Dispersive Liquid-Liquid Micro-Extraction Of Steroidal Hormones And Determination In Wastewater Using High Pressure Liquid Chromatography – Charged Aerosol Detector

By

Cecilia Oluseyi Osunmakinde

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Supervisor: Prof. Mathew M. Nindi

Co-supervisor: Prof. Simiso Dube

October 2014

CERTIFICATION

As the candidate's supervisors, we have approved this dissertation for submission.

Signature ----------

Date ----------

Signature ----------

Date ----------

Student number: 48829331

DECLARATION

I declare that Dispersive Liquid-Liquid Micro-Extraction of Steroidal Hormones and Determination in Wastewater Using High Pressure Liquid Chromatography – Charged Aerosol Detector is my own work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references.

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Cecilia Oluseyi Osunmakinde Date

DEDICATION

I dedicate this work to God Almighty, the Father of all and the Beginning of all good and perfect things in life.

ABSTRACT

Steroid hormones belong to a group of compounds known as endocrine disruptors. They are hydrophobic compounds and are categorized as natural and synthetic estrogens. Some common household products have been implicated as estrogen mimics. Exposure effects of these compounds are felt by human and wildlife, such reproductive alterations in fish and frogs. They mainly introduced into the environment through veterinary medicines administration to animals and the discharges from wastewater treatment plants (WWTPs).

In this study, a new alternative analytical procedure that is simple, rapid and fast for the determination and quantification of five steroidal hormones: estriol (E3), beta estradiol (β-E2), alpha estradiol (α-E2), testosterone (T), progesterone (P) and bisphenol A (BPA) using the High pressure liquid chromatography coupled to a charged aerosol detector (HPLC-CAD). These compounds were studied because of their strong endocrine-disrupting effects in the environment.

Under optimum conditions, a linear graph was obtained with correlation coefficient (R^2) ranging from 0.9952 - 0.9996. The proposed method was applied to the analysis of water samples from a wastewater plant and the results obtained were satisfactory. The limits of detection (LOD) for the target analytes in wastewater influent was between 0.0002 – 0.0004 µg/L and the limit of quantification (LOQ) was 0.001 µg/L respectively for each of the analytes. Enrichment factors of 148- 258, and extraction efficiency 84- 102% were obtained for the target analytes; relative standard deviations (% RSD) for m = 6 were between 2.8 and 7.6%. The concentration of the EDCs in environment sample was between 0.2 - 2.3 µg/L.

Keywords:

Steroid, hormones, endocrine, wastewater, plant, detection, sample, preconcentration, DLLME, Gas, Chromatography, Spectrometry, Enrichment

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LIST OF ABBREVIATIONS

BPA	=	Bisphenol A
CAD	=	Charged aerosol detector
CE	=	Capillary electrophoresis
CPE	=	Cloud point extraction
DNA	=	Deoxyribonucleic acid
DAD	=	Diode array detection
DDT	=	Dichlorodiphenyltrichloroethane
DLLME	=	Dispersive liquid-liquid microextraction
FLD	=	Fluorescence detection
E1	=	Estrone
E3	=	Estriol
EDCs	=	Endocrine disruption chemicals
EF	=	Enrichment factor
ER	=	Estrogen receptors
EE	=	Extraction recovery /efficiency
ESI	=	Electrospray ionization
ESLD	=	Evaporating scattering light detection
FLD	=	Fluorescence detection
GC MS	=	Gas chromatography - mass spectrometry
GC MS/MS	=	Gas chromatography - tandem mass spectrometry
GLU	=	Glucuronide
HF-MMLE	=	Hollow fiber micro porous membrane liquid extraction
HF-SLME	=	Hollow fiber supported liquid microextraction
HPLC	=	High pressure liquid chromatography
IUPAC	=	International Union of Applied and Pure Chemistry
LLE	=	Liquid-liquid extraction
LC-MS	=	Liquid chromatography - mass spectrometry.
LC-MS/MS	=	Liquid chromatography-mass spectrometry/mass spectrometry
Log K_{ow}	=	Octanol water partition coefficient
LOD	=	Limit of detection
LOQ	=	Limit of quantification
OP	=	Organic pollutants
PAH	=	Polycyclic aromatic hydrocarbon
P	=	Progesterone

pK_a	=	Acid Dissociation Constant
PPCPs	=	Pharmaceuticals and personal care products
PR	=	Progesterone receptors,
RI	=	Refractive index
RSD	=	Relative Standard Deviation
SPE	=	Solid-phase extraction
SPME	=	Solid phase microextraction
SPE-MIP	=	Solid phase microextraction-molecular imprinted polymer
STP	=	Sewage treatment plants
SUL	=	Sulfates
T	=	Testosterone
UV	=	Ultraviolet
WWTP	=	Waste water treatment plant
WHO	=	World health organization
β-E2	=	Beta estradiol
α-E2	=	Alpha estradiol
μg/L	=	Micro gram per litre
ng/L	=	Nano gram per litre
mg/L	=	Milli gram per litre

CHAPTER 1

INTRODUCTION

1.1 Background

Environmentalist and analytical chemists are concerned about the presence of emerging organic pollutants (OP) which include pharmaceutical personal care products (PPCPs), endocrine disrupting chemicals (EDCs) and some chemicals that are deliberately added into our aquatic environment. Most of these compounds display estrogenic activities causing undesirable adverse effects in humans, wildlife and the environment after long exposure periods. Conventional wastewater treatment plants (WWTPs) are not designed to remove steroid hormones or their metabolites present in wastewater [1-4], thus affecting the consumer confidence about the quality of the water. Studies have shown that some hormones are responsible for feminization of fish in aquatic environment [5, 8]. The EDCs causing these effects are the natural, synthetic and other compounds that have the ability to mimic the estrogenic activity. This increasingly publicized presence of these compounds has led authorities to seek water treatment solutions and sensitive analytical methods proactively, despite the fact that many OPs are not regulated.

Over the last decade, the presence and activity of EDCs in the environment has been a major concern. These compounds alter the production and activities of endogenous hormones by interacting with the endocrine system, presenting a potential threat to aquatic life and human health [6]. According to Damstral and World Health Organization (WHO) [9], “any substance or mixture that has the ability to alter the function(s), or to disrupt the synthesis, secretion, transport, binding, reproduction, development or the behavior of an organism or its progeny in the endocrine systems and consequently causes adverse health effects in an intact organism

has been defined as an EDCs". EDCs have been categorized from various literatures mainly into the following categories [6, 10];

- Pesticides and its metabolites (DDT, deldrin)
- Industrial and household chemicals (paints, detergents, UV sunscreen)
- PPCPs (clofibrates, sulfamethoxazole, ibuprofen)
- Polycyclic aromatic hydrocarbon (PAHs)
- Heavy metals (lead, cadmium)
- **Steroid hormones (estrogens)**

In this study, steroid hormones and bisphenol A will be our main focus as these compounds have been reported to have ability to interfere with the hormonal systems of humans and animals for over 80 years [11]. Some of the reasons why this class of compounds has received more attention are their abundance in the human body and their estrogenic potency, as well as the extent of their use in contraceptive pills and as growth promoters in animals.

Steroid hormones are mainly excreted from the body as estrogen conjugates, which comprise sulphate (SUL) and glucoronide (GLU) in the urine [12-14]. Likewise, Hoffmann [15] reported that female cattle also excrete mainly SUL and GLU estrogen conjugates. Conjugated estrogens can also be found in some prescribed drugs as SUL estrogen salts, which can be used in the treatment of hormonal imbalances, post-menopausal symptoms and osteoporosis [12]. Steroid hormones have a strong endocrine disrupting effect, which interferes with the reproductive functions of aquatic organisms [5, 10, 13, 16]. They have been reported to cause testicular, prostate, and breast cancer [17-19]. Their presence has also been reported to decrease sperm counts leading to reproductive disorders in men [18]. In fish, their presence has been reported to be responsible for the reduction of fertility (decreased sperm number and quality, or egg number), feminization in fish and frog abnormalities [15], effects on the development of the gonads in male and juveniles yolk synthesis [20-22], and

reproductive alterations [15, 17]. Morphological changes have also been reported in teleost fish and vertebrate's thyroid gland as a result of exposure to organic pollutants [23-25].

The abilities of WWTPs in removal of steroid hormones have drawn a great deal of attention. The removal rates of steroid hormones can vary from one WWTP to the other as the removal of these compounds depends on the type of the treatment process and the physicochemical properties. Recently, relative success has been achieved in the use of advanced technologies such as granular activated carbon (GAC), membrane technology, ozonation, and ultraviolet radiation in the removal of some PPCPs from wastewater [24, 26-27]. The removal of EDCs falls into three categories; physical removal, biodegradation and chemical advanced oxidation (CAO) [28]. The removal efficiency for estrone (E1), estradiol (E2) and ethylinestradiol (EE2) in Germany, Canada and Brazil was studied using the activated sludge and was reported to be 83%, 99.9% and 78%, respectively [14, 28]. In Korea, conventional drinking water treatment methods were relatively inefficient for contaminant removal. The efficient removal for (E1) was 99% and was achieved by GAC [26]. In some other studies, activated sludge treatment steps removed hormones efficiently up to 77-79% for estriol (E3), progesterone (P) 95%, estradiol (E2) 59%, and 17 alpha estradiol (17α-E2) 98% [14, 31- 32]. As much as these studies show the ability of the steroids to be removed from the treated water, it is clear that the WWTPs have no capacity for 100 % removal efficiency. Thus the WWTPs need improved treatments to completely remove these compounds from water systems. The water industry is thus faced with the challenge to better understand the mechanisms and methods of removal of EDCs to be able to offer cost effective solutions for their removal in wastewater treatment.

1.2 Problem statement

There is much interest on the effects of steroid hormones on animals and human endocrine system. Various types of steroid hormones and metabolites are introduced into the environment through the discharges of WWTPs directly or indirectly, agricultural practices,

household and hospitals discharges. These activities and processes have increased the release of steroids into the aquatic environment. These have affected the water body and water cycle and hence a global concern. The whole world is struggling to keep up with the high demand for water and its scarcity, with no alternative source of water, treatment plants tend to recycle wastewater for drinking purposes [32]. In the process of recycling, some of the steroid hormones might survive water treatment and then move to the underground water which might pose some health effects. Determination of natural hormones such as estriol, progesterone and estradiol has become of great interest due to the frequent detection in treated and untreated WWTPs at low concentrations range of ng/L to μg/L. The natural and synthetic steroid hormones have great higher estrogenic potentials. This is due to the fact that many of their constituents are excreted unchanged as well as metabolites [12-14]. Inactive hormones could be converted back in the environment to their active forms; this is due to the fact that steroid hormones are not completely degraded biologically. This has led to increase in development of preconcentration and analytical methods that can detect these compounds at such concentration levels.

Numerous analytical methods have been used in the determination of these steroid hormones in the environment [1, 11, 16, 25, 33] but some come with limitations. Currently, the analytical determination of steroid hormones is dominated by gas chromatography- mass spectrometry (GC-MS), and gas chromatography-tandem mass spectrometry (GC-MS/MS) [6, 12, 13, 34- 37]. Due to the poor volatility of these compounds, derivatization steps are prerequisite. This approach is unfortunately time consuming and labor intensive.

The use of liquid chromatography-tandem mass spectrometry (LC-MS/MS) has gained more popularity for the analysis of steroid hormone due its ruggedness and sensitivity [6, 12, 34, and 37]. An advantage of using LC-MS/MS is that it has the ability to analyze steroid hormones in the environment without derivatization, or the need to hydrolyse the conjugated form. However, using LC/MS/MS as an analytical tool is not without difficulties such as

matrix interference [37]. However, this approach is expensive for upcoming research and development laboratories in emerging economies.

High pressure liquid chromatography (HPLC) with diode array (DAD) and other detectors is also a preferred method of analysis of steroid hormones (12, 42). It is a fast, easy and simple to operate. The concentration of analytes reported with DAD and the analytes were 0.2-1.6 µg/L [12, 42-44]. HPLC ultraviolet (UV) was able to detect at concentration levels of 0.3-1 µg/L [12, 18, 45-46]. HPLC has also been combined with mass spectrometry.

In this study, we propose an alternative technique for the determination of steroid hormones in wastewater with the use of high pressure liquid chromatography coupled to a charged aerosol detector (HPLC-CAD). CAD can be used in the determination of semi-volatile, basic, neutral or acidic compounds without need for derivatization of the target analytes before analysis. The mode of operation is by producing a signal that is proportional to the mass of the sample and the response is independent of chemical structure [47- 49]. It detects compounds electrically by ionizing them with charged nitrogen gas, an advantage CAD has over the other detectors. When compared to other HPLC detectors, such as ultraviolet (UV), diode array (DAD), fluorescence detector (FLD), there is a need for a derivatization step prior to analysis. [47-51]. Evaporative light scattering detection (ESLD), is another type of detector which is similar to the CAD in the mode of operation, it is able to detect steroid hormones without a derivatization step prior to analysis.

Different preconcentration methods and techniques have been developed and adopted for the extraction of steroid hormones in environmental matrices. Some of the procedures include, cloud point extraction (CPE) [18, 37], solid phase microextraction (SPME) [36-37, 44], hollow fiber micro porous membrane liquid extraction (HF-MMLE) [37, 52], solid phase extraction using imprinted polymers [53], both online and offline solid-phase extraction (SPE) [37, 40-42, 54]. Sample preconcentration is described as the backbone in the detection

and quantification of steroid hormones in the environment. It will also be addressed by using a more environmentally friendly approach, a preconcentration technique known as dispersive liquid-liquid microextraction (DLLME). It is less time-consuming, inexpensive and simple procedure which uses microliter volume of solvent and easy to operate [45-46, 55-56]

In this study, we intend to develop a new and alternative method for the determination of steroid hormones in wastewater samples using HPLC-CAD. The main choice of this technique is that there is no need for the derivatization of the compounds before analysis and a preconcentration technique which uses less sample volume, fast and lower solvent consumption with higher extraction efficiency will be used to extract the target analytes.

1.3 Research objectives

Several analytical methods are available for the determination of steroid hormones in the environment but they come with some limitations. The ultimate goal of this research was to develop an alternative, cost-effective method for the determination and quantification of five steroid hormones and bisphenol A (BPA) in wastewater.

The aim of this investigation was achieved through the following specific objectives:

- To develop HPLC-CAD analytical method for steroid hormones and bisphenol A.

- Validate the developed HPLC-CAD analytical method based on the following validation parameters: (a) Limit of detection (LOD); (b) Limit of quantification (LOQ); (c) Linearity; (d) Accuracy/Recovery; (e) Selectivity (f) Robustness; (g) Precision.

- To develop and optimize a pre-concentration method for the extraction of five steroid hormones and BPA using DLLME.

- To validate the DLLME method based on the specified validation parameters above.

- To apply the DLLME method on real water samples from a WWTP

1.4 Contributions and dissertation outline

The major contributions of this research are as follows:

- The validation of an alternative method for the determination and quantification of five steroid hormones and bisphenol A in wastewater using HPLC-CAD.
- The development and optimization of a preconcentration technique for the extraction of five steroid hormones and BPA in waste water using HPLC-CAD.
- Experimental application of DLLME to samples obtained from a wastewater treatment plant (WWTPs).

The dissertation is presented in five chapters. The first chapter is an introduction which provides the general background, problem statement and rationale, aims and objectives of the research.

Chapter 2: Extensive review was conducted of the available literature on steroid hormones and their detection, with detailed information on the physiochemical properties, usage, sources, occurrence, fate, transport and ecotoxicity, as well as the general analytical methods that have been developed and used in the determination and quantification of these analytes from various matrices.

Chapter 3: Experimental section. This chapter outlines the procedures of the validation of the developed separation method using HPLC-CAD. Development and optimization of DLLME was designed and described. Finally the application of the proposed method on real water samples is described.

Chapter 4: This chapter presents the main findings and gives a detailed overview of the results and graphs of the research

Chapter 5: The chapter provides the conclusions of this research, followed by recommendations on future work.

1.5 Declaration of recent publications

The following recent articles have been presented and sent for review under this research work.

Refereed journal publication

- Osunmakinde, C.O., Dube, S and Nindi, M.M. Development of Dispersive Liquid-Liquid Microextraction for the determination of six endocrine disruptors in wastewater using High Pressure Liquid Chromatography-Charged Aerosol Detector**.** Journal of analytical methods in chemistry. (*Accepted ISI journal:*171739)

Refereed conference publications

- Osunmakinde, C.O., Nindi, M.M., and Dube, S. Method development for the determination of steroid hormones in waste water using HPLC with charged aerosol detector. Book of abstract 12th ICCA conference, Pretoria, South Africa 2013.

- Osunmakinde, C.O., Nindi, M.M., and Dube, S. Investigation of steroids and hormones in wastewater using HPLC with a charged aerosol detector. Book of abstract of the 4th SEANAC International Conference, Maputo, pg 73, 2012.

- Osunmakinde, C.O., Nindi, M.M., and Dube, S. Screening for steroids and hormones in wastewater using HPLC with charged aerosol detector (CAD)**.** Book of abstracts

at SACI YCS Symposium, 2012.

- Research Reports presented, submitted and accepted by the Water Research Commission South Africa K5/2094//3 (July, 2013).

CHAPTER 2

STEROID HORMONES AND THEIR DETERMINATION

Steroid hormones belong to a class or group of compounds known as the EDCs. They are biologically active organic compounds whose structure consists of a phenolic and hydroxyl group. They are non-volatile, with moderate to high hydrophobicity [1]. Steroid hormones are classified mainly into three main groups; natural, synthetic and xenoestrogens [6].

