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Method development for determination and removal of the selected steroids from water sources in selected areas around the Vaal River in South Africa using High performance Liquid Chromatography, *Macadamia* Activated Carbon and Solid Phase Extraction.

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This dissertation is presented in fulfilment of the requirements for the degree Magister Technologiae: Chemistry at Vaal University of Technology.

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DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the Degree Magister Technologiae to the Department of Chemistry, Vaal University of Technology, Vanderbijlpark. It has not been submitted before for any degree or examination to any other University.

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LIST OF ABREVIATION

HPLC	High Performance Liquid Chromatography
SPE	Solid Phase Extraction
AC	Activated carbon
E3	Estriol
E1	Estrone
E2	β-estradiol
EDC	Endocrine Disrupting Compounds
q_e	Amount at equilibrium
q_t	Amount at time
C_e	Concentration at equilibrium
EPA	Environmental Protection Agency
WHO	World Health Organisation
RSD	Relative Standard Deviation
%R	Percentage Recovery
MAC	Macadamia Activated carbon

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ABSTRACT

A simple and rapid method for determination of estrone (E1) and β-estradiol (E2) was developed and validated using high performance liquid chromatography (HPLC). The solutions of standards and sample were prepared with distilled water. HPLC separation was performed in isocratic method 50/50 (water/methanol) using 4.6 mm x 250 mm id (film thickness 5 µm) XDB-C18 capillary column, detector DAD, UV on 254 nm, temperature 20 ºC with flow rate of 2 mL/min, sample volume 20 µL and run time of 10 min. Calibration curves were linear between concentration range 1.0 - 15.0 ppm.

The method was validated for limit of detection and quantification, linearity, precision, trueness and specificity. Also the method was applied to directly and easily to the analysis of the E1 and E2. Adsorption experiments were carried out in batch mode using multistirrer in a series of Erlenmeyer flasks of 50 ml capacity covered to prevent contamination having concentration ranges of E1 and E2 from 1 to 10 mg/L with adsorbent dose range 0.01 to 1 g at pH range 1 to 10 and temperature range 15°C to 35°C, placed on multistirrer. The results of the batch studies showed that simultaneous adsorption shows the maximum percent (91%) removal of E_1 and (86 %) E2 at optimum temperature 25 °C of adsorbent dose 0.1 g, and pH 7. The mechanism, isotherms and kinetics of removal of two endocrine disrupting chemicals, estrone (E1) and β-estradiol (E2) by activated carbon adsorption were investigated in an agitated non-flow batch adsorption studies. Mathematical models were used to describe the adsorption phenomenon with the kinetic and thermodynamic parameters evaluated using the adsorption equilibrium data at varying temperatures.

Higher adsorption rates were achieved at acidic to neutral pH ranges, with the sorption kinetic data showing a good fit to the pseudo second order rate equation and the Langmuir adsorption isotherm model for both E1 and E2. The Gibbs free energy were –16.68 kJ/mol and –17.34 kJ/mol for E1 and E2 respectively. The values of enthalpy for both E1 (84.50 kJ/mol) and E2 (90 kJ/mol) indicated a chemical nature of the sorption process. Both the isotherm and thermodynamic data obtained all supported the mechanism of adsorption of E1 and E2 to be mainly chemisorption's supported by some physical attractions.

CHAPTER 1

1.1 Introduction

The presence of a large number of chemicals in the environment described as endocrine disrupting chemicals (EDCs) like estrone (E1) and 17β-estradiol (E2) are among the most intoxicating EDCs found in environmental waters. There have been various researches in and around the world attempting the removal of these chemicals in river and wastewater treatment processes since the conventional treatment methods seem not to be efficient in removing the compounds to levels below their potentially non effect concentrations. Various methods have been tried including granular activated carbon (GAC) adsorption (Ifelebuegu et al., 2006), and other novel absorbents which have shown potential (Rossner et al., 2009). Of all the methods tried, use of activated carbon is favoured by many as there is no concern of by-product formation as with most advanced chemical oxidation processes (Rossner et al., 2009). In this study the kinetics and thermodynamic properties of the adsorption of E1 and E2 unto macadamia activated carbon was investigated to inform the modelling and design of full scale adsorption processes for river water treatment applications.

