobile phase the elements were efficiently retained as their interaction with water were minimal.

5.4.2 Detector

The DAD detector was chosen because steroids absorb in the UV/VIS region and therefore can be detected using a DAD. The DAD was also used because of its availability and accessibility despite the many disadvantages associated with its use for determination of trace elements in complex environmental samples. The DAD has high limit of detection and therefore inefficient when analyzing toxicants of very low concentrations. The calculated limit of detection from the calibration curve was 0.0106 ng/ml for estradiol and 0.1875 ng/ml for progesterone. Diode array detector also has a limitation of low selectivity. The ideal
detector for the analysis of trace levels of steroids is a LC–MS/MS but due to its unavailability, the DAD was used.

5.5 Presence of Steroids in Mukuvisi River Sediments

The presence of estradiol was investigated using effect directed analysis. The concentration of estradiol in Mukuvisi River from the Matapi sample was determined to be 0.156 ng/ml. Despite the variability in the levels of estradiol in sediment samples, its concentration is a function of the characteristics of human population (sex composition, age and contraception practices), the size of the sewers and degradation rates occurring in the sewers (Eljarrat et al., 2009). The presence of detectable levels of estradiol can be attributed to the high levels of contamination of the river due the high population in Matapi, poor sanitation practices as well as the presence of illegal inhabitants in the Mukuvisi River banks. Inefficient waste treatment systems also contribute to the high levels of estradiol in the Mukuvisi river sediments as noted by bursting sewer pipes which are washed down into the river.

Apart from the diverse sources of contamination of the river water, the presence of estradiol in the river can also be explained in terms of the physicochemical properties of the steroid itself. It has been observed that estradiol is a hydrophobic organic compound of low volatility thus sorption on soil or sediment is a significant factor in reducing aqueous phase concentration. The slow degradation rates of estradiol as discussed make them persistent environmental contaminants which can accumulate to high concentrations.

Estradiol was not detected in any of the three other samples because of various reasons. This could be because of the high limit detection of the HPLC-DAD system versus the low concentration levels of the estradiol in the environmental sample. In Harare CBD, the levels of pollution are lower since it is not a residential area thus there is less flow of waste in the sewer systems. Contamination levels are also closely monitored by the city council.
Along Magaba area, the levels were also below limit of detection because the area is male-dominated due to the type of business conducted in that area hence the levels of estradiol are low. Microbial degradation can also explain the results from the Mufakose pasteurized land. Treatment of the sludge with bacteria can increase the rate of degradation of estradiol sorbed onto sludge.

The levels of Progesterone were below the limit of detection of the HPLC-DAD system which was 0.1875 ng/ml hence they were not detected. Progesterone is a natural hormone responsible for the thickening of uterus during pregnancy therefore it is only produced by pregnant women in significant amounts. The concentration of progesterone in the environment is therefore expected to be lower when compared to the levels of estradiol which is produced by every girl as soon as they reach puberty and every woman until they reach menopause. Progesterone also has a higher solubility than estradiol of 314 mg/L and a lower log \( K_{ow} \) of 3.87. This means that less of progesterone is likely to be sorbed to sediments. The limit of detection for the determination of progesterone was very high reducing the efficiency of detecting lower levels of the steroid in complex sediment samples.

The values of estradiol detected were in good agreement with previous work. Labadie and Hill, 2006, detected levels E2 ranging from 0.34-3.30 ng/g dry. However the DAD is not usually used in the analysis of steroids and reported work is that performed using LC/MS or LC-MS/MS systems. Barceló et al, 2006, detected levels ranging from 0.05-22.8 ng/g using an LC/TOF system.

**5.6 CONCLUSION**

Effect Directed analysis was used to determine steroids in Mukuvisi River Sediments at ng/ml level. The level of estradiol in Mukuvisi sediments along Mbare was determined to be 0.156 ng/ml. In three other samples estradiol was not detected. Progesterone was not detected
in any of the samples. The limit of detection for estradiol analysis was 0.0106 ng/ml. LOD for Pg was 0.1875 ng/ml.

5.7 RECOMMENDATIONS

Although the analysis of steroids in Mukuvisi River was the main aim of this project, the lack of specific literature specific on domestic wastewater treatment and reuse in Harare has required this project to be based predominantly on international literature.

The following recommendations can help acquire information on occurrence of emerging organic contaminants in Harare.

5.7.1 Presence of steroids in wastewater and sewage sludge

Traditional end-of-line treatment of wastewater by WWTPs is increasingly being recognised as inefficient in removing emerging organic contaminants and their metabolites. There is therefore need to study and implement other effective alternative routes of wastewater treatment. Given the current evidence that aerobic biodegradation is the main pathway for elimination of steroids from sludge, the effect of microbial agents on the degradation process should be investigated and possibly applied in treating wastewater. This detail is also important on the implications on the application of these bio-solids on land for agricultural purposes.

5.7.2 Analytical Methodologies

Comprehensive analytical method validation is a crucial factor in trace level analyses of organic pollutants in environmental samples. This is especially true in cases where matrix interferences can contribute to the overall reliability of the method sensitivity and reproducibility (Shareef et al., 2008). Therefore there is need to develop and optimise analytical methodologies for the determination of emerging organic contaminants in our environment. There is need to develop cost effective monitoring analytical methods for analysis of trace elements in complex environmental samples.
5.7.3 Environmental Monitoring

Due to the lack of appropriate instrumentation (e.g. LC –MS/MS, GC-MS) and expertise in Zimbabwe as well as financial constraints, emerging organic contaminants have not been monitored in the environment. Therefore there is need to educate the public on the effects of pollution and the endocrine disrupting properties of steroids when they accumulate in the environment. If rate of pollution is reduced then accumulation in the environment is also reduced.
7. REFERENCES


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APPENDIX A

CHROMATOGRAMS FOR SAMPLES AND STANDARDS