Natural steroid hormones are the bases of all sex hormones such as estradiol, estrone, estriol and progesterone. They are produced in human beings, animals as well as in some plants. Natural hormones are secreted by the adrenal cortex, testis, ovary, placenta and the endocrine gland in human beings and animals [7] and they travel through the bloodstream. They can bind to specific receptor sites in various organs and tissues and regulate a variety of biological functions in mammals, such as controlling metabolism and reproductive function, as well as maintaining blood pressure, glucose and ion levels, muscle and nervous system functions [8]. Synthetic hormones such as 17α-ethyinylestradiol, menstranol and its metabolites/conjugates are chemically synthesized. They are the main active agents in hormone therapy, birth and oral contraceptive pills [6, 17].

The term xenoestrogens is used to describe compounds that have shown to act like estrogens in terms of their interference with the endocrine system. They comprise a multitude of chemicals, which affect the endocrine (hormone) system of animals and humans. Some of these compounds are the main active ingredient in pharmaceuticals and are used in manufacturing industries, agriculture and households [6, 10, 12, 14]. They can mimic the physiological processes such as growth, sexual differentiation or reproduction by binding to

the estrogen receptor. An example of such an estrogenic chemical compound is Bisphenol A (BPA) [6, 10, 12, 20], Which is used in the production of plastics used for food product packages, bottles for water, bottles for infant food and kitchen utensils [6, 45, 57-58]. Because of its polarity, persistence and solubility, BPA is often able to pass through treatment plant operations and enter the aquatic environment [57]. BPA has also been demonstrated to exhibit estrogenic activity and it has been classified as an endocrine disruptor [6, 8, 10, 57].

2.1 Mechanisms of steroid hormones

Steroid hormones are built from cholesterol, produced and secreted naturally or synthetically by endocrine glands in the body. The endocrine system is an integrative system that controls the cell function and activities of mammals, amphibians, birds, fish and various invertebrates by communicating through chemical messengers called hormones [60]. The endocrine or hormone system plays a critical role in development and growth, metabolism, reproduction, natural defenses to stress, stimuli and nutritional balance of the blood. This system consists of several glands (e.g. adrenal, pituitary, gonads, and ovaries) that synthesize and secrete hormones. Steroid hormones are transported throughout the body via the circulatory system to the target cells. On reaching a target cell, the hormone binds to a specific hormone receptor, and then the receptor/hormone complex attaches to a specific segment of deoxyribonucleic acid (DNA) called the response element to activate or inhibit gene expression, ultimately leading to protein synthesis [60].

2.1.1 Molecular structure and physicochemical properties of steroid hormones

Steroid hormones have a tetracyclic molecular structure, which is derived from cholesterol. It comprises four rings: a phenol group, two hexamethylene groups and a cyclopentane group. Numbering and labeling of the four rings (A, B, C, D) as shown in Figure 2.1 are presented in accordance with the International Union of Applied and Pure Chemistry's (IUPAC) recommendations for the nomenclature of steroids.

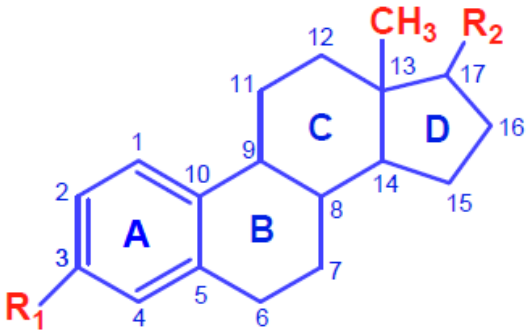
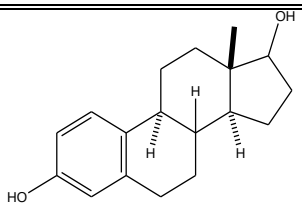
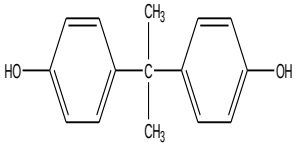
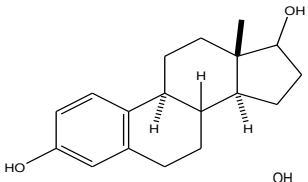
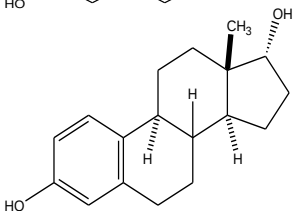
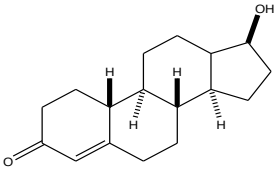
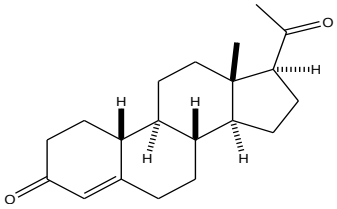


Figure 2.1 Typical structure of a steroid hormone [60]

Natural steroid hormones have relatively high hydrophobicity owing to their ringed structure, higher water solubility of approximately 13 mg/L, whereas synthetic estrogenic steroids have much lower solubilities of 4.8 mg/L. All steroid hormones have very low vapour pressures ranging from 2.3×10^{-10} to 6.7×10^{-15} mm Hg [3]. The difference between these compounds lies in the configuration of the D-ring at positions C16 and C17. Table 2.1 shows the physical and chemical properties of the five steroid hormone and BPA used in this study. The chemical nature and high molecular weight suggest volatilization to be of low importance for their environmental behaviour. The octanol-water distribution coefficients (*K*ow) indicate that free estrogens have a very strong tendency to accumulate in organic matter. These physicochemical properties have an important impact on the compounds' behaviour in environmental matrices.

Table 2.1 Physiochemical properties of five steroid hormones and BPA [37]

COMPOUND	STRUCTURE	CAS no	MW (g/mol)	pK_a	Log K_{ow}
ESTRIOL (E3) $C_{18}H_{24}O_3$		50-27-4	288.4	10.25	2.5
BISPHENOL A (BPA) $C_{15}H_{16}O_2$		80-05-7	228.29	10.29	3.641
BETA ESTRADIOL (β- E2) $C_{18}H_{24}O_2$		50-28-2	272.38	15.06	5.09
ALPHA ESTRADIOL (α- E2) $C_{18}H_{24}O_3$		57-91-0	272.4		4.106
TESTOSTERONE (T) $C_{19}H_{28}O_2$		58-22-0	288.42	15.05	3.179
PROGESTERONE (P) $C_{21}H_{30}O_2$		57-83-0	314.46	-	3.827

2.2 Sources, usage and occurrence

2.2.1 Sources

Steroid hormones are bioactive compounds that are introduced into the environment as complex mixtures via a number of routes. The principal method by which steroid hormones and their metabolites are transferred into the environment or terrestrial body is usage, disposal

and excretion by humans and farm animals (cattle and pigs). A combination of metabolized and unmetabolized steroid hormones is excreted by humans. These are discharged from private households, industrial waste waters, farm effluents and disposal systems of hospitals and eventually enter municipal WWTPs [14]. Some steroid hormones can also enter the wastewater via regular use during showering or bathing, and applications on the body [12, 14, 60].

Steroid hormones are also introduced into the environment either after extensive treatment in WWTPs, mainly the urban domestic wastewater, or after minimal treatment from agricultural wastes, e.g. irrigation water effluents from ponds, direct waste excretion by grazing livestock without treatment. Thus, effluent from WWTPs becomes the most important source of pollution. Conventional WWTPs have historically been used to improve dissolved oxygen levels in water bodies, reduce nutrient loads and remove some biodegradable organic compounds. Therefore, by design, they are not equipped to remove emerging contaminants such as steroid hormones or any other PPCPs [2-4]. Hence, steroid hormones and their by-products are discharged directly into surface waters, again without prior treatment at WWTPs [57- 60].

Another significant source of steroid hormones in the environment that is common and unregulated is the discharges from manufacturing, health facilities, and household disposal of expired and unused medicine via toilet flush or domestic trash [2, 58, 61]. The release of untreated sewage from WWTPs directly into the surface waters and the surroundings is also another point. This is a major challenge particularly for plants or treatment facilities that are designed to deal with large amounts of storm water, or erosion and other natural disasters that result in the release of large amounts of untreated effluent into water bodies. Figure 2.2 shows the mode of entry of steroid hormones into the environment. Finally, the introduction of steroid hormones into the environment has no geographic or climatic boundaries.

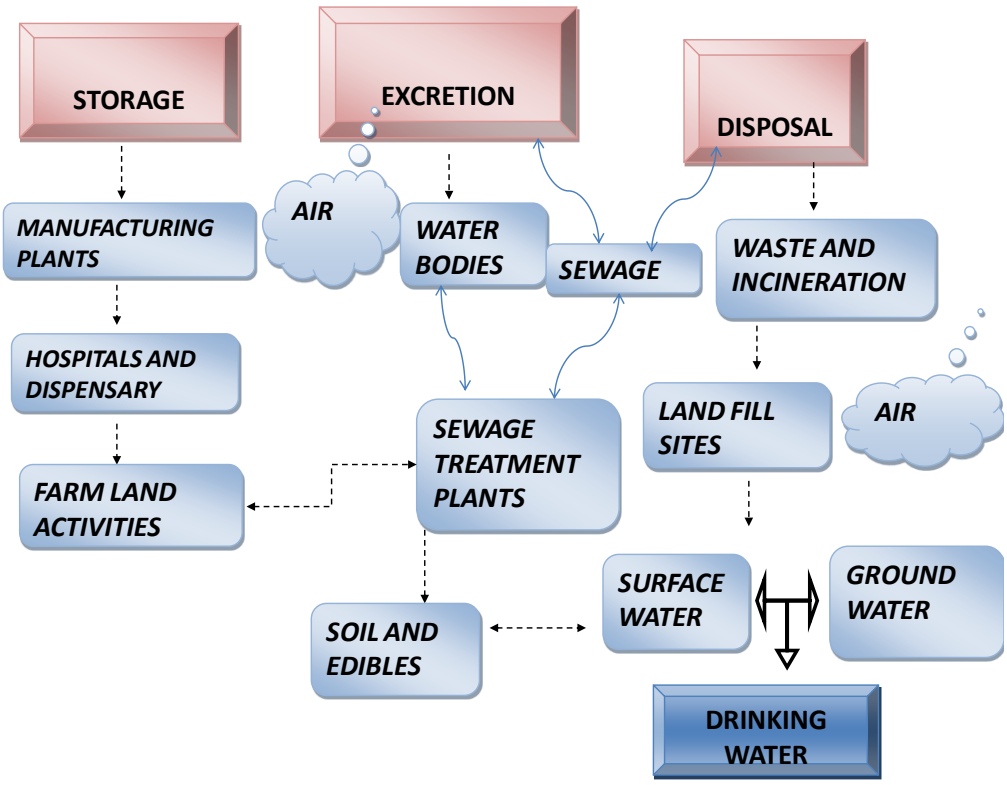


Figure 2.2 Sources of Steroid hormones in the environment adapted from Kummer [62]

2.2.2 Usage of steroids

A pharmaceutical (also frequently referred to as a drug or a medicinal product) can broadly be defined as a compound of known chemical structure, which when administered into a host organism produces biological effects. The US Food and Drug Administration classifies a substance as a "drug" if it is intended to diagnose, treat, cure, mitigate or prevent diseases [9-10]. Steroids are known to be endogenous and they comprise a wide group of substances that mediate various types of biological responses and have been synthesized by chemists over the years [63]. Cholesterol is the most widely spread steroid hormone in the body, which is derived from dietary intake, but can also be synthesized in the body [12].

The natural steroid hormones estrone (E1), 17-beta-estradiol (E2) and estriol (E3) are responsible for female sex organ development and functioning [6, 9, 12, 14]. It can also be

used in the treatment of various diseases such as allergic reactions, arthritis, some malignancies and diseases resulting from hormone deficiencies or abnormal production [10, 12]. The synthetic steroid hormone such as 17-alpha ethinylestradiol (EE2) is used in female contraceptives and in hormone replacement therapy [9, 12, 16-18]. In addition, synthetic steroid hormones such as EE2 are used for therapy in treating menstrual syndrome, as a hormone supplement, or as the main component of contraceptives [10, 12, 16, 20]. Other synthetic steroids that mimic the action of progesterone are widely used as oral contraceptive supplements and are also designed to stimulate the synthesis and muscle-building action of protein in the body [63- 67].

Steroid hormones are used legally in veterinary medicine as growth promoters and for animal fattening purposes. They have the capacity to increase weight gain and reduce the feed conversion ratio [68]. Steroids are used in the agricultural sector for food production, mainly for boosting the mass and quality of animal carcasses in food production for economic reasons [68]. Anabolic steroids may also be used in sport or entertainment in order to improve performance in competitive humans and animals [69]. These steroids may enhance performance through a number of mechanisms, including increased muscle mass, enhanced recovery from training, raised red blood cell count and heightened aggression [69-70]. Large amounts of steroid hormones and synthetic hormones are illegally used, because of their potential anabolic effects [69-70]. Natural and synthetic have been reported to be the most potent of the EDCs [5].

2.2.3 Occurrence

The occurrence of steroid hormones and their metabolites has been studied in various matrices over the years, owing to their possibly adverse effect on humans and wildlife [62]. Steroid hormones have been detected in various samples and their occurrence has been documented in Europe, United Kingdom and China [14, 17, 19]. Their presence has been reported in drinking water and bottled water at low concentration level of 0.01- 0. 9 ng/L [10,

73, 76], concentration levels of 0.2-114 ng/L have also been confirmed at WWTPs [7, 14, 65, 77-79]. In surface water, 0.3 – 1.7 ng/L [14, 17, 79-81], 2.6- 9.5 ng/L in sea water [10, 76], and 6- 66 ng/L were detected in ground water [7, 76, 78, 82]. In the river water, 0.3 – 32.9 ng/L has been observed [60, 75, 83-84], 0.08 - 0.89 ng/g in the soil [7, 85-87], 0.9- 49 ng/g in sediment and sludge [33, 76, 85, 87], and 0.6- 26 ng/g in some fish samples [88]. Finally the presence of steroid hormones has also been confirmed in hospitals and industrial units, as well as intensive animal breeding farms [7, 78].

2.3 Transportation of steroid hormones

The transport of steroid hormones is determined with respect to their partitioning into different environmental compartments. They are non-volatile organic compounds and their disposal or mode of entry into the environment is mainly by aqueous transport. The partitioning into aqueous or solid phase depends on the compound-specific distribution coefficients. Compounds that tend to stay in the dissolved phase are transported into surface waters through the effluent discharge from WWTPs, while those with high sorption capabilities end up in terrestrial environments, particularly if the treated sludge is used for land applications. Pharmaceutical products residing in soil environments may reach ground water upon leaching [88] or enter surface water again after run-off events. Figure 2.3 shows the fate and transport pathways in which steroid hormones undergoes.

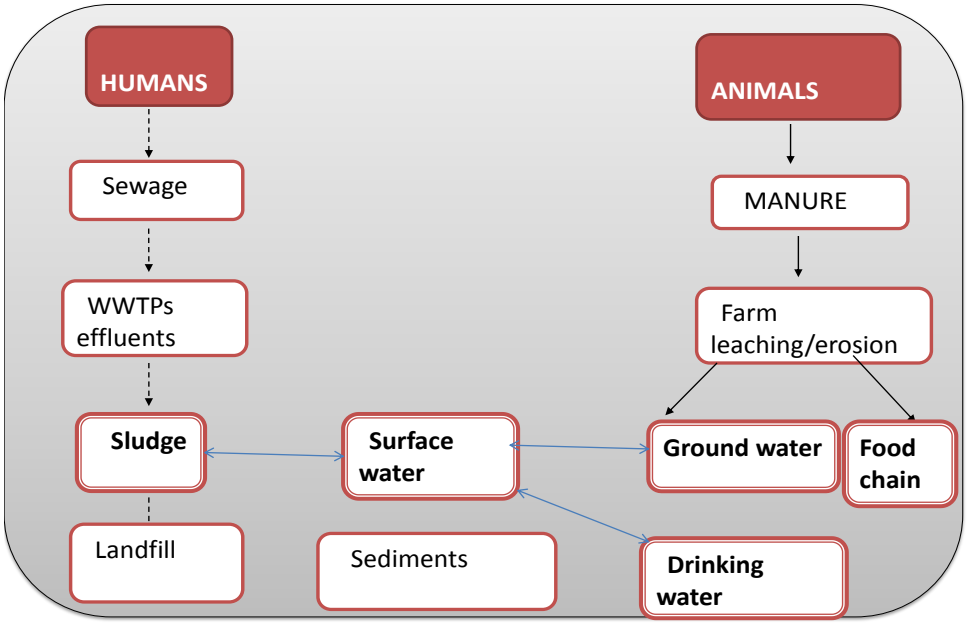


Figure 2.3 Transportation and exposure of steroid hormones in the environment. Adopted from Scherr [60]

2.4 Environmental fate of steroid hormones

Steroid hormones are absorbed by humans and animals that ingest them. They undergo some metabolic processes and the end product is then excreted from the body. The behavior of steroid hormones in the environment is of concern because these compounds have endocrine disruption potential. The main elimination of estrogens finally takes place when they arrive at WWTPs. However, the removal of steroid in WWTPs is a complicated process, which includes the cleavage of conjugates, sorption, photolytic degradation and microbial degradation [60, 87- 90]. Since steroid hormones are structurally similar and often carry multiple polar functional groups with higher solubility, environmental fate or alterations in the compound are produced by biotic or abiotic processes or a combination of the two [88-91].

2.4.1 Abiotic transformations

An abiotic transformation is any process in which a substance is converted into simpler products by a physical or chemical mechanism. Abiotic transformation of PPCPs products in surface or wastewater may occur by hydrolysis and photolysis. Steroid hormones are usually resistant to hydrolysis, whereas direct and indirect photolysis is a primary pathway for their transformation. Direct photolysis of a compound is caused by the direct absorption of solar light and indirect photolysis involves the use of natural photosensitizers, e.g. nitrate and humic acid [90-92]. Steroid hormones and their metabolites undergo two major kinds of abiotic transformations, these include photodegradation and sorption.