Steroid hormones are class of drugs that are administered for different human and veterinary purposes. They have the ability to stimulate and alter human and animal bodies. For example, 17β-estradiol is widely used as a human contraceptive pill for birth control, and as a growth stimulator in feedlot ration of livestock animals (Daughton & Ternes 1999; Caminada et al., 2006). A number of different steroid hormones are

used as veterinary drugs, and as components of livestock animal feed, such as poultry, cattle and pigs. According to (Roig & Touraud, 2010), significant portions of steroid hormones administered to human and animals are not fully metabolized by the body. The excess is spilled over and end up being passed out as waste (Daughton 2001; Díaz-Cruz et al., 2009).

A considerable amount of consumed steroid hormones, especially estrogens leave the system through excretion, either in the form in which they are administered or as derivatives of the parent compound (Diaz-Cruz et al. 2003). As they are excreted, the steroid hormones find their way into the environment via the discharge of domestic and industrial wastewater, farm wash water discharge, farm wastes, abattoir process water and careless disposal of unused or expired prescribed and non-prescribed steroid hormones (Jobling & Sumpter 1993; HollingSorensen et al., 2002); Heberer et al., 2002); Goel et al., 2006); Arditsoglou & Voutsa 2008; (Jafari et al., 2009). Most of these steroid hormones residues end up in wastewater, wastewater treatment plant effluents

(WWTPs), fresh surface water systems and groundwater (Figure 1) (Arditsoglou & Voutsa 2008; Jafari et al., 2009; Aufartova et al., 2011).

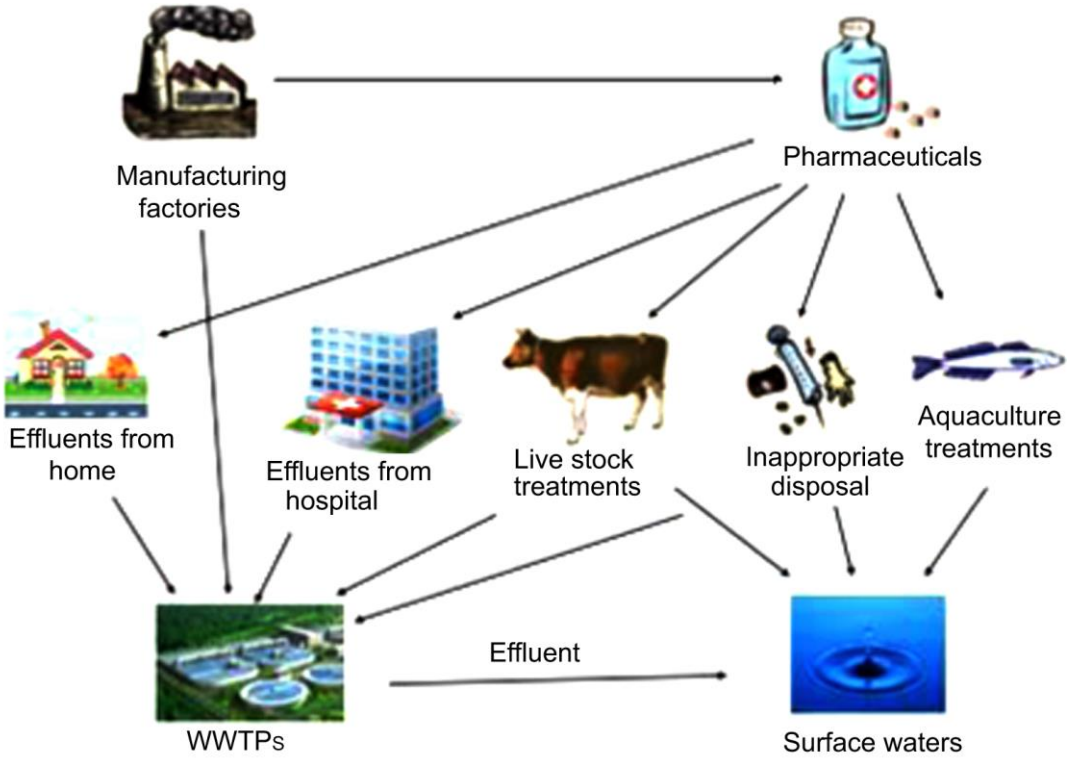


Figure 1.1: Schematic diagram of sources of steroidal hormones into water sources (Jafari et al., 2009).

Due to the challenge posed by the presence of steroids and other pollutants in water, researchers are working to develop low cost materials that can be used to remove unwanted pollutants from water. This has led to the usage of biomaterials from agricultural waste. Biomaterials according to literature have been used to remove various pollutants (Gupta et al., 2015) like dyes (Mittal et al., 2010; Dawood & Sen, 2012), lead (Pholosi et al., 2013), zinc (Paduraru et al., 2015), cadmium (Gupta &

Nayak, 2012). However, no report was found on their usage for the removal of steroid hormones from water.