Photo degradation is a major transformation process for steroid hormones in the environment, both in WWTPs and natural waters. In natural surface waters, photochemical reactivity is strictly limited to the photic zone, i.e. the uppermost region of the water column, which is affected by depth and attenuation [91-92]. Surface water with a high algal content or sediment loading will have a very shallow photic zone because of light absorption and scattering. In addition, humic substances can absorb or attenuate sunlight, which also decreases the depth of light penetration, while colored dissolved organic matter is the main UV-absorbing constituent in surface water and controls UV light penetration [89, 93].

Solar degradation of organic compounds can occur either directly or indirectly. In direct photolysis, a compound absorbs appropriate radiation depending on the absorption spectrum of the compound directly from the sunlight in which it becomes excited. After the excited state, it will then undergo a chemical transformation, which enables it to generate a different product based on the structure of the compound [88-93]. Indirect photolysis occurs when the dissolved photosensitizers (nitrates, humic acids etc.) generate free radicals in surface water, which then absorb solar radiation to get to an excited state [89-91]. Photo degradation

depends on various factors, such as the intensity of radiation, dissolved organic matter, nutrient loads and water depth [93-94].

Adsorption or sorption occurs when suspended solids, particulates, sediments, colloids or natural organic matter is a major elimination pathway of steroid hormones and metabolites in WWTPs as well as in surface waters [91]. This results in the distribution of organic contaminants between the aqueous and solid phases, which is governed by equilibrium properties and non-hydrophobic mechanisms such as electrostatic interactions [90-95]. The distribution of steroid hormones in the environment is based mainly on its Log K_{ow} values (solubility), which are the critical chemical parameters. Large Log K_{ow} values (> 4) suggest large hydrophobic molecules and will tend to dissociate from solid organic matter, i.e. high sorption potential, while smaller Log K_{ow} values (< 2.5) suggest low sorption potential [66]. Steroid hormones are hydrophobic substances and are readily absorbed onto the surface of solid materials. They pose some risks to the environment, which includes soil, sediment, aquatic and non-aquatic species [91]. Different studies have shown that sorption of steroid hormones in the soil and sediment of aquatic environments is moderate to high [87, 89, 91-92].

2.4.2 Biotic transformation (microbial degradation)

Biological transformation of pharmaceuticals occurs in two different ways in living organisms: through the action of enzymes, e.g. cytochromes, and by the microorganisms present in the digestive tract [67]. These transformations are aided by different species of bacteria in the environment. Metabolism of drugs in the body is supported by different phases of reactions such as hydrolysis, oxidation, hydroxylation and reduction [76]. The first phase of the products generated is less toxic than the parent drug, but there are instances where the metabolic product could be more toxic [89]. Hydrophilicity of the first phase products generated is further enhanced by second phase conjugation reactions with the help of

glucuronic acid, SUL, acetyl, glutathione and amino acids. The highly polar conjugated products are then excreted through urine and feces. Therefore, the extent to which a parent compound or its metabolites are excreted depends on the compound-specific metabolism [94-95]. High proportions of administered drugs are sometimes excreted unchanged [96-97]. The excreted compounds in the second phase, which are the conjugated products, are sometimes converted back to the parent compound with the aid of various treatment processes that occur in WWTPs [97-100].

Microbial degradation constitutes an important process for PPCPs products. This degradation is often part of the secondary treatment in WWTPs' activities and involves the use of aerobic activated sludge treatments and anaerobic sewage sludge digestion [28]. It is often desirable that biodegradation results in complete mineralization. However, in addition to the differences in the chemical characteristics of pharmaceutical compounds, the extent of biodegradation (low, moderate or high) and hence the removal efficiency, depends on a variety of factors including sludge age, hydraulic retention times, season, differences in WWTPs' construction and treatment technologies, amount of biomass, temperature, pH and oxygen saturation levels [14, 64, 77, 89, 96, 98-99]. Conjugated estrogens are hydrolyzed into free estrogens and GLU and sulfuric acid in the presence of fecal bacteria - Eschericia coli [13, 92, 101]. Very limited information is available on environmental parameters such as nutrient levels, pH and other recognized variables affecting microbial activity that influence or inhibit the degradation of steroid hormones in the environment [13, 101-102].

2.5 Environmental significance and effects

2.5.1 Environmental significance

The presence of steroid hormones in the aquatic and terrestrial environments has increased based on the effects of estrogens on target and non-target organisms. They interfere with normal endocrine function by mimicking and blocking hormonal function [6, 10, 12, 24, 103].

The endocrine and reproductive effects of environmental contaminants are believed to be due to:

(1) Mimicking endogenous hormones such as estrogens and androgens,

(2) Antagonizing normal, endogenous hormones,

(3) Altering the pattern of synthesis and metabolism of natural hormones, and

(4) Modifying hormone receptor levels.

The ecological relevance of endocrine disruption in wildlife is, however, difficult to quantify, as there is limited understanding and few reports on how physiological changes affect the individual animal and how individual responses affect the population and community [10, 103]. Furthermore, a major challenge faced by environmental biologists is the need to place endocrine disruption into context with other environmental pressures faced by wildlife populations, for example global warming.

There has been increasing evidence of endocrine disruption in fish and frogs due to estrogens in the environment and several field and laboratory studies have reported inter-sex conditions in fish exposed to estrogen compounds [16, 23]. Estradiol and 17α-ethinylestradiol (EE2) is the active ingredient in many contraceptive pills and birth control methods. It induces the synthesis of the yolk protein precursor vitellogenin, a specific and sensitive indicator of exposure to xenoestrogens in fish at concentrations 1 600 000-fold lower than the concentration necessary to cause the death of 50% of the test fish population [10, 105].

2.5.2 Environmental effects

Steroid hormones have interactions with the endocrine system mechanisms, however little has been studied about the environmental effects. They are a potential risk for wildlife and humans through the consumption of contaminated food or water. Some of the alterations in the endocrine system can lead to disturbances of homeostasis, miscarriage, failures in

development and reproduction and even death [10, 12, 16, 102-106]. Steroid hormones may affect not only the exposed individual but also the children and subsequent generations. These effects may be transmitted not because of mutation of the DNA sequence, but rather through modifications to factors that regulate gene expression, such as DNA methylation and histone acetylation [103-104]. A detailed overview of the effects that estrogens may cause in the environment is not the purpose of the current report. However, from an analytical perspective, the concentration levels of the compounds in the environment are of interest since such data determines the sensitivity requirements for analytical methods.

In humans, some steroid hormones have been linked to declining male reproductive health, by both decreasing sperm counts and affecting quality leading to reproductive disorders in human males [5, 10, 21, 103-106]. Women are normally exposed to estrogen, but the effects on females are more difficult to track because of the estrous cycle and the resulting huge differences in circulating hormone concentrations at different stages of the cycle [105-106]. The presence of estrogen-mimicking compounds in adult women can impair reproductive capacity by interfering with natural hormone cycles, potentially rendering women unable to conceive or to maintain pregnancy, and may promote abnormal cell growth [105- 109].

In aquatic environments, fish are considered the most sensitive of all species. Studies have shown that steroid hormones EDCs have influenced the sexual development of fish in the waters of the United Kingdom as far back as 15 years ago [107-109]. The blocking and development of frog egg maturation [12, 16, 23, 108 -114], decline in alligator populations [8, 23, 110] and an increase in male fish possessing male and female characteristics [111-112]. Altered levels of circulating thyroid hormones and morphological changes in the thyroid gland have been demonstrated in sea bass exposed to organic pollutants (OPs) [110-112]. A number of studies have shown that male fish exposed to estrogens at ng/L level will exhibit estrogenic responses, such as vitellogenin (VTG; precursor to yolk, a female-specific protein) production [25, 109- 114].

2.6 Analytical determination of steroid hormones

The occurrence of steroid hormones in the environment is gaining wide attention owing to their effects fate, transport and behavior. Modern and sensitive techniques are being developed worldwide for their determination. Since steroid hormones and their metabolites are found in the environment at lower concentrations, there is a need for sensitive analytical methods that are reliable and reproducible. The most common advanced analytical techniques used in the determination and quantification of steroid hormones are GC-MS, GC-MS/MS, LC-MS, LC-MS/MS, HPLC and immunoassays [36-37,71. However, steroid hormones cannot be analyzed directly without a sample preparation technique which is predominately the most critical step in the analytical process. Figure 2.4 shows the process involved during the determination of steroid hormone in the environment.

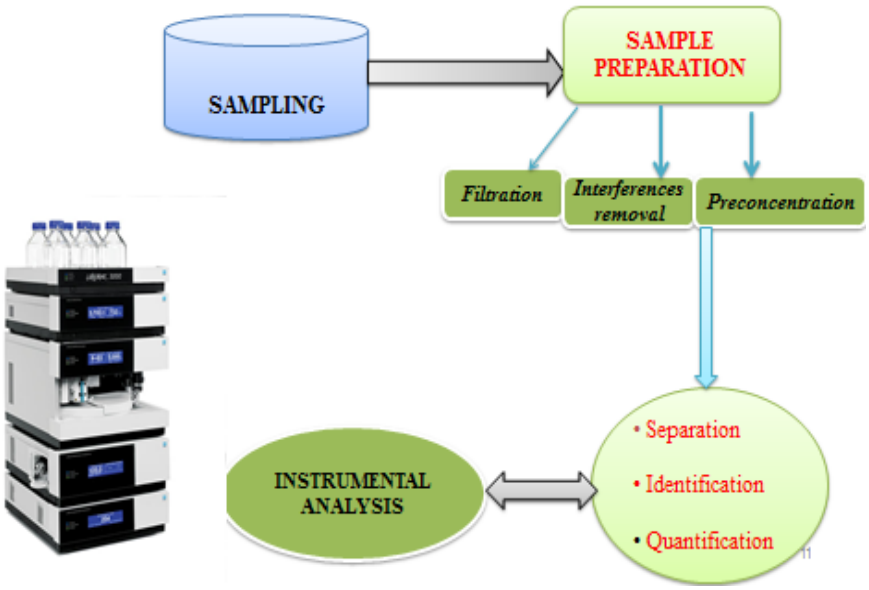


Figure 2.4 Schematic for the analysis of steroid hormone

2.6.1 Sample pre-treatment and clean-up methods

Sample pre-treatment and clean-up is usually a difficult step and time consuming segment in the analysis of steroid hormones or other OPs in the environment. Due to the low level concentrations in which they exists, as well as the complexity of the matrices in which they are detected. However, sample preparation is necessary to convert the sample into a form that is suitable for the analysis to be performed without the loss of the secondary sample [37]. The process of analyzing a sample is based on the type of matrix and concentration level at which the analytes are detected. It plays a major role in the data generated, as it can influence the results in various ways.

Adequate optimization procedures and conditions are necessary to enhance sensitivity and interference from the matrix. The application of sample preparation with high enrichment achieves a low limit of detection (LOD) [37]. Sample preparation steps, such as filtration, extraction, purification, and evaporation are employed before the final determination is performed by bioassays or GC or HPLC.

2.6.1.1 Sampling and storage

The value of any data generated in the laboratory depends solely on the integrity of how the sample was taken. Sample must be collected in such a way that nothing is added or lost during the process. To examine the quality of wastewater discharged into the environment, the characteristics of the water in question must be defined chemically or biologically. There are different approaches in sampling organic pollutants in wastewater, such as grab sampling, composite sampling and also the polar organic chemical integrative sampler (POCIS). Samples are kept in ice-packs containers during transportation to the laboratories to avoid further degradation of the target analytes.

2.6.1.2 Filtration

Because wastewater usually contains a high load of organic material and suspended particles, filtration is usually the first step in sample preparation as the suspended solids easily clog the absorbent bed. Similarly, when the analysis is performed by immunochemical assay, filtration helps to avoid undesired adsorption onto antibodies. The filtration step is performed in numerous ways, but most of the studies reviewed employed glass filters [90].

2.6.1.3 Isolation

Sample extraction from heavily contaminated samples (manure, soil and to some extent waste water) often requires further clean-up before analysis [60, 90]. This has been achieved using different techniques such as liquid-liquid extraction and solid-phase purification on C18 columns. Some of the purification procedures were developed to isolate the estrogenic active fractions from the wastewater extract for further identification of the compounds responsible for such activity, rather than for simple clean-up of the extract [90]. Good detection limits without purification have only been reported by studies using biological techniques for analysis or graphitized carbon black adsorbents as SPE [90].

2.6.1.4 Extraction

Extraction of natural and synthetic steroid hormones from wastewater is usually performed using numerous techniques. Cloud point extraction (CPE) has been used as an alternative to the liquid-liquid extraction (LLE) method in the determination of steroid hormones with LOD values of 0.23-5.0 ng/L, and recoveries between 81-99.5% [18, 37]. In CPE technique surfactants such as triton are normally used as the extraction solvent. The disadvantage of this method however lies in the compatibility of the surfactant during analysis of the analytes with instruments such as GC and HPLC. Also CPE is a time-consuming procedure [18, 37, 56, 113-115]. Hollow fiber micro porous membrane liquid extraction (HF-MMLE) has been developed for determination of estrogens in sewage-water samples. The HF-MMLE is been

referred to as a green technique [37, 52]. This technique showed LOD as low as 1.6–10 ng/L with high enrichment factors and extraction efficiency, but long processing time of 180 min [37, 52]. Solid phase micro extraction (SPME) is a solvent free, fast and simple procedure which was introduced by Pawliszyn for the extraction of environmental samples [36-37, 44]. It is a very fast technique and could be automated. The technique can also be coupled to HPLC or GC [37, 8, 47, 53, 57, 62]. Using SPME as a preconcentration technique, the instrumental detection of steroid hormones in environmental was as low as 6-100 ng/L with recoveries of 88-116%. The limitation that comes with this technique is that it is expensive and some of the fibers are fragile with limited life time. The desorption temperature and sample carry over is also a major challenge during the procedure [37, 56, 115].

Solid phase extraction (SPE) could either be online or offline [37-38, 40-41, 54]. In most cases discs and often specific cartridges have been employed for the use of extraction of steroid hormones using SPE from wastewater. Both discs and cartridges have been used with advantages and disadvantages. Cartridges have the advantage of easy system automation, because devices are available for automated washing, conditioning, sample loading, drying and elution of a large number of samples [37, 90]. It is time consuming and an expensive technique which requires special instrumentation [37, 115]. Another disadvantage is that it requires specific cartridges for target analytes, and these cartridges are not reusable. Analytes were detected in water samples with LOD of 0.02-40 ng/L and recoveries of 79- 95% recovery [38, 54]. Due to the various drawbacks of the existing methods, ongoing research has led to the development of more preconcentration procedures to overcome these limitations.

2.7 Dispersive liquid-liquid microextraction

The use of small amount or the elimination of solvent has led to the development of dispersive liquid-liquid micro extraction (DLLME). DLLME was first reported by Assadi and

his co-workers in 2006 [55]. DLLME has also been used for the extraction of natural, synthetic hormones as well as some EDCs [45-46, 56]. It is a liquid-phase microextraction technique based on a ternary component solvent system in which appropriate mixture of dispersing and extracting solvent are rapidly injected into an aqueous sample containing the analytes of interest. The mixture of (water/disperser solvent/extraction solvent) is shaken in order to produce a cloudy solution in the conical test tube. Centrifugation is done for a few minutes, the fine particles of extracting solvent are sedimented at the bottom of the conical test tube, and this is referred to as the sedimented phase. DLLME is a technique that can be used for analytes with high or moderate lipophilic properties [47, 56-57,114-115].

DLLME uses an extracting solvent such as the chlorinated solvents that has a high density than water such as carbon disulfide, carbon tetrachloride, tetrachloroethylene, chlorobenzene, chloroform and dichloromethane [55-56]. The disperser should be miscible in water and extracting solvent to help increase the surface area between the extracting solvent and aqueous phase [114-115] thus ensuring that equilibrium is achieved quickly. The applicability of the DLLME is based mainly on the distribution coefficient (k) which is defined as the ratio of the analyte concentration in extraction solvent and sample solution [115]. The value of the distribution coefficient should be greater than 500 to achieve a suitable application of DLLME. However, for the acidic or alkaline analytes, distribution coefficient could be increased by controlling the pH value of sample solution; making the analytes existing in nonionic state [115-116]. Steroid hormones are largely neutral analytes, they have a great potential to be extracted from the aqueous phase into the organic phase, and this makes them a good candidate for this type of preconcentration procedure.

In 2011, DLLME was used in the determination of 17β-estradiol and diethylstilbestrol in aqueous water samples using HPLC. The enrichment factors and extraction recoveries were 71.0 – 78.5 and 85.2 – 94.2 respectively. The limit of detection for 17β-estradiol was 0.010µg/L and diethylstilbestrol was 0.008 µg/L [56]. Bisphenol A has also been extracted in

water sample using DLLME and determined using HPLC-UV. Under optimum condition the limit of detection was 0.07µg/L [45].

The method has some advantages over other existing ones, such as being economical, very fast, simplicity and high extraction efficiency [55-56]. Figure 2.5 below describes the procedure for dispersive liquid-liquid microextraction in DLLME.

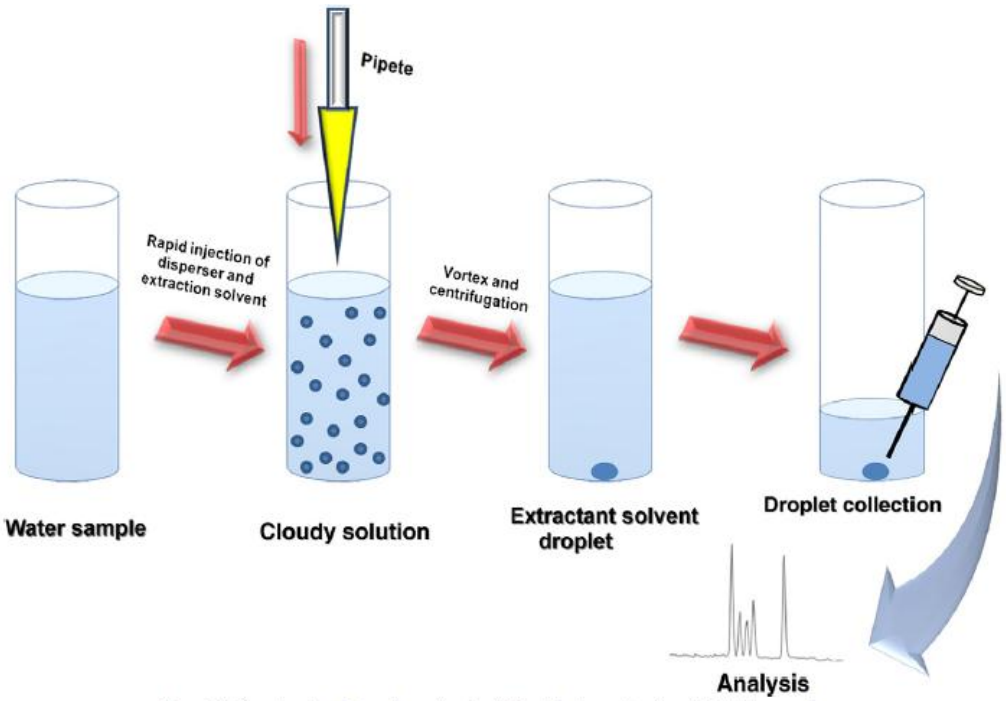


Figure 2.5 Procedure for dispersive liquid-liquid microextraction [115]

2.8 Analytical techniques

Numerous analytical methods have been used for the determination of steroid hormones in the environment. Nowadays, the most important and common methods used for steroid determination are GC and LC. GC is a chromatographic technique that is used for the detection of polar, volatile compounds because of its sensitivity. The analytical determination of natural and synthetic estrogens in various matrices has been dominated by the use of GC-MS and GC-MS/MS. These are well-established technique that offers extremely robust

instrumentation that is widely available in most laboratory settings [21]. Various derivatization agents can be used for this process, but each of these steps can influence the accuracy of the method. However, for the determination of concentration levels of conjugated steroid hormones or metabolites a further step of enzymatic hydrolysis is needed and loss of analytes can occur, which makes the derivatization reaction incomplete [6, 12-13, 76]. GC-MS methods have recorded LODs ranging from 0.1- 1.5 ng/L [36], GC-MS/MS recorded LOD values of 0.1-2.4 ng/L [17, 34-37, 58].The main drawback of GC-MS and GC-MS^2 analysis is the need for derivatization in the analysis of compounds. It is labor-intensive and requires long analysis time and suitable for steroid hormones derivatives.

Currently, LC-MS and LC-MS/MS have become widely used techniques for the determination of steroid hormones in environmental samples because of their sensitivity and specificity. LC-MS uses the direct method during the analysis of steroid hormones unlike the GC-MS. Liquid chromatography coupled to mass spectrometry enables the determination of both conjugated and non-conjugated estrogens without a derivatization step or hydrolysis [10, 34-41]. However, LC-MS as an analytical technique comes with some limitations such as interferences [6, 12, 20, 37-41]. Matrix effects occurring in LC-MS can result in ion suppression or enhancement of the signal of target analytes. Reported LODs on different types of water samples vary between 1-20 ng/L [36-37], 0.05-3 ng/L [13, 17, 34, 37].

High performance liquid chromatography (HPLC), coupled to a wide variety of detection systems has gained in popularity for analysis of steroid hormones. However, many LC–MS methods have been developed to measure steroid hormones and hormone-like substances in environmental samples [70] due to its high selectivity, specificity and sensitivity [12, 43]. However, many LC-MS methods have been developed to detect steroid hormones and hormone like substances in environmental samples [37, 68]. HPLC has been combined with detectors such as diode array (DAD), the concentration of analytes were 0.2-1.6µg/L [12, 42-43]. HPLC ultraviolet (UV) was able to detect as low as 0.3-1µg/L [12, 18, 45-46].

Other methods that have also been used in the determination of Steroid hormones are capillary electrophoresis (CE) and immunoassay. CE has been explored in the analysis of polymers, monitoring of endocrine disruptors and for complex mixtures [12, 117-118]. Different types of detection modes can be coupled with the CE. The CE can be coupled to a UV, FLD, MS and immunoassay. The use of MS detection with CE is quite sensitive, but it is expensive and tedious process. CE has been used in the determination of steroid hormones in different water samples, and it was able to detect estriol, estrone and 17β-estradiol with detection limits of 8.9×10^{-8}, 6.7×10^{-8}, and 1.1×10^{-7} mol/L respectively [118]. The limitations of CE application in the determination of EDCs involve sensitivity, sample matrix interferences, high detection limit and sample ionic strength [12, 117].

In the field of environmental analysis, immunoassay techniques are getting more and more attention because of their high sensitivity, ease of use, short analysis time cost-effectiveness (they are cheaper than the LC-MS/MS) simplicity and the possibility of analyzing large sets of samples even in the field [13, 119-120]. Positive results obtained by immunochemical methods usually lead to more comprehensive analyses using sophisticated methods, such as GC-MS, GC-MS/MS, LC-MS, and LC-MS/MS [68]. Immunochemical measurements can be carried out directly, but sample pretreatment is sometimes necessary, as natural organic matter may interfere with the analysis [120-122]. Immunoassays rely on the recognition reaction between a specific antibody and the determinant in antigen [123]. Immunoassays are fast and therefore suitable for screening a large number of samples. Disadvantages are the cross-reactivity and sometimes scarceness of a specific antiserum. The detection limits vary between 3 and 37 pg/ml [119-123].

2.9 High pressure liquid chromatography coupled to a charged aerosol detector

The charged aerosol detector (CAD) offers a new approach for the determination of proteins, lipids, steroids, carbohydrates and weak chromophore compounds used in pharmaceuticals. CAD can also be used in the determination of semi-volatile, basic, neutral or acidic compounds without the need for derivatization of the target analytes before analysis. CAD offers some advantages over traditional detection methods such as RI, evaporative light scattering detection ELSD, UV/Vis absorbance, and fluorescence, especially for the components of dispersed-phase systems. CAD is also suitable for detecting non-chromophoric or non-fluorescent molecules. CAD detector is mass dependent and the response generated does not depend on the spectral or physiochemical properties of the compound like the UV detector which is concentration dependent [49, 50]. Figure 2.6 shows the internal schematic of CAD

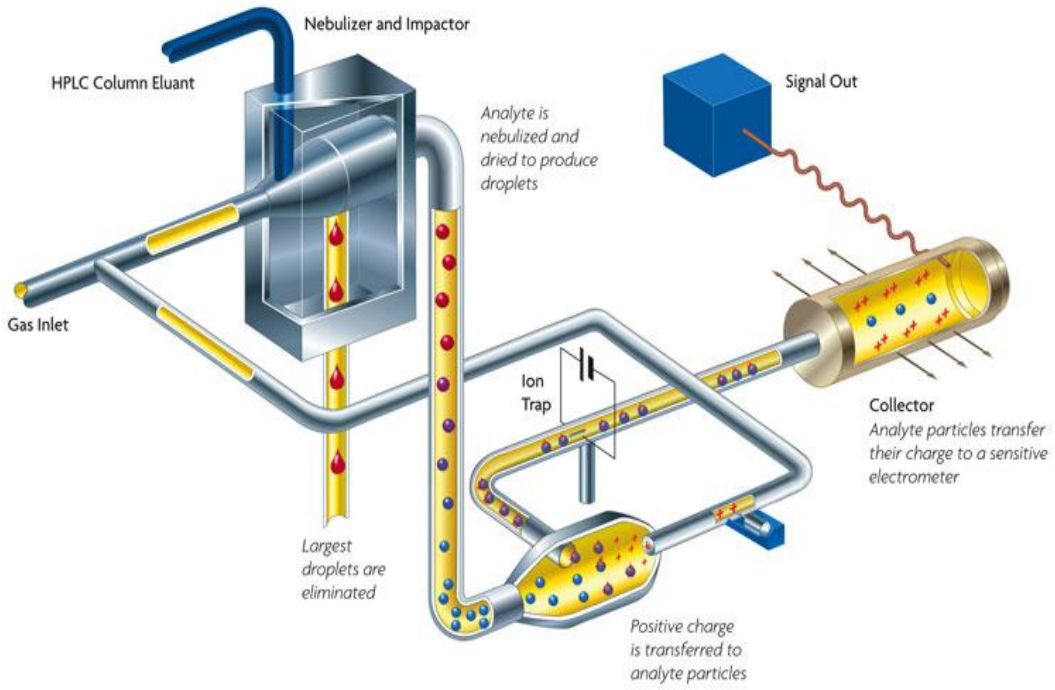


Figure 2.6 Schematic diagram of a charged aerosol detector [49]

Charged aerosol detection was introduced by Dixon and Peterson and this is based on aerosol detection mode. The detection mechanism involves a three main stage namely: nebulization, evaporation and detection [50]. Detection of analytes starts after the analytes moves through the separation column, the HPLC eluent is transferred to the CAD where it is nebulized by means of a small pressure effect produced by nitrogen (a carrier gas). In this situation, nitrogen flows in a two or three dimensional way to the mobile phase eluting from the chromatographic column.

The nebulized eluent is transforms the liquid phase into small droplets which are produced into a heated drift tube. In the drift tube, a secondary stream of nitrogen becomes positively charged as it passes through a high-voltage, platinum corona wire. This charge transfers to the opposing stream of analyte particles and is then transferred to a collector where it is measured by a highly sensitive electrometer as shown in figure 2.6. The detection of the resultant analyte takes place through the electrical charging of the aerosol and the charged particles using an electrical aerosol analyser The signal intensity generated by a charged aerosol detector (CAD) is said to be directly proportional to the analyte concentration [49, 50]. CAD does not depend on certain functional groups or moieties within the molecules when compared with other detection method like UV, and FLD. Therefore a similar detector response can be expected for different compounds [50], a considerable advantage when screening for impurities where no structural information is available or where the detector response is unknown.

CAD appears to be more sensitive, with a greater linear dynamic range, more consistent mass response factor, and easier operation enabling quantification across a range that exceeds four orders of magnitude. It has a more consistent analyte response; it has a compatible gradient mode. Unlike UV/Vis, CAD detects analytes with or without chromophores and no derivatization step is need before analysis of target compounds. CAD also has the same limitation as other types of detector such as the response is affected by density and viscosity

of the mobile-phase composition. Higher organic content in the mobile phase leads to greater transport efficiency of the nebulizer, which results in a larger number of particles reaching the detector chamber and hence affecting the signal [50]. This problem has been resolved by introducing secondary stream nitrogen to the mobile phase of exactly reverse composition, this is provided by a second pump, this ensures a constant mobile-phase composition at the detector inlet. Due to this procedure, a constant response that is independent of the mobile-phase composition is usually obtained [50]. This procedure known as inverse gradient compensation is done in high pressure liquid chromatography and supercritical fluid chromatography. However, more studies are needed to fully investigate the effects of these solvents.

According to Andruch in 2013 [116], various analytical techniques of HPLC, GC and LC have been used in the determination of steroid hormones in environmental samples. To the best of our knowledge, this is the first time that HPLC-CAD has been used in the determination of steroid hormones in wastewater samples.

CHAPTER 3

EXPERIMENTAL

3.1 Chemicals and reagents

The five steroid hormones studies in this work included estriol (E3), beta estradiol (β-E2), alpha estradiol (α-E2), testosterone (T) and progesterone (P). Bisphenol A was also included in the study because it has been classified as an EDC since it has the ability to mimic estrogenic activities in the body. Table 3 provides information about the compounds used in this study, in addition to their names, CAS numbers, purity and molecular weight. They were all purchased from sigma Aldrich (Steinheim Germany). Ultra high purity (UHP) water of 18.2 Ω cm resistivity was generated from the MilliQ system, Millipore (Billerica, MA, USA). The UHP water was used to prepare all standards in this study. All reagents used as extracting solvents in the experiments were of analytical grade and were purchased from Sigma Aldrich, (Steinheim, Germany). These include methanol, acetonitrile, acetone, chloroform ($CHCl_3$), tetrachloroethylene ($Cl_2C=CCl_2$), chlorobenzene (C_6H_5Cl), dichloromethane (CH_2Cl_2) and 1, 2-dichloroethane ($C_2H_4Cl_2$). All glassware was washed with distilled water and rinsed with UHP water, dried in the oven and allowed to cool prior to use.

Table 3.1 Properties of standard compounds used in this study

Compound	CAS	Purity (%)	Molecular Weight (g/mol)
ESTRIOL (E3)	50-27-4	99.9	288.4
BISPHENOL A (BPA)	80-05-7	99.9	228.29
BETA ESTRADIOL (βE2)	50-28-2	99.9	272.38
ALPHA ESTRADIOL (αE2)	57-91-0	99.9	272.4
TESTOSTERONE (T)	58-22-0	99.9	288.42
PROGESTERONE (P)	57-83-0	99.9	314.46

3.2 Instrumentation

For chromatographic separations, a Dionex UltiMate 3000 HPLC series (Dionex Corporation Sunnyvale, CA), consisting of a Dionex UltiMate 3000 series quaternary pump and an autosampler was used. The HPLC was connected to a charge aerosol detector (CAD) (Thermo Scientific™ Dionex, Corporation Sunnyvale, USA) and the Chromelon version 6.0 software was used for instrument control and data processing. Figure 3.1 shows a pictorial image of an HPLC-CAD. The separation of steroid hormones was achieved using a Zorbex Eclipse analytical column XDB-C8 (4.5 mm x 150 mm, 5μm particle size), using an isocratic mode with a mixture of methanol and water as the mobile phase (70/30, v/v). The injection volume was 5 μL and the separation was done at a flow rate of 1.2 mL/min.

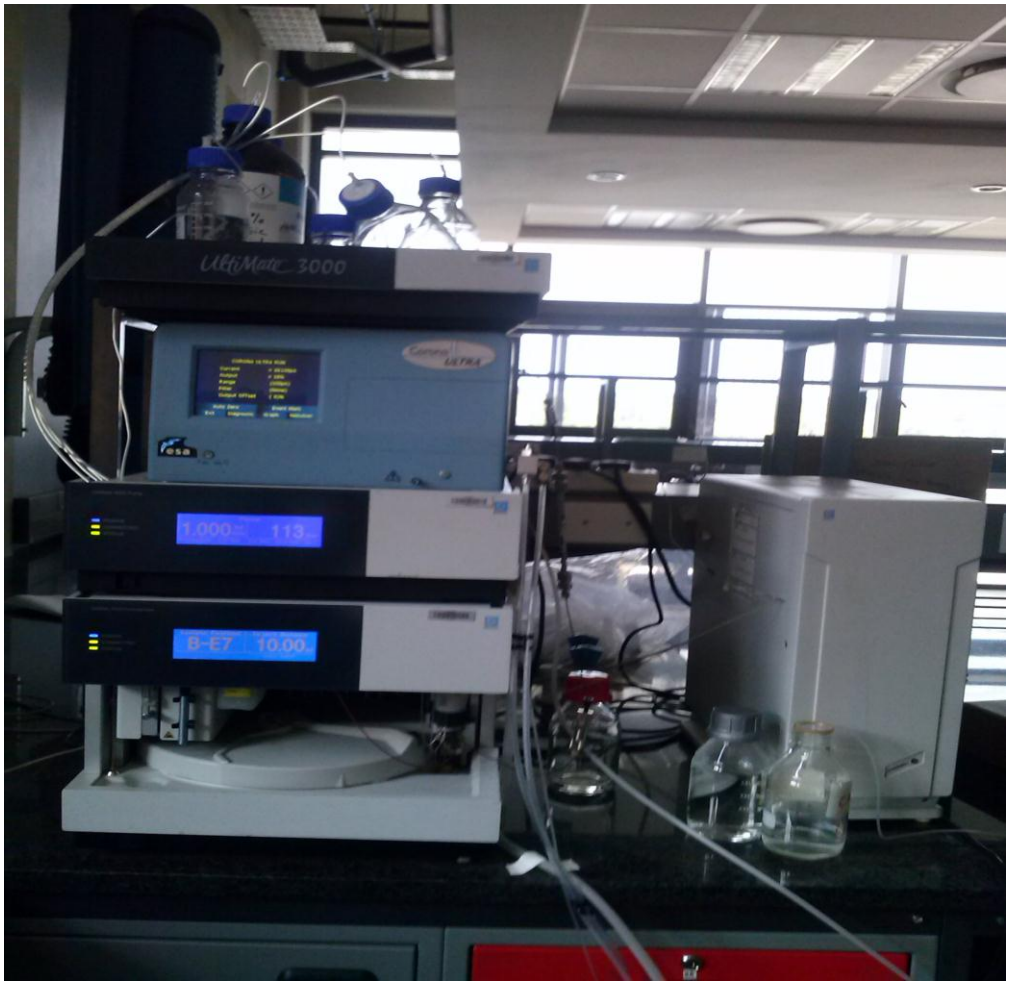


Figure 3.1 Picture of the HPLC-CAD

For sample preparation and drying purposes, a Thermo Electron centrifuge (Thermo Electron Corporation, Massachusetts, USA) and a Thermo Electron SpeedVac concentrator (Model SPD131DDA) coupled to a refrigerated vapor trap (Model RVT4104), and vacuum pump (Model OFP400) (Thermo Scientific, CA, USA) were used.

3.3 Preparation of standard solutions

A stock standard solution of a concentrate on of 1000 mg/L was prepared by mixing 1 mg of accurately weighed five steroid hormone standards and BPA (on a micro balance-Mettle Telodo XP6U). The accurately weighed standards were dissolved and diluted to volume with a mixture of methanol and water in the ratio (50/50 v/v). It was sonicated for 10 minutes to dissolve the analytes. Working standard solutions were then prepared by appropriate serial

dilutions of the stock solution and adjusted to volume with methanol and water mixture. These were all stored at 4^0C until ready for use.