Agricultural waste materials are composed of lignin, cellulose and hemicellulose which are usually responsible for exchange and complexation properties of this class of adsorbents (Ofomaja & Ho, 2007). These adsorbents have shown promising performance for the removal of pollutants from wastewater. However, some limitations encountered in their usage include their low uptake capacity when used in their raw form and the release of organic components leading to a high chemical and biological oxygen demand and total organic carbon in water (Abdolali et al., 2014).

In order to overcome these limitations, lignocellulosic materials are chemically activated to advance their properties and performance. This is done by using several types of chemical reagents including basic solutions such as sodium hydroxide, calcium hydroxide and sodium carbonate and inorganic acid solutions such as hydrochloric acid, nitric acid, sulfuric acid, tartaric acid, citric acid and thioglycollic acid (Ngah & Hanafiah, 2008). Chemical treatment of biomaterials does not only assist in the extraction of soluble organic compounds from lignocellulosic materials, it also modifies the surface chemical properties of the final product.

In this research a method was developed using HPLC for the determination and quantification of estrone and17β-estradiol. An attempt was also made to remove the selected steroid hormones from water using activated macademia nutshell charcoal. Information and data concerning the screening and quantification of occurrence level and characterization of steroid hormones in different water sources from the Vaal Triangle area are scarce and scanty. Baseline information such as this may be of great importance in understanding the levels of selected steroid hormones in the water sources in and around the Vaal area of South Africa.

1.2 What are steroids?

Steroid hormones are biologically active compounds synthesized from cholesterol, with the common cyclopentano- perhydrophenanthrene ring in common (Cook-Botelho, French 2017). The three major naturally occurring estrogens in women are estrone (E1), estradiol (E2), and estriol (E3). Estradiol is the predominant estrogen during reproductive years both in terms of absolute serum levels as well as in terms of estrogenic activity. Steroid hormones have three six-member rings (A, B, C) and one five-member ring (D) called a cyclopentanophenophenanthrene ring system (Fig. 2). All hormones have an oxygen at C3 on the A ring either as a double bonded oxygen (=O) or a hydroxyl group (−OH). Methyl groups ($-CH_3$) are typically found at C10 and C13.

The functional groups on C17 upon the D ring vary significantly. Many steroid hormones have the same molecular weight but different functions due to the six centers of asymmetry resulting in 64 possible stereoisomers (Cook-Botelho et.al,

2017). Steroids include progestogens, glucocorticoids, mineralocorticoids, androgens, estrogens (Raven & Johnson, 1999). Natural steroids are secreted by the adrenal cortex, testis, and ovary, placenta in human, mamals and other animals. The estrogens estriol, estradiol (E2) and estrone (E1) (Figure 1.2), predominantly female hormones, are responsible for maintenance of reproductive organs and tissue, breast, skin and brain. Over the years, steroids have been detected in sewage treatment plant effluents and in surface water (Andrasi et al., 2013). Their eventual presence in the environment poses a significant potential problem of interference with normal function of the endocrine systems, and can thus affect reproduction and development in wildlife. The steroids of major concern in the aquatic environment, due to their endocrine disrupting potential, are mainly the estrogens.

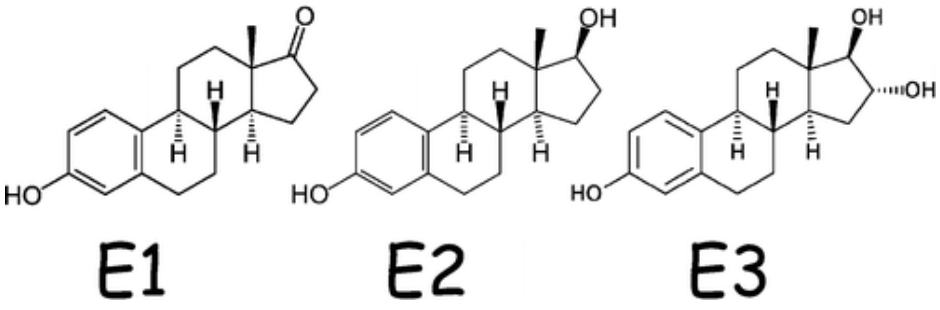


Figure 1.2: The structures of estrogens (estrone (E1), β-estradiol (E2), and estriol (E3)