3.4 Sample collection

In analytical chemistry, sampling is an important experimental procedure in the determination of organic contaminants in wastewater. Wastewater samples (influent and effluent) were collected from Daspoort, a WWTP located in Pretoria, one of the major cities in Gauteng Province, South Africa. Figure 3.2 shows the map of the sampling site of the WWTP. The Daspoort WWTP discharges into a neighbouring Apies river in its surroundings. Grab sampling method was used and the samples were collected into amber glass bottles, which had previously been washed and rinsed with UHP water and then flushed at least thrice with wastewater before collection. Each sample (influent and effluent) was collected in duplicate into 2.5 L bottles. All water samples were ice-packed during transportation to the laboratory. The samples were vacuum filtered immediately through the Whatman (MN 615. 125 mm) filter paper and extracted immediately to avoid degradation. The samples were then stored at < 4 ^{o}C in the dark until ready for analysis.

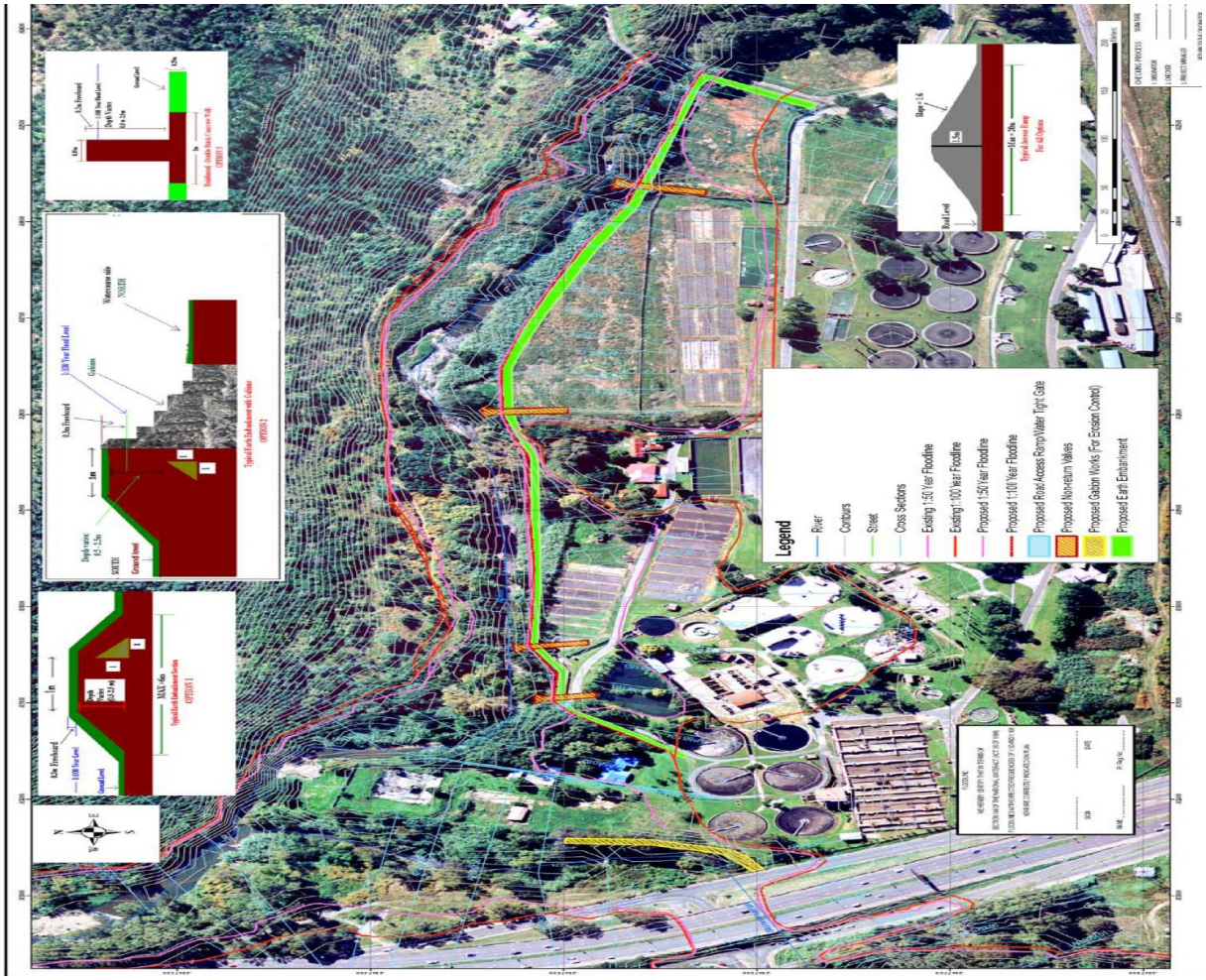


Figure 3.2 A map showing the locality of the Daspoort WWTP in Pretoria

3.5 Calibration standard solutions

Standard solutions for the calibration curve were prepared in a mixture of methanol and water (50/50 v/v %) at a concentration range of 0.1 to 100 mg/L. The calibration standards were made by serial dilution of the stock solution and the calibration curves were calculated using the linear regression model.

3.6 Validation of the HPLC-CAD method

The developed separation method for the five steroid hormone and BPA was achieved using a Zorbex Eclipse analytical column XDB-C8 (4.5 mm x 150 mm, 5µm particle size), using isocratic mode with a mixture of methanol and water as the mobile phase (70/30, v/v). The

developed method was evaluated by using the following parameters: linear range, correlation of determination (R^2), limit of detection (LOD), limit of quantification (LOQ) and intra-day precision. For recovery experiments, MilliQ, tap water and wastewater (effluent) were spiked with a mixture of all the analytes at three different concentration levels (5, 10 and 25 mg/L).

In this work the limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected but not necessarily quantified. LOD was calculated using the signal to noise ratio (S/N =3), which was then multiplied by the standard error (S) obtained from the regression equation of the calibration curve. The value obtained was divided by the slope (b) of the line of regression of y = bx + c in the calibration curve. It is represented mathematically as;

$$LOD = 3 * S/b \quad (1)$$

Limit of quantification (LOQ) is the minimum concentration that can be quantified at a specific concentration level. LOQ in this study was calculated using the signal to noise ratio (S/N =10), and then multiplied by the standard error (S) obtained from the calibration. The value obtained was divided by the slope (b) of the analytes in the calibration curve. It is represented mathematically as;

$$LOQ = 10 * S/ b \quad (2)$$

An eight point calibration line was prepared at different concentration level. The recovery experiments were carried out by spiking steroid hormone standards into MilliQ, tap water and wastewater at different concentrations levels in triplicate.

3.7 Developing a DLLME method for the extraction of steroid hormones

A 5.0 mL water sample was placed in a 10 mL capped tube with a conical bottom and spiked with 0.01 mL of a mixture of five steroid hormone standards plus BPA. Extracting solvents such as; 1, 2 dichloroethane, chlorobenzene, chloroform, dichloromethane and

tetrachloroethylene were used during the preliminary extraction experiment. Acetonitrile, acetone and methanol were also investigated as suitable dispersive solvents during the preliminary stages. Figure 3.3 below shows the extraction of target analytes using DLLME procedure, and a cloudy solution was produced.

Under optimized conditions a 35 µL aliquot of tetrachloroethylene (extracting solvent) and 0.50 mL of methanol (dispersal solvent) were injected rapidly into the sample solution. A cloudy solution was formed in the conical test tube and then centrifuged for 5 min at 4000 rpm. The lower/organic phase was withdrawn with a micro syringe and transferred into a 2 mL centrifuge tube for evaporation of the solvent using a SpeedVac. The residue was reconstituted in 50 µL of HPLC grade methanol and injected into the HPLC-CAD for analysis. All the experiments were performed in triplicates.

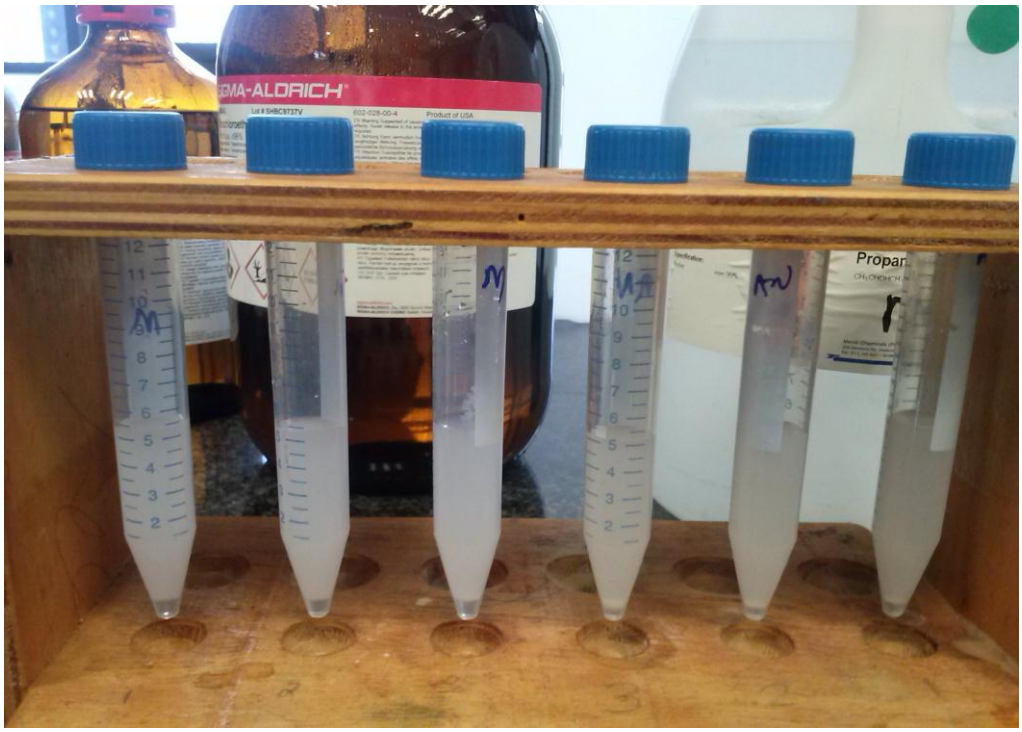


Figure 3.3 Cloudy solution produced during DLLME procedure

3.7.1 Optimization of DLLME Method

The extraction efficiency of DLLME is influenced by several factors such as type and volume of extraction and disperser solvent, extraction time, sample amount, pH, and salt addition [55-56]. In this study the parameters optimized were the type and volume of extracting and dispersing solvent. A series of preliminary experiments were performed using five extraction solvents and three dispersive solvents to determine which best system was favorable for the target analytes. The extracting solvents must have a density greater than water; the disperser must also be soluble in the extracting solvent and miscible with water. The optimal volume of extractants (35 µL) and disperser (500 µL) were also investigated to ensure high extraction efficiency.

3.7.2 Validation of the DLLME method

The proposed DLLME method was validated under optimized conditions for the following analytical characteristics: the LOD, LOQ, and coefficient of determination (R^2) and linearity. Relative standard deviation was used to express repeatability. The extraction recoveries and enrichment factors were also determined. The validation data is presented in Table 4. Linearity was established and the LODs and LOQs were calculated as described in Section 3.6.

3.8 Analysis of real wastewater samples

The developed and validated DLLME and HPLC-CAD methods were applied to influent and effluent wastewater samples collected from Daspoort WWTP, in Gauteng South Africa. Sampling was done during the winter season, and samples were collected in duplicates. Wastewater samples were first vacuum filtered using the Whatman filter paper. 5 mL of the water sample was placed in a 10 mL capped tube with a conical bottom. The target analytes were extracted under optimized DLLME conditions. 35 µL aliquot of tetrachloroethylene (extracting solvent) and 0.50 mL of methanol (dispersal solvent) were injected rapidly into

the sample solution. A cloudy solution was formed in the conical test tube and then centrifuged for 5 min at 4000 rpm. The lower/organic phase was withdrawn with a micro syringe and transferred into a 2 mL centrifuge tube for evaporation of the solvent using a SpeedVac. The residue was reconstituted in 50 µL of HPLC grade methanol and injected into the HPLC-CAD for analysis.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Method validation of chromatographic separation of steroid hormones using HPLC-CAD

A previously developed chromatographic separation method was validated for the determination of five steroid hormones and BPA in wastewater. The analytes were separated using a Zorbex Eclipse C8 column (4.5 mm x 150 mm, 5µm particle size) and a mixture of methanol and water as the mobile phase (70/30%, v/v) at a flow rate of 1.2 mL/min. Figure 4.1 shows a typical chromatogram obtained for the separation of five steroid hormones and BPA using the HPLC-CAD. The conditions for the corona detector include a gas pressure at 35.0 psi, with a range of 100p-A, and the nebulizer heat was on.

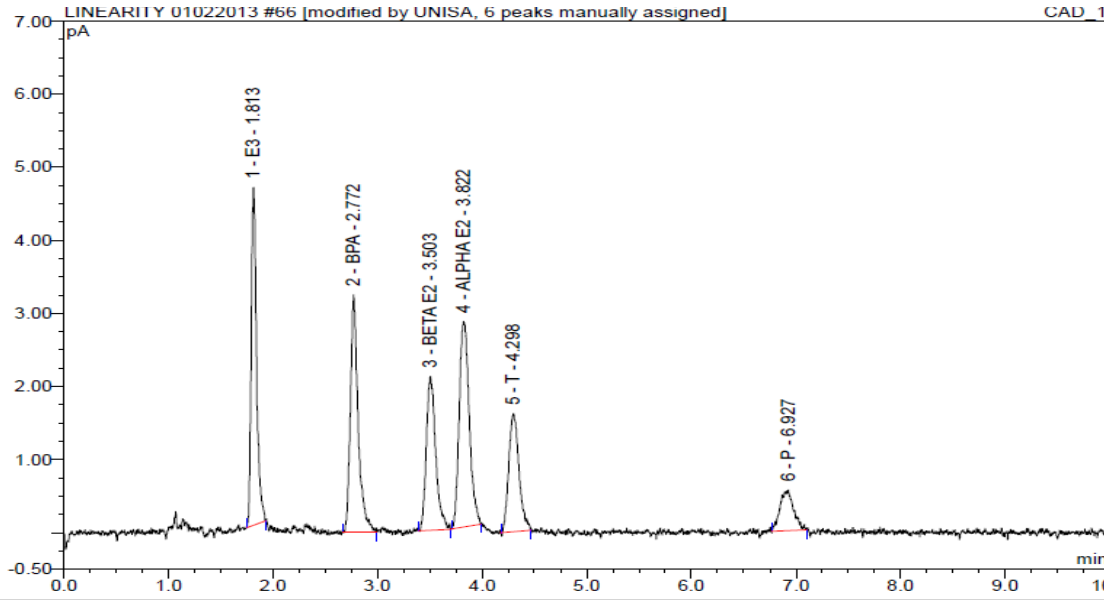


Figure 4.1 Chromatographic separations of five steroid hormones and BPA using Eclispe XDB-C8 4.6 x 150 mm, 5 µm. Mobile phase: methanol/water; (70/30 v/v %), at a flow rate of 1.2 m L/min for 10 minutes at a 10 mg/L concentration level

For method validation, linearity, coefficient of determination (R^2), limit of detection, limit of quantification, and reproducibility were determined and the results are summarized in Table 4.1**.** The LOD was determined based on a signal-to-noise ratio (S/N) of 3, and the LOQ was defined as a signal-to-noise ratio (S/N) of 10**.** The % RSD was used to express the precision and repeatability of the method.

4.1.1 Linear range and working range

A range is the interval between the upper and lower concentration of analytes in the sample. Calibration solutions of the working range were made by serial dilution of the stock solution. The working range of this method was between 0.1 to 100 mg/L. Linearity of an analytical procedure is the region of a calibration curve within which a plot of the concentration of an analyte versus the response of that particular analyte remains linear and the correlation coefficient of the line is approximately 1. The method was found to be linear at concentration level of 0.3 – 20 mg/L. Linearity of the method was tested with standard steroid hormone mixtures containing E3, BPA, β-E2, α-E2, T and P using eight concentration levels. The number of data points or concentration level was represented as n (i.e. n = 8*)*, number of the replicates of the concentration level was denoted by m (i.e. m = 6*)*, and the number of injection of each concentration level as x (i.e. x = 3*)*. The calibration graphs were linear at 0.3 – 20 mg/L, this shows a good relationship between concentration and peak areas over a concentration range for all the target analytes. Above the 50 mg/L or higher concentration level, the response of the analytes signal versus concentration tends to deviate from linearity. The coefficient of determination for the compounds ranged from 0.9955 - 0.9997 for the target analytes.

4.1.2 Limit of detection

The LOD was obtained from the peak areas of the compounds analyzed at different concentration levels. It was defined as 3* S/ b, where S is the standard error and b is the slope

of the calibration curve. Calibration was performed on a mixture of six standard solutions of the compounds of interest. The calibration curves were constructed using the eight concentration levels (i.e. n = 8). A sample solution at each concentration level was prepared 6 times (m= 6) and it was injected in triplicate (mx_3). The calibration plots were approximated by the linear regression equation, y = bx + c, where y is the peak area and x is the concentration of determination compounds in mg/L. The results for the limit of detection are summarized in Table 4.1. The LOD for E3 and P were 0.1 mg/L, while BPA, β-E2, α-E2 and T was 0.2 mg/L. These values obtained are very high and at this concentration levels steroid hormones will not be detected in the environmental samples. When these values were compared to the detection limits obtained from a GC-MS or LC-MS, it is deduced that there is a need for a cost friendly sample preconcentration technique, since the target analytes are only found at lower concentration levels of µg/L or ng/L in the environment.

4.1.3 Limit of quantification

LOQ is defined as the minimum concentration that can be quantified at a specific level. It was defined as 10 * S/b, where S is the standard error and b is the slope of the calibration curve. Calibration was performed on a mixture of six standard solutions of the compounds of interest. The calibration curves were constructed using the eight concentration levels (i.e. n = 8). A sample solution at each concentration level was prepared eight times (m= 8) and it was injected in triplicate (mx_3). The calibration plots were approximated by the linear regression equation, y = bx + c, where y is the peak area and x is the concentration of determination compounds in mg/L. The results for the limit of quantification are summarized in Table 4.1. The LOQ for E3 was 0.3 mg/L, 0.7 mg/L for BPA, β-E2 was 0.8 mg/L, α-E2 was 1.1 mg/L, T was 0.9 mg/L and P was found at 0.8 mg/L. With these high values obtained, the target analytes cannot be quantified in the environment because they are found at lower quantification levels. This suggests that there is a need for a preconcentration procedure that will help to increase the sensitivity of the method on the HPLC-CAD.

Table 4.1 Validation parameters for the proposed HPLC-CAD method

Compound	*Range (mg/L)*	*Regression equation*	R^2	*SD*	*(LOD) (mg/L)*	*(LOQ) (mg/L)*
E3	0.3- 20	y = 0.1224x + 0.128	0.9955	0.01	0.1	0.3
BPA	0.3- 20	y = 0.086x + 0.0056	0.9997	0.01	0.2	0.7
β-E2	0.3- 20	y = 0.0377x + 0.0175	0.9994	0.01	0.2	0.8
α-E2	0.3- 20	y = 0.0281x + 0.0469	0.9992	0.02	0.2	1.1
T	0.3- 20	y = 0.0488x+ 0.0092	0.9993	0.01	0.2	0.9
P	0.3- 20	y = 0.0935x + 0.0154	0.9992	0.01	0.1	0.8

4.1.4. Accuracy and recovery

Accuracy of an analytical procedure refers to the closeness of agreement between the conventional true value or an accepted reference value and the value found. Percentage recoveries were obtained from the difference between the peak area response of the spiked samples and unspiked samples from the calibration curve. The experiment was carried out using different concentration levels of steroid hormone standards in wastewater, tap water and MilliQ. The mean recovery obtained for MilliQ was 81.45 – 101.0%, for tap water 83.92 – 104.1, and for wastewater it was between 88.71 and 110%. This indicated that the method is very accurate due to the fact that all the target analytes were recovered totally almost above 80% in the various matrices that they were applied into. The results showed that the different matrices used had no interference effect on the recoveries at 5, 10 and 25 mg/L. The performance of the proposed method in the extraction from the spiked genuine water samples was acceptable, which also demonstrates the feasibility of the proposed method for environmental aqueous sample analysis, but the overall analysis was not significantly affected. The average results obtained are presented in Tables 4.2 – 4.4 for each of the concentration levels.

Table 4.2 Steroids hormones recovered after spiking water samples at 5 mg/L (m = 6)

COMPOUND	*MILLIQ Mean Recovery %*	*TAP Mean Recovery %*	*WASTE Mean Recovery %*	*%RSD*
E3	81.41 ± 2	83.92 ± 4	88.71 ± 6	1.1
BPA	95.15 ± 5	99.0 ± 5	101.4 ± 8	1.2
β-E2	96.44 ± 5	100.2 ± 4	99.9 ± 5	2.3
α-E2	96.80 ± 2	99.1 ± 5	99.7 ± 5	2.5
T	85.0 ± 5	88.2 ± 3	90.1 ± 8	1.7
P	101.0 ± 3	104.1 ± 4	110.0 ± 8	1.4

Table 4.3 Steroids hormones recovered after spiking water samples at 10 mg/L (m = 6)

COMPOUND	*MILLIQ Mean Recovery %*	*TAP Mean Recovery %*	*WASTE Mean Recovery %*	*%RSD*
E3	100.04 ± 3	116 ± 3	98.2 ± 5	2.1
BPA	101.2 ± 4	112 ± 3	94.1 ± 5	2.3
β-E2	108 ± 4	107.2 ± 4	95.2± 8	1.1
α-E2	100.01 ± 3	98 ± 6	92.4 ± 6	1.6
T	85.45 ± 3	95.2 ± 5	98.44 ± 6	1.1
P	98.5 ± 5	101 ± 5	112.1 ± 5	1.1

Table 4.4 Steroids hormones recovered after spiking water samples at 25 mg/L (m= 6)

COMPOUND	*MILLIQ Mean Recovery %*	*TAP Mean Recovery %*	*WASTE Mean Recovery %*	*%RSD*
E3	106.04 ± 3	110 ± 4	99.18 ± 6	2.7
BPA	103 ± 3	106 ± 3	109 ± 6	2.8
β-E2	102 ± 4	101 ± 4	103.1 ± 5	1.1
α-E2	101.1 ± 3	103 ± 5	99.92 ± 6	1.2
T	98.22 ± 4	105 ± 5	104.8 ± 5	1.5
P	95.11 ± 3	102 ± 5	110 ± 5	1.5

4.1.5 Precision

Precision is the agreement between a set of replicate measurements under a prescribed condition. Precision may be classified into three levels: repeatability, intermediate precision and reproducibility. The repeatability of this method was expressed as the precision obtained when the experiment was performed under the same operating conditions over a period of

time. Table 4.5 shows that the relative standard deviation (% RSD) of the mean values found were 1.7 – 2.2%. This RSD value indicates that the method repeatability is satisfactory.

The intermediate precision was obtained when the experiment was performed on different days. The values obtained, which are reflected in Table 4.6, revealed that the method was precise and rugged with RSD values below 2.5. Peak areas were measured on different days at the same concentration. The compounds were quite stable and decomposition was only observed after 3 months of preparation.

Table 4.5 Repeatability (Intra-day precision)

ANALYTE	MEAN PEAK AREA	SD	%RSD
E3	0.72	0.01	1.7
BPA	0.72	0.01	1.8
β-E2	0.71	0.01	2.0
α-E2	0.42	0.02	2.2
T	0.60	0.01	1.7
P	0.46	0.02	2.1

Table 4.6 Repeatability (Inter-day precision)

ANALYTE	*DAY 1*	*DAY 2*	*DAY 3*	*DAY 4*	*DAY 5*	*MEAN*	*SD*	*%RSD*
E3	0.7163	0.7222	0.6538	0.7222	0.7222	0.71	0.03	1.2
BPA	0.7146	0.7352	0.7352	0.7351	0.7351	0.73	0.01	1.2
β-E2	0.7065	0.7138	0.6812	0.7138	0.6978	0.70	0.01	1.9
α-E2	0.4172	0.4247	0.3970	0.3970	0.3839	0.40	0.01	1.4
T	0.5987	0.6088	0.5427	0.5987	0.5535	0.58	0.03	1.1
P	0.4632	0.4674	0.4481	0.4380	0.4122	0.45	0.02	1.9

4.1.6 Selectivity of the HPLC-CAD method

Selectivity is the ability to distinguish and quantify the response of the target analytes from the response of all other compounds present in the medium. Under the optimal chromatographic conditions used in the method development, the target analytes selectivity was evaluated based on the retention times of the analytes of interest (E3, BPA, β-E2, α-E2, T and P). Even during the optimization of the DLLME step (i.e. with the addition of other species), the retention time of each of the analytes were still the same, the addition of other species didn't have effect on the separating time. The findings are expressed in Table 4.7.

It was observed that the retention time of each compound and peak area responses were consistent based on the values of relative standard deviation.

Table 4.7 Selectivity Evaluation by comparing the retention time, n = 10 injections

COMPOUND	RTs DAY1	RTs DAY2	RTs DAY3	RTs DAY4	RTs DAY5	MEAN RTs	SD	%RSD
E3	1.82	1.83	1.85	1.85	1.85	1.85	0.01	0.5
BPA	2.88	2.84	2.87	2.89	2.88	2.88	0.03	1.2
BETA E2	3.57	3.64	3.67	3.73	3.68	3.65	0.06	1.6
ALPHA E2	3.89	3.95	4.01	4.06	4.02	3.98	0.06	1.6
T	4.38	4.45	4.52	4.58	4.53	4.49	0.07	1.3
P	7.12	7.18	7.31	7.42	7.32	7.25	7.14	1.9

4.1.7 Robustness

Ruggedness or robustness is a measure of an analytical procedure's capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. For the determination of the robustness of this method, some parameters, such as flow rate, injection volume and mobile phase, were varied. The quantitative influence of the flow rate and injection volume variables was determined and is shown in Table 4.8 – 4.9. When the flow rate was adjusted there was only a slight change in the retention times. When the injection volume was increased, it was observed that there was no impact on the retention time as long as the plate number stays constant and there is no over loading; therefore the separation remains the same. However with a small injection volume, it is assumed that all the sample molecules must arrive at the head of the column at the same time. Thus when large volumes samples are injected, the peak area and peak height are expected to grow in proportion to the injected volume. When the injection volume becomes high, the front edge of the peak behaves normally, but the back edge of the peak is shifted thus resulting to a reduced resolution and tailing peaks. Figures

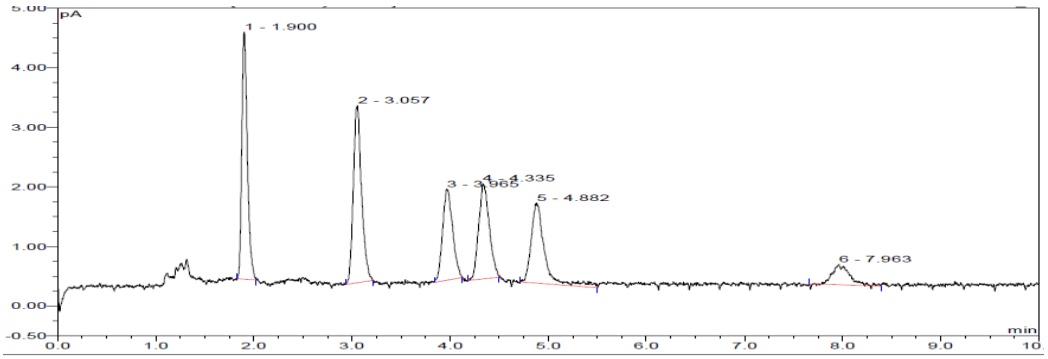
4.2a and 4.2b shows the chromatograms obtained during this experiment. The % RSD obtained showed the robustness of the method, which was satisfactory.

Table 4.8 Variation of the flow rate from 0.7 mL to 1.0 mL (*m = 6)*

Compound	*0.7mL (Av. peak .area)*	*RTs 0.7mL*	*% RSD*	*RTs 1.0 mL*	*1.0 mL (Av. peak area)*	*% RSD*
E3	0.16	2.31	0.7	2.26	0.15	0.4
BPA	0.21	3.22	1.0	3.66	0.36	1.0
β-E2	0.44	5.20	1.3	4.77	0.39	1.1
α-E2	0.55	6.78	1.5	5.22	0.45	1.4
T	0.63	7.43	1.2	5.86	0.38	1.1
P	0.54	8.36	1.0	9.51	0.19	1.3

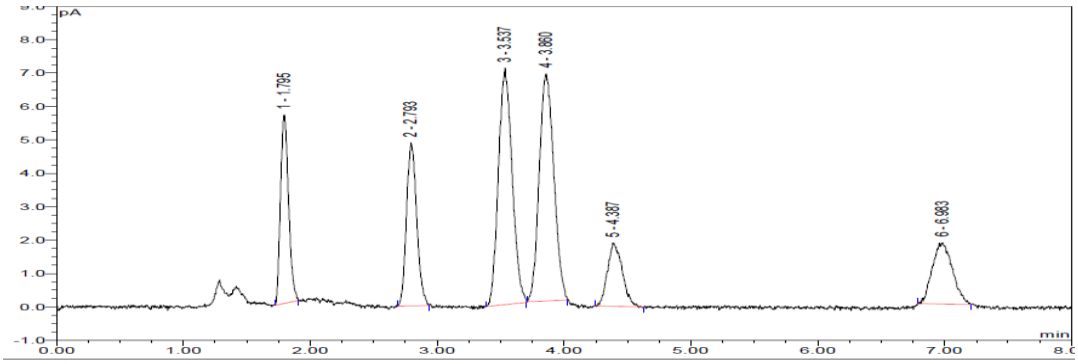
Table 4.9 Variation of the injection volume between10 µL and 20 µL *(m = 6)*

Compound	*10µL (Av. peak)*	*RTs (10 µL)*	*% RSD*	*20µL (Av. peak)*	*RTs (20 µL)*	*% RSD*
E3	0.2766	1.90	0.3	0.4651	1.89	0.5
BPA	0.2881	3.06	0.9	0.4051	2.93	0.2
β-E2	0.1792	3.97	1.1	0.8042	3.86	0.7
α-E2	0.2001	4.34	1.2	0.900	4.23	0.6
T	0.2133	4.88	0.9	1.289	4.74	1.2
P	0.0954	7.96	1.1	0.314	7.74	0.9



No.	Ret.Time min	Peak Name	Height pA	Area pA*min	Rel.Area %	Amount	Type
1	1.90	n.a.	4.152	0.276	22.54	n.a.	BMB
2	3.06	n.a.	2.974	0.288	23.53	n.a.	BMB
3	3.97	n.a.	1.533	0.179	14.62	n.a.	BMB
4	4.34	n.a.	1.601	0.200	16.36	n.a.	BMB*
5	4.88	n.a.	1.345	0.213	17.39	n.a.	BMB*
6	7.96	n.a.	0.347	0.068	5.55	n.a.	BMB*

Figure 4.2a: Chromatogram obtained by injecting 10µL volume sample



No.	Ret.Time min	Peak Name	Height pA	Area pA*min	Rel.Area %	Amount	Type
1	1.80	n.a.	5.658	0.418	12.83	n.a.	BMB
2	2.79	n.a.	4.885	0.457	14.03	n.a.	BMB
3	3.54	n.a.	7.097	0.885	27.16	n.a.	BMB
4	3.86	n.a.	6.815	0.907	27.84	n.a.	BMB
5	4.39	n.a.	1.898	0.254	7.79	n.a.	BMB*
6	6.98	n.a.	1.841	0.338	10.36	n.a.	BMB*
Total:			28.194	3.260	100.00	0.000	

Figure 4.2b: Chromatogram obtained by injecting 20µL volume sample

4.2 DLLME determination for steroid hormones

Despite the advances in analytical instruments in recent years, sample preconcentration procedures must first be considered before the instrumental determination of steroid hormones in environmental samples. DLLME was introduced by Assadi in 2006 and is an extraction method based on a ternary component solvent system like the homogeneous liquid-liquid extraction (HLLE) and cloud point extraction (CPE). It is a simple and fast microextraction technique based on the use of an appropriate amount of high-density

extraction solvent such as chlorobenzene, tetrachloroethylene, chloroform or carbon tetrachloride and a dispersive solvent such as methanol, acetonitrile, ethanol or acetone with high miscibility in both extraction organic solvent and aqueous phase [55-56]. The extraction solvent is fully dispersed into the aqueous sample as very fine droplets by gently shaking and a cloudy solution is formed, into which the analytes are enriched. Owing to the considerably large surface area between the extraction solvent and the aqueous sample, the equilibrium is achieved quickly and the extraction is independent of time, which is the principal advantage of DLLME. After centrifugation of the cloudy solution, the organic phase that is enriched with analytes settles at the bottom of the conical test tube and it is then collected for instrumental analysis.

Due to its important advantages such as negligible or micro litre volume of extracting solvent, equilibrium state is obtained within a short time. The enrichment factor (EF) and recovery are due to high phase ratio of the donor (aqueous sample) and the acceptor (extraction solvent). DLLME has been widely used by many researchers for the determination of many kinds of PPCPs in the environment. Steroid hormones are detected in the environment in concentrations ranging from micro (µg/L) to nano (ng/L). Based on the HPLC-CAD developed method, the instrumental limit of detection was in the range (0.1- 0.2 mg/L) and the instrumental limit of quantification (0.3- 1.1 mg/L). The above limit of detection does not have the ability or potential to determine the analytes of interest in environmental samples. Incorporation of a preconcentration procedure is required to increase the sensitivity of analytical method in other to achieve a lower concentration level [37].

This setback has led to the choice of DLLME as a preconcentration method due its attractiveness of high enrichment factors [45-46, 55-56]. DLLME has been used in the determination of BPA, and recovery of 93.4 % was obtained. The LOD of BPA in the water sample was 0.07 µg/L [45]. In another study, estrone, 17β-estradiol and diesthylstilbestrol were determined in aqueous sample using DLLME. The enrichment factors and extraction

recovery were 71-79 and 85-94 respectively and LOD of 0.08 for estrone and 17β-estradiol, while diesthylstilbestrol was detected at 0.10 µg/L [46].

Steroid hormones are nonpolar hydrophilic compounds. The water solubility of steroid hormones is low and range from 0.3-13 mg/L with the natural steroids having the highest solubility and high solubilities in organic solvents. The synthetic steroids such as 17-ethinylestadiol and menstranol have the highest octanol-water partition coefficients (log Kow) of 4.15 and 7.5 respectively. The natural steroid hormones have lower log Kow of 2.81 for estriol, 3.94 for 17β-estradiol and 4.01 for 17α-estradiol. All steroids have very low vapor pressures of 2.3-6.7 $x10^{-10}$ mm Hg and relatively high pKa-values above 10 [37, 115]. Steroid hormones are neutral compounds that can be readily extracted from the aqueous phase into the organic phase using DLLME procedure.