1.3 Estrogen transformation cycle

Estrone (E1), estradiol (E2), and estriol (E3) are found in the joining metabolic pathways (Casey et al., 2003); (D'Alessio et al., 2014; Duncan et al., 2015;

Goeppert et al., 2014 & Goeppert et al., 2015). Microorganisms living in aerobic conditions are able to convert one estrogen to another (Fig. 3). for example, some microbes (e.g. nitrifying bacteria), can convert E1 to E3, and others reduce E1, E2 and EE2 (e.g *Novosphingobium* sp. in activated sludge) (Ma et al., 2016). On the other hand, the synthetic EE2 can be converted to E1 by *Sphingobacterium* sp. (Haiyan et al., 2007).

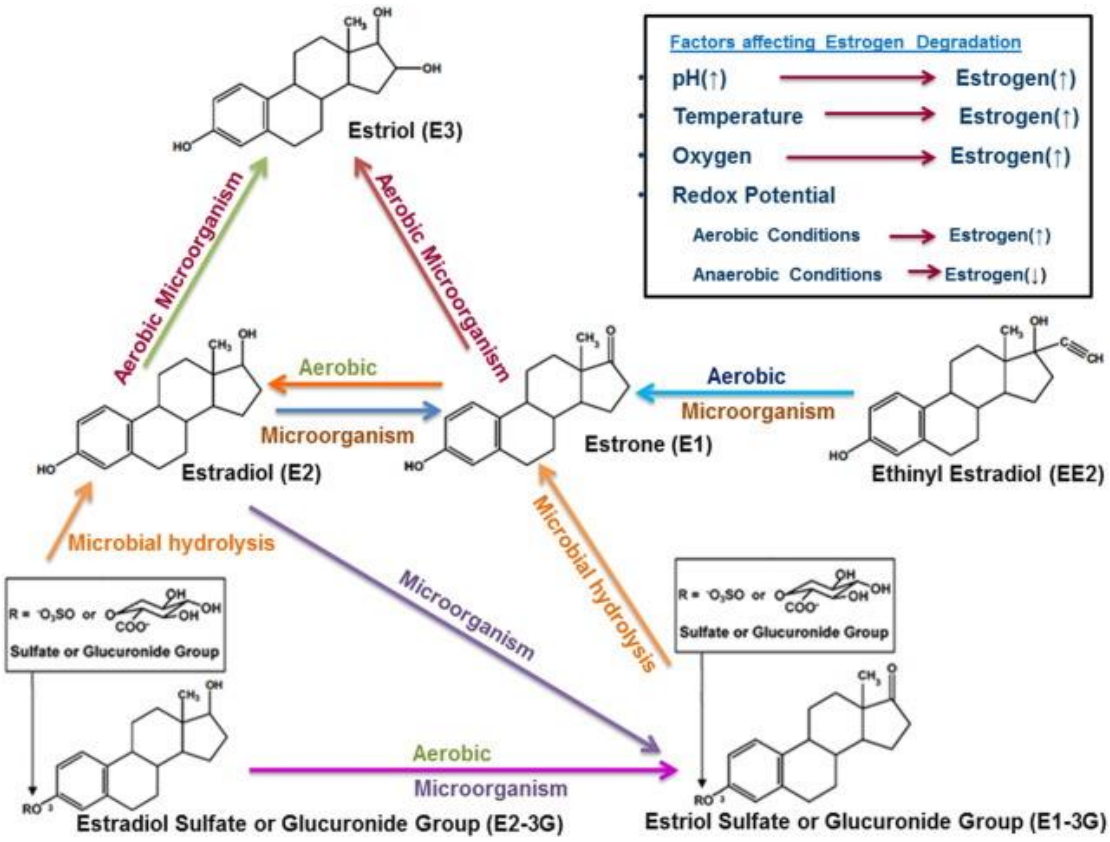


Figure 1.3: Interconversion pathways of natural and synthetic estrogens (Adeel et al., 2017).

1.4 Effects of steroidal estrogens on fish and domestic animals

Previous studies done by other researchers showed that high concentrations of natural and synthetic estrogens converts male fish to female fish, that is, they reduce testes size (Arnold et al., 2014 & Tetreault et al., 2011), affect reproductive fitness (Rose et al., 2013), lower sperm count, induce the production of vitellogenin (Kidd et al., 2007) and alter other reproductive characteristics (Van Donk et al., 2016). Furthermore, E2 caused a considerable reduction in fish biomass and cause some disturbances in the aquatic food chain (Hallgren et al., 2014). On the other hand, E2 does have severe harmful effects on other forms of aquatic life).