4.3 Parameters affecting the extraction efficiency of DLLME

4.3.1 Selection of extracting solvent

The first step in the development of the DLLME method was to identify a suitable extraction solvent. A suitable extraction solvent should possess the following characteristics: ability to extract the analytes of interest, good chromatographic capabilities and a density higher than water, which will allow the extraction solvent to separate from the aqueous phase during the centrifugation process [47, 56-57,116-117]. In order to evaluate and optimize the DLLME method the enrichment factor (EF) was calculated and it was defined as the ratio of the analyte concentration in the sedimented phase (C_{sed}) to the initial concentration of the analyte in the aqueous solution (C_o) [55-56].

$$EF = \frac{C_{sed}}{C_o} \quad (1)$$

The C_{sed} of each analytes was obtained from the calibration curves. The extraction efficiency (EE %) was defined as a percentage of the total analyte amount, extracted to the sedimented phase.

$$EE = \frac{C_{sed} \times V_{sed}}{C_o \times V_o} \times 100 \quad (2)$$

where V_{sed} and V_o are volumes of the sedimented phase and the initial volume respectively.

Based on the above conditions, five solvents that meet desired requirements were identified and investigated. These included dichloromethane, ρ = 1.33 g/cm^3, chloroform, ρ = 1.48 g/cm^3, tetrachloroethylene, ρ = 1.62 g/cm^3, chlorobenzene, ρ =1.11 g/cm^3, and 1,2-dichloroethane ρ = 1.253 g/cm^3. For each solvent, three replicate extractions were performed. Figure 4.3 clearly shows the extraction efficiencies of each of the extracting solvent on the target analytes. Dichloromethane, chloroform and chlorobenzene gave very low extractions efficiencies of 15-28%, 19-38% and 30-60%. Chloroform and chlorobenzene have been used elsewhere as extraction solvents in the DLLME extraction of BPA in water samples and extraction efficiencies of 33.9% and 45.2% respectively were obtained [45]. These values are comparable to our own finding in as much as a smaller volume of the extracting solvent was used in the current study. 1,2 dichloroethane and tetrachloroethylene gave higher extraction efficiencies with tetrachloroethylene giving the highest values ranging from 65- 85% for most of the analytes. This was probably due to the solubility of the target analytes in tetrachloroethylene which is the organic phase. This is probably due to the higher solubility of the target analytes in tetrachloroethylene in comparison to the other extracting solvents. The target analytes were readily extracted more into the organic phase. This led to the choice of tetrachloroethylene as the best extracting solvent.

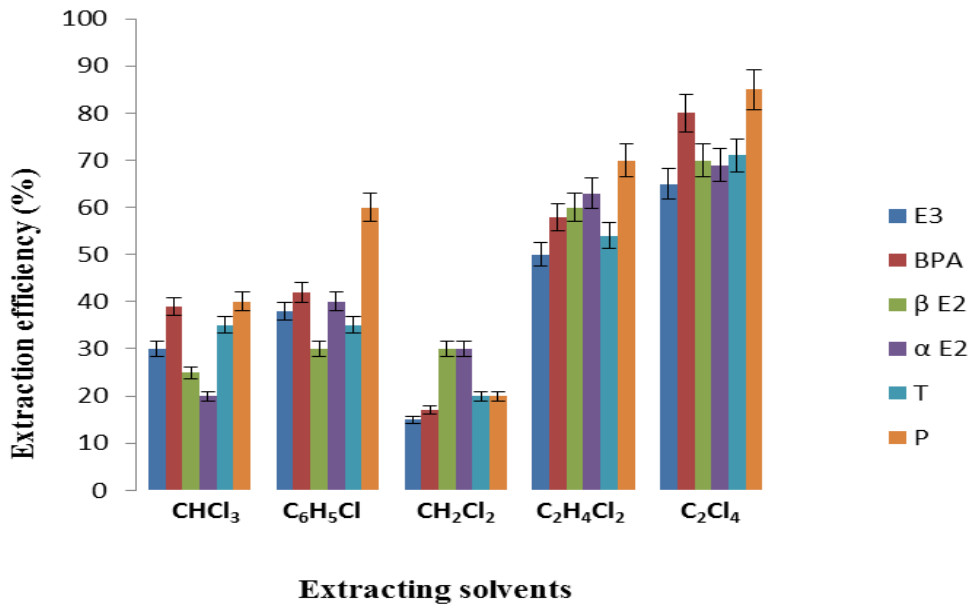


Figure 4.3 Effect of different extracting solvents on the target analytes m= 4.

4.3.2 Selection of disperser solvent

The choice of a disperser solvent, which aids in dispersing the extraction solvent into fine droplets, must obey some criteria, such as dissolving in both the extraction solvent and aqueous phase to produce a cloudy solution [55-56]. Based on these criteria, three solvents were tested, namely acetone, acetonitrile and methanol. For this experiment 100 µL of tetrachloroethylene was used as the extraction solvent, while varying the type of dispersive solvent at 750 µL. Similarly, the experiment was carried out in triplicate. Figure 4.4 shows the results of the extraction efficiency using the three solvents. Acetone did not disperse the analytes completely into the organic phase, which led to poor extraction efficiency. In previous study, acetone has been used as a dispersive solvent for BPA extraction during DLLME and a large volume of acetone (2.0 mL) was used to disperse the analyte of interest [46]. In another application 0.5 mL of methanol was used in the dispersing solvent while 25 µL of extracting solvent was used in the determination of estrone and 17β estradiol in water samples [56]. This is supported by our findings where methanol and acetonitrile displayed better extraction capabilities with methanol giving the highest extraction efficiencies.

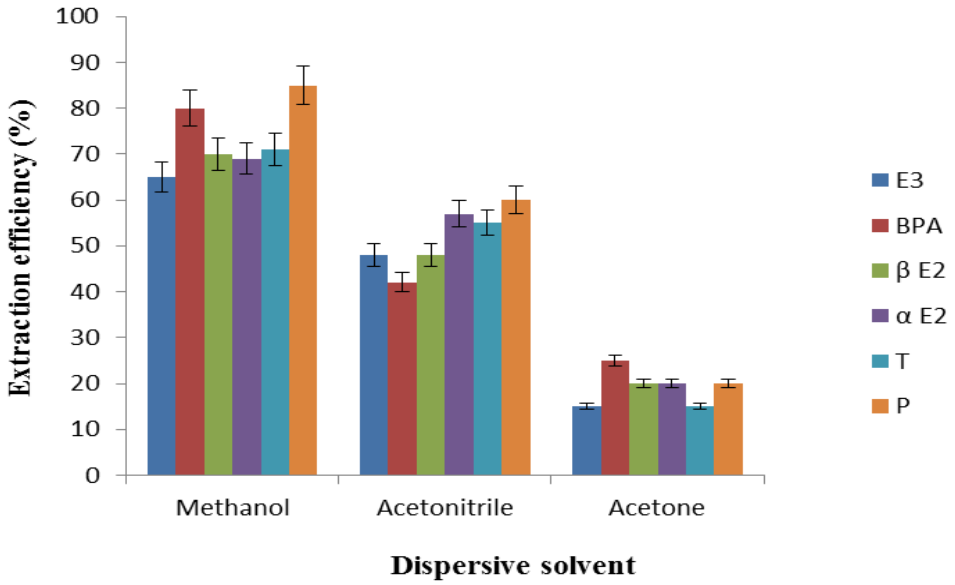


Figure 4.4 Effect of different dispersive solvents on the target analytes m= 4

4.3.3. Determination of optimal volume of the extracting solvent

The extracting solvent volume also has great effects on the enrichment factor of the analytes and it directly determines the volume of the lower/organic phase. The extraction solvent volume is expected to be minimal, while at the same time allowing the target analytes to be dispersed into the aqueous solution. To investigate this, different volumes of tetrachloroethylene (between 10 and 50 µL) were mixed with a fixed volume of methanol (750 µL) and rapidly injected into the sample. Increasing the volume of tetrachloroethylene from 10 to 50 µL led to an increase in the volume of the sedimented phase from 30 - 105 µL. The EF initially increases as the volume of the extracting solvent increases and gradually decreases at volumes above 35 µL. Figures 4.5 show the extraction efficiency of each analyte in the organic phase. At optimum volume of 35 µL, Progesterone showed the highest extraction efficiency of 102%. E3 and testosterone had extraction efficiencies of 84%, β-E2 was 87% while BPA and α-E2 was 89%. Figure 4.6 shows the enrichment factor obtained for the target analytes at optimal volume to be between 148 -258. In literature, DLLME has been used in the determination of BPA, and recovery of 93.4 % was obtained. The LOD of BPA in the water sample was 0.07 µg/L [45]. In another study, estrone, 17β-estradiol and

diesthylstilbestrol were determined in aqueous sample using DLLME. The enrichment factors and extraction recovery were 71-79 and 85-94 respectively and LOD of 0.08 for estrone and 17β-estradiol, while diesthylstilbestrol was detected at 0.10 µg/L [46].

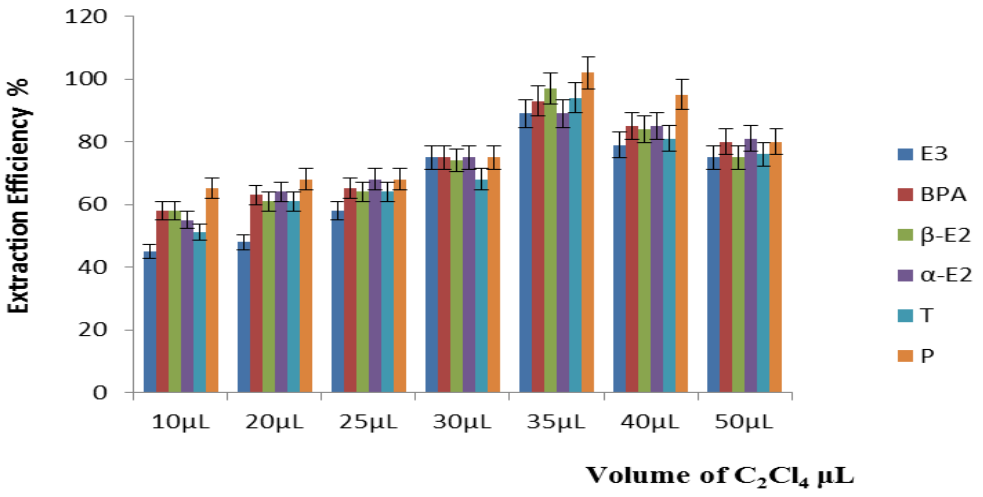


Figure 4.5 Effect of volume of the extracting solvents on the extraction efficiency m=4

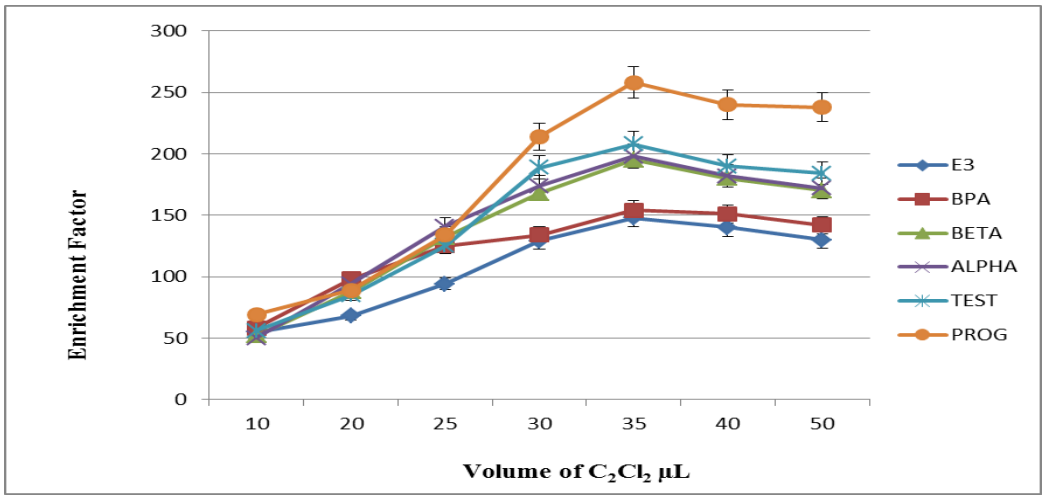


Figure 4.6 Effect of extracting solvent volume on the enrichment factor m = 4

4.3.4. Determination of optimal volume for the dispersive solvent

The volume of the dispersive solvent is another parameter in the DLLME that requires optimization. The effect of volume was studied by varying the volume of methanol (100 µL –

1500 µL) and a constant volume of tetrachloroethylene (35 µL) was added to the sample solution to produce a significant increase in the sedimented phase. The results are shown in Figure 4.7. It was observed that the EE increased with the increase of methanol from 100 - 500 µL and then there was a gradual reduction when the volume was increased above 750 µL. This is because at low volume, the disperser did not disperse the tetrachloroethylene properly and the cloudy solution was not well formed. At higher volume above 500 µL the efficiency values decreases due to the fact that the target analytes are further diluted. The extraction efficiency ranged from (84 - 102%). Based on the results, a volume of 500 µL of methanol was used in subsequent experiments.

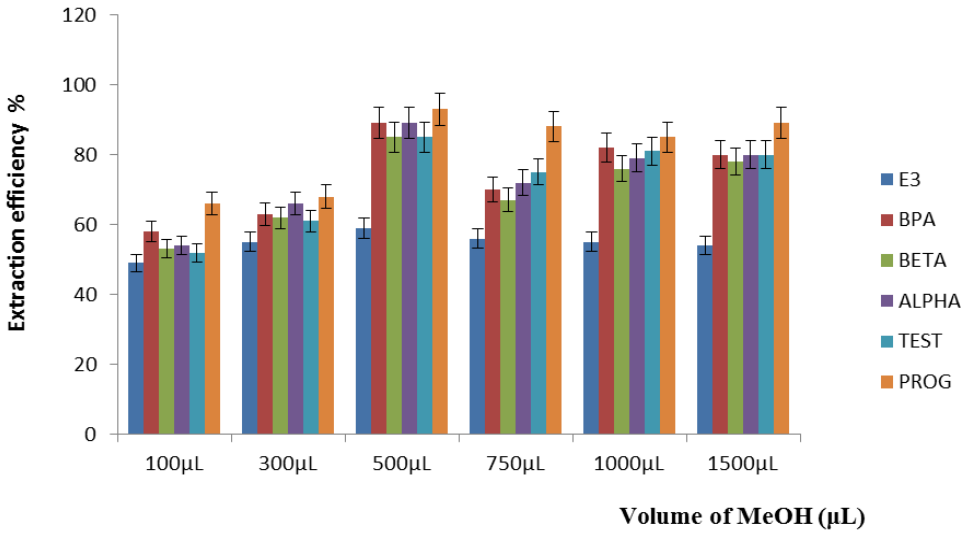


Figure 4.7 Effect of volume of the dispersive solvents on the extraction efficiency

4.4 Validation of the DLLME method

Using optimized conditions the performance of the proposed DLLME method was evaluated for linearity, coefficient of determination, limit of detection limit (LOD), limit of quantification (LOQ), and repeatability. 5 mL of water sample was spiked with 0.01mL standards at different concentration levels and the effective concentration was used for the calibration curve. The summary of the validation results are presented in Table 5.0. A good

linear relationship was obtained with R^2 ranging from 0.9963 – 0.9990 for the target analytes and a relative standard deviation (% RSD) for six replicates of between 1.4 – 2.4 %. The LOD for the EDCs investigated in this study were very low and was in the range of 0.0002 to 0.0004 µg/L and the LOQ was 0.001 µg/L for each of the analytes. Based on these findings it was observed that, after preconcentration of the water samples and instrumental analysis on the HPLC-CAD, we were able to detect the analytes of interest at the µg/L. When compared with other more sensitive methods such as the GC-MS and LC-MS/MS, it can be concluded that this technique can be used as an alternative approach in the determination of steroid hormones, especially to laboratories that cannot afford expensive instruments. DLLME has the advantage of giving higher enrichment factors and extraction efficiencies [45, 56-57].

Table 5.1 shows the recoveries obtained with different water samples (MilliQ, tap water and wastewater) which were spiked at three different concentration levels (1, 5 and 10 µg/L) and the recoveries obtained ranged from 95 to 112 %. This indicated that the DLLME method was not affected by matrix interferences. The higher recovery values obtained from the wastewater samples indicates the presence of the analytes already in the sample.