Phytoestrogens which are similar to 17β-E2 in terms of their function cause developmental abnormalities in domestic animals. Cows will in some cases show changes in the teat length and colour of the vulva (Burton & Wells, 2002). Some plants naturally contain enough concentrations of estrogens which somehow lead to reproductive alterations in domestic animals. For example, if by any chance sheep graze on the clover plant, which contains potent levels of phytoestrogens, such sheep may develop permanent infertility, which is called “clover disease” (Hotchkiss et al., 2008).

1.5. Problem Statement

Presence of steroids, namely, estrone (E1) and β-estradiol (E2) have become an emerging and serious concern, particularly in the water sources in the Vaal Triangle area of South Africa. Concern has been expressed regarding the entry of these steroids into the human food chain. Steroids have a variety of harmful health effects and an ability to disrupt normal endocrine system functions after exposure to concentrations so small that they are difficult to detect in the environment. Reported effects of steroids include masculinization of females, altered sex ratios and reduced fertility. In addition, little is known about additive effects or bio accumulative effects over a prolonged period of time of these chemicals, therefore attention should be paid to detecting and eliminating endocrine disruption sources if two or more are found in water. Until now, steroids cannot or can only insufficiently be removed by conventional techniques and methods of water treatment (Andersen et al., 2003; Ying et al., 2002; Zhang & Zhou, 2005). In order to solve this problem, great amount of research need to be conducted to identify tough new methods of purifying water at lower cost and with less energy, while at the same time minimizing the use of chemicals and impact on the environment (Shannon et al., 2008) hence in this study macademia nutshell activated carbon was used.

1.6. AIM

The aim of this research was to develop HPLC method for the determination of the concentrations of estrone (E1), β-estradiol (E2) and their removal using SPE and activated macademia nutshell charcoal from water source around Vaal Triangle area of South Africa using HPLC.

1.7. OBJECTIVES OF THE STUDY

The objectives of this research is to:

- Develop a suitable HPLC method for the simultaneous quantitative determination of estrone (E1) and β-estradiol (E2) water sources around Vaal Triangle area of South Africa.
- Optimize the activated macadamia nutshell charcoal (pH, temperature, adsorbent dose, time and concentration) for the removal of estrone (E1) and β-estradiol (E2), from different water sources around Vaal Triangle area of South Africa.
- Determine the actual concentrations of estrone (E1) and β-estradiol (E2) from selected water sources.

CHAPTER 2

2.1 Literature Review

Steroidal estrogens are one class of moderately hydrophobic compounds that exist widely in the aquatic environment. Estrogens are the primary female sex hormones secreted by all vertebrates. Natural estrogens are present in free form and as glucuronide or sulphate conjugates when excreted in urine. Glucuronide conjugates undergo cleavage by b-glucuronidase enzymes to re-form the initial biologically active estrogens. These substances are extremely potent compounds. Their estrogenic effects on fish have been observed in laboratory studies down to 0.2–1 ng/L which were lower than those commonly detected in the aquatic environment (Legler et al., 2002; Campbell et al., 2006; Labadie & Budzinski, 2006; Zha et al., 2008).

The concentrations of some of the steroid estrogens have been reported in rivers, waterways and ground water receiving treated effluents from wastewater treatment plants. The concentration to read concentrations they ranged from 0.1-17 ng/L for estrone E1 and 0.1-5.1 ng/L for estradiol E2. In earlier work, (Swart & Pool, 2007) reported the presence of estrone, estradiol and estriol in surface water and surface water/sewage effluent mixtures in the Kuils River water catchment areas of South Africa. Oestrogenic activities of selected endocrine-disrupting compounds such as 17β-estradiol, BPA and many others in some water sources in South Africa have also been reported (AneckHahn et al., 2005, 2007, 2008, 2009); (Mahomed et al., 2008).

Several authors have determined steroid concentration in water by using gas chromatography coupled with mass spectrometry (GC-MS), high performance liquid chromatography coupled to mass spectrometry (HPLC-MS).

Tabata et al. (2001) conducted an extensive survey of estrogenic steroids in 109 Japanese rivers and found E2 with a mean concentration of 2.1 ng/L. E1, E2, E3 and EE2 were found in Tiber river water, while in Italy it was found with a concentration of 1.5, 0.11, 0.33 and 0.04 ng/L (Baronti et al., 2000). Similarly, In seven French rivers, estrogens were detected in the range from 0.8–3.9 ng/L for E1, 0.8–3.6 ng/L for E2, 0.6–3.1 ng/L for E3 and 0.6–3.5 ng L^{-1} for EE2 (Cargouet et al., 2004). Also there were high levels of estrogens in polluted rivers. For example, (Kolpin et al., 2002) surveyed 139 polluted streams and rivers in the US and found the maximum concentrations of 112 ng/L for E1, 200 ng/L for E2 and 51 ng/L for E_3. The largest concentration of E1 is up to 30 ng/L in the Jalle D'Eysines River of France (Labadie & Budzinski, 2005).

Gas chromatography coupled with mass spectrometry (GC–MS) and GC–MS/MS have been developed and used to analyze estrogen levels in water samples. Although estrogens can be detected at the ng/L level by these techniques, the methods require sample derivatization prior to analysis, which is very time consuming. Liquid chromatography coupled with mass spectrometry (LC–MS) and LC–MS/MS were used to directly analyze estrogens without prior derivatization of the sample, but the instrument used for this was very expensive (Alum et al., 2004).

Early methods of steroid analysis involved extraction as the first step and such extractions are normally carried out using solvents. The main drawback of liquid–liquid extraction is the formation of emulsion. Before chromatography gas or liquid analysis, sample preparation techniques must be carefully selected and optimized because the low concentration of steroids and pharmaceutical drugs can make the detection difficult in environmental samples but the determination is becoming even more challenging when target analytes are degraded and then their concentration are as low that analytical signal become undetectable reliably in treated effluent. The solid phase extraction (SPE) is found to be one of the alternatives methods frequently used for this purpose, since it isolates the analytes from liquid sample and preconcentrate the analytes leading to improved detection (Almeida & Nogueira, 2009).

Hajkova et al.(2005) tested three different SPE adsorbents: ENVI-Carb and Supelclean LC-18, both provided by Supelco; and, Oasis HLB, provided by Waters, and different elution solvents for clean-up of sediment extracts in 10% acetonitrile-water. While elution of the target compounds failed with the ENVI-Carb cartridge under the chosen conditions, Oasis HLB, in combination with an acetonitrile-1% ammonium hydroxide mixture (95:5, v/ v), achieved the best recoveries as well as good repeatability. Furthermore, the same authors reported that gel permeation chromatography (GPC) on Bio-Beads SX3 with cyclohexane: ethyl acetate as mobile phase, failed to separate the targeted estrogens from impurities. However, GPC was used successfully by other researchers (Zhang & Zhou, 2007).

In recent years, Assadi and co-workers demonstrated a novel micro-extraction method called dispersive liquid–liquid micro-extraction (DLLME). DLLME utilizes an extraction solvent, the density of which is higher than water, and a dispersive solvent that can dissolve in both phases to produce a cloudy solution. Due to the high contact area between the organic solvent and water sample, the extraction time of DLLME is very short. DLLME becomes a very popular technique for preconcentration. (Labadie & Hill, 2007) performed a microwave-assisted solvent extraction of steroids from sediment samples (Tan et al., 2007).

HPLC with UV detection and auto sampler is a fast, simple, easy-to-use and widely available technique. Moreover, the assay time for estrogens is short. Only one research group reported the classical method for extraction of solid matrices, namely soxhlet extraction, which seems to be replaced by the other methods. Clean-up techniques are essential after extraction of soils, sludge or sediments. Following extraction, SPE was the clean-up method of choice in most studies. In this research, acid activated macademia nutshell was used to replace SPE since it is cheaper and easily available.

2.2. Method validation

Method validation, according to ISO 9000 standard series, can be defined as a confirmation through the provision of objective evidence that the requirements for a specific method have been fulfilled. Validation parameters include Limit of detection

(LOD), Limit of Quantitation (LOQ), linearity, linearity verification, precision, trueness and statistical significance testing (Landis, 2007).

A typical method validation document must specify the intended use of the method, define the analytical performance requirements and most importantly provide reliable analytical data from validation experiments. In-house validation, as opposed to inter-laboratory assays have an advantage in that they cover performance parameters such as linearity, matrix effects, selectivity and limits of detection (Kelly et al., 2008).

2.2.1 Limits of Detection (LOD)

The limit of detection can be calculated