Table 5.0 Validation parameters for the developed DLLME method for five steroid hormones and one estrogenic compound

Analytes	***Range (µg/L)***	***R^2***	***Regression equation***	***LOD (µg/L)***	***LOQ (µg/L)***	***%RSD***	***SD***	***EF n=6***	***ER % n=6***
E3	0.0004 – 10	0.9990	y= 30.313x + .0231	0.0003	0.001	1.4	± 2.3	148	84
BPA	0.0004 – 6	0.9990	y= 33.506x + .0043	0.0002	0.001	2.3	± 3.2	154	89
β-E2	0.0004 – 6	0.9986	y= 15.247x + 0.021	0.0003	0.001	1.4	± 4	195	87
α-E2	0.0004 – 6	0.9963	y= 43.324x +0.0285	0.0004	0.001	1.8	± 4.2	198	89
T	0.0004 – 6	0.9966	y= 21.553x + 0.022	0.0004	0.001	1.1	± 3.1	208	84
P	0.0004 – 6	0.9988	y= 52.433x +0.0519	0.0003	0.001	2.4	± 6	258	102

Table 5.1 Recovery of steroid hormones from MilliQ, tap and waste water samples spiked at three different concentrations (1, 5, and10 µg/L)

		MilliQ water	**Tap water**	**Waste water**	
Analytes	**Spiking level (µg/L)**	**Recovery % (m = 6)**	**Recovery % (m = 6)**	**Recovery % (m = 6)**	**RSD% (m = 6)**
E3	1	71 ± 3	83 ± 6	88 ± 6	2.1
	5	78 ± 3	89 ± 6	98 ± 6	2.4
	10	88 ± 2	90 ± 5	99 ± 7	2.1
BPA	1	85 ± 4	89 ± 5	101± 7	3.2
	5	75 ± 3	81 ± 6	94 ± 8	3.8
	10	79 ± 3	88 ± 5	109 ± 7	3.2
β-E2	1	86 ± 2	90 ± 5	99 ± 8	4.5
	5	86 ± 3	90 ± 4	95 ± 6	3.5
	10	82 ± 4	92 ± 5	103 ± 6	4.5
α-E2	1	72 ± 4	93 ± 5	99 ± 6	3.8
	5	88 ± 5	93 ± 6	92 ± 8	3.1
	10	90 ± 4	93 ± 5	99 ± 8	3.8
T	1	75 ± 5	74 ± 6	90 ± 7	2.4
	5	75 ± 5	78 ± 6	98 ± 8	2.1
	10	77 ± 4	88 ± 5	104 ± 8	2.4
P	1	88 ± 4	94 ± 5	110 ± 7	2.3
	5	90 ± 3	95 ± 5	112 ± 7	2.6
	10	92 ± 3	95 ± 4	110 ± 5	2.3

Highly reliable methods are required for the detection and quantification of compounds with estrogenic activities because they are detected at low concentration levels in the environment. The importance of performing the DLLME technique on the HPLC-CAD was to enhance sensitivity in this study. The instrumental detection limits for the target analytes before the DLLME procedure was 0.1 mg/L for E3 and P, while BPA, β-E2, α-E2 and T was 0.2 mg/L. The concentration level for the MilliQ water sample that was used in the DLLME method

development was at 0.0002 mg/L i.e. 0.2 µg/L. Figure 4.8.1 below shows a chromatogram that was obtained before the preconcentration procedure. No peaks were identified and only a background noise was visible when a sample concentration of 0.1mg/L was injected. However by including a DLLME step, analytes were enriched and they were clearly detected, our findings are shown in Figure 4.8.2 below. The DLLME technique enhanced the enrichment and sensitivity of the compounds of interest after preconcentration.

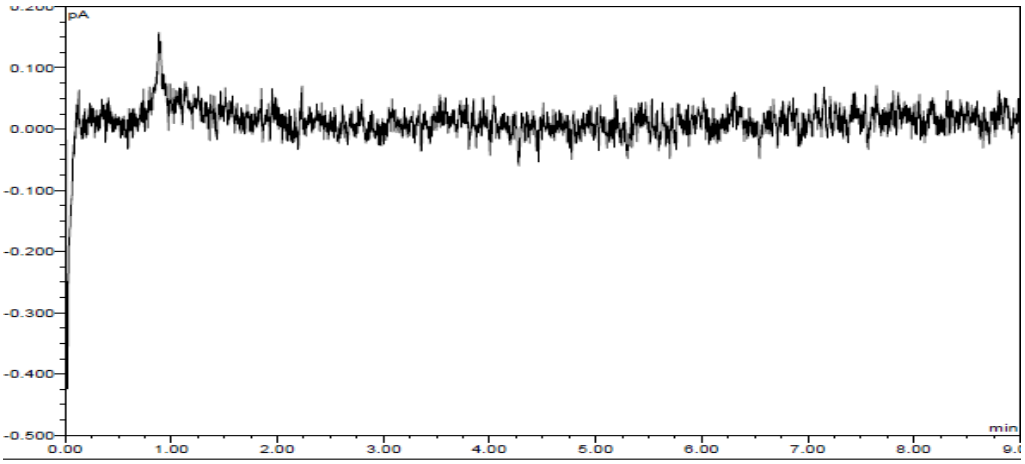


Figure 4.8.1 Chromatogram obtained before preconcentration at concentration level 0.1mg/L

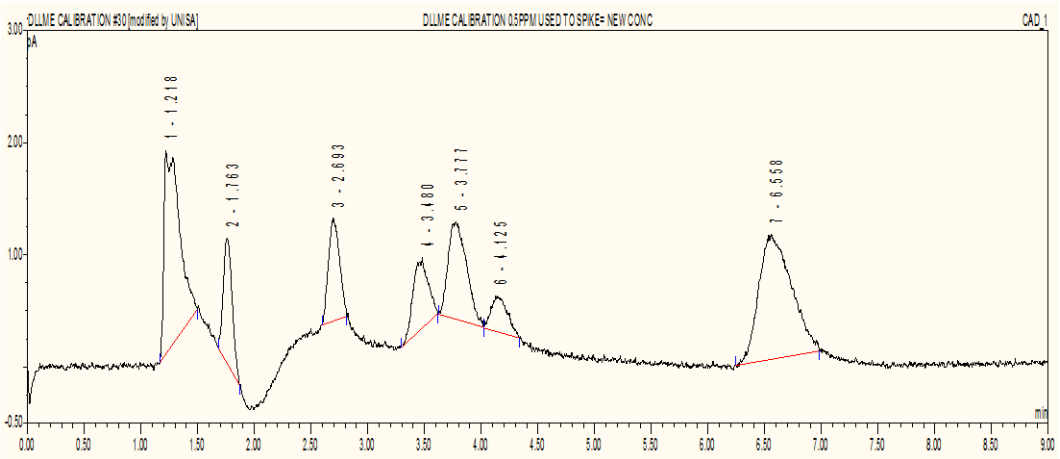


Figure 4.8.2 Chromatogram obtained after preconcentration when 5mL of water was spiked with 0.1mg/L standard solution, effective concentration was 0.2 µg/L

4.5 Environmental water sample analysis

The developed and validated DLLME and HPLC-CAD method was applied to wastewater samples from Daspoort WWTP, in Gauteng, South Africa. Prior to analysis, the wastewater samples were filtered and analysed directly using the DLLME/HPLC-CAD. Linearity of the method was calculated at seven different concentration levels. The correlation of coefficient, LOD and LOQ were calculated on the basis of signal to noise ratio. Recovery of the analytes was tested using two concentration levels (1 and 6 µg/L) in other to assess the matrices effect. The analytical results are summarized and presented in Table 5.2.

The six target analytes were all found in the influent water sample, the result showed that all of the EDCs studied were detected above the detection limits of the method. In the influent water sample, the target analytes were confirmed by spiking with steroid standards to assess matrix effects. Figure 4.9 shows the overlaying of chromatograms obtained before the influent water sample was spiked with steroid hormones standard (1) and after the influent was spiked with standard of steroid hormones (2). The concentration of the five EDCs and BPA in the influent water sample was calculated from the calibration curve equation of $y = bx + c$. They were found at levels which ranged from 0.2 - 2.3 µg/L respectively in the influent water sample. The experiment showed that the analytes of interest were not detected in the effluent samples before and after the preconcentration step. Based on the detection limits obtained after the preconcentration step, it was concluded that the DLLME step had the capability to extract the target compounds from the aqueous matrix into the organic phase. The developed DLLME/HPLC-CAD method can be used as an alternative approach apart from the GC-MS and LC-MS methods for the determination of steroid hormones and BPA in wastewater. The repeatability of the method was ascertained in six replicates of extractions under optimal extraction conditions. The relative standard deviations (RSD %) were in the range of 2.8 - 7.6%. Studies have shown that BPA can be determined using DLLME/HPLC, with a detection limit 0.07 µg/L [45], for 17β-E2 and diethylstibesterol detection limit of 0.008 and 0.10 µg/L was obtained [46]. In another study 17β-E2 and estrone were

investigated, LOD was 0.2 and 0.1 ng/L. Samples were analyzed in replicate for the whole analytical procedure at optimized DLLME/HPLC-CAD conditions. The proposed method was successfully used for the sensitive determination of steroid hormones and estrogenic chemical in wastewater.

Table 5.2 Quantitative determination of steroid hormones concentration in wastewater samples, and recovery using DLLME

Analyte	***R^2***	***LOD (µg/L)***	***LOQ (µg/L)***	***Spiked level (µg/L)***	***Conc found (µg/L) ± SD n=5***	***Recovery (%)***	***%RSD***
E3	0.9966	0.004	0.02	1 6	0.4 ± 0.3	98.4 102.3	7.6
BPA	0.9952	0.004	0.01	1 6	0.2 ± 0.1	94.9 105.2	5.3
β-E2	0.9992	0.005	0.04	1 6	1.2 ± 0.5	97.5 103.1	6.3
α-E2	0.9996	0.002	0.02	1 6	0.7 ± 0.4	98.5 101.2	3.5
T	0.9991	0.005	0.02	1 6	1.2 ± 0.4	91.1 98.4	3.3
P	0.9989	0.006	0.02	1 6	2.3 ± 0.1	93.6 96.9	2.8

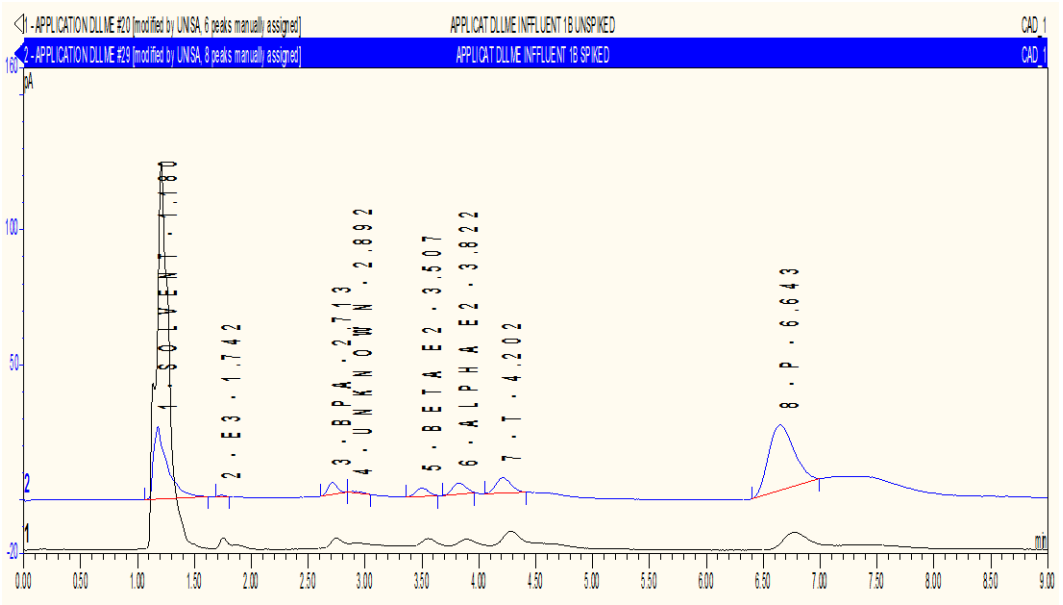


Figure 4.9 chromatogram obtained (1) influent unspiked (2) Influent unspiked with target analytes

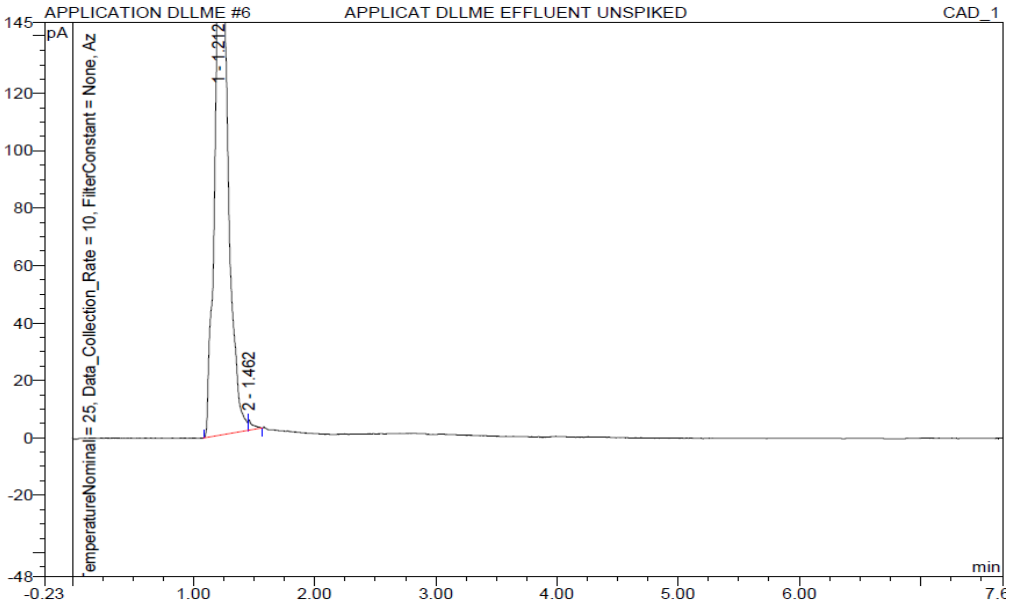


Figure 5.0 Chromatogram obtained when effluent was not spiked with steroid hormones samples

CHAPTER 5

Conclusions and Future Work

5.1 Introduction

In this final chapter, a summary of the research study is provided by revisiting the research objectives and goals that inspired the study. In addition, the main contributions arising from the proposed DLLME/HPLC-CAD analysis are highlighted. Furthermore the research limitations and challenges identified in the research study are also highlighted. Finally future research work that could address some of the identified challenges are also discussed. In this study, an alternative analytical method was developed for the determination five steroid hormones and BPA.

5.2 Research Objectives

This section describes the approach in which the objectives presented in Chapter 1 section 1.3 were addressed and accomplished in the study. The already developed analytical separation method was validated in order to confirm that the method or procedure was suitable for the detection of five steroid hormones and BPA wastewater samples. Based on the validation parameters, the results obtained showed that the method was accurate, had good repeatability and was very robust. The LODs obtained using HPLC-CAD for the target analytes were as follows: E3 and P were 0.1 mg/L, while BPA, β-E2, α-E2 and T was 0.2 mg/L. The recoveries obtained for the analytes in wastewater was between 88.71 - 110%. From our validation results, it was concluded that the method could separate the five steroid hormones and BPA, but it would not be able to detect the target analytes in environmental without a preconcentration step, since they are found in the environmental wastewater samples at low concentration levels.

A preconcentration procedure known as DLLME was developed, optimized and also validated. The optimization of the different conditions helped to enhance better sensitivity of the analytes in environmental samples. Under optimum DLLME conditions, 35 µL of tetrachloroethylene and 500 µL of methanol were used as extracting and dispersing solvent, this led to higher extraction efficiencies of about 84- 102% for the analytes. Enrichment of the analytes into the organic phase was also achieved at optimal values. The optimal volumes helped to reduce the further dilution of the analytes of interest. After preconcentration, the LOD values for the six analytes were found to be as low as 0.0002 µg/L for BPA. E3 and β-E2 were detected at 0.0003 µg/L, while α-E2 and T were detected at 0.0004 µg/L respectively. Based on the detection limits obtained after the preconcentration step, it was concluded that the DLLME step had the capability to extract the target compounds from the aqueous matrix into the organic phase and produce lower detection limits. Hence, it was concluded that the DLLME/HPLC-CAD could be used as an alternative method for the quantification of steroid hormones in the environment.

Finally the developed and optimized DLLME method was used as a preconcentration step in the determination of five steroid hormones and BPA in a wastewater sample obtained from a WWTP. The real wastewater sample (influent and effluent) were analysed using the DLLME/HPLC-CAD technique. The concentration levels at which these analytes are found in our own environment in South Africa, Pretoria using the HPLC-CAD were also reported.The concentration of each of the target analytes in the influent sample was 0.4µg/L for E3, α-E2 was 0.7 µg/L, BPA was 0.2 µg/L. Others found at higher concentration level include β-E2 and T and was detected at 1.2 µg/L. P was found to be with the highest in the environment with concentration level of 2.3 µg/L.

The target analytes were not detected in the wastewater effluents. Satisfactory recoveries for the spiked tap water (95%), MilliQ water (92%) and wastewater (112%) were obtained. Precision was quantified by relative standard deviation (% RSD) which was less than 4%.

Studies have shown that BPA can be determined using DLLME/HPLC, with a detection limit 0.07µg/L [45], for 17β-E2 and diethylstibesterol detection limit of 0.008 and 0.10 µg/L was obtained [46]. In another study 17β-E2 and estrone were investigated, LOD was 0.2 and 0.1 ng/L. Based on our findings, determination of five steroid hormones and BPA in wastewater sample was performed using DLLME/HPLC-CAD, the method was simple, fast, easy, and more cost friendly for the extraction and preconcentration of the target analytes. The analysis of the target analytes was achieved without any derivatization step prior to determination on the HPLC. When DLLME/HPLC-CAD was compared with other methods for the extraction and determination of EDCs on HPLC, the presented method has low limit of detection, high recoveries and a short extraction time. This is the first time that five steroid hormones and one estrogenic chemical BPA will be determined and quantified on an HPLC-CAD.

5.3 Limitation and Recommendation

During the process of this research project, the determination and quantification of steroid hormone has been increasing globally and will continue to increase, since the intake of steroids hormones is still taking place. The continuous release of steroid hormones and their metabolites into the environment needs to be understood better and more scientific documentation is necessary. The overall objectives of the study set out in Chapter 1 of the dissertation were accomplished. However, the proposed DLLME/HPLC-CAD has the potential to be improved as more analysis is carried out on the instrument. Despite recent progress, there are still many gaps in scientific knowledge about the fate and behavior of steroid hormones in the environment and the present dissertation provides some recommendations for future research:

- More preconcentration methods that are suitable for the determination of steroid hormones and its metabolites need to be developed for the determination of steroid hormone and its metabolites in the environment.

- Improvement in analytical methods and instrumentation should be reviewed, as some may not reveal the presence of steroid hormones, but they currently remain undetected in the environment.
- Monitoring of steroid hormones at different WWTPs should be done at regular intervals to determine the extent of the release of these compounds in the environment.
- However, surrogate standard should be included in the future work of this method in order to give a clear indication of the possibility of analyte loss during sampling of water samples.
- Finally, the exact amount of steroid hormones that would be tolerated by human beings should also be documented for future purposes.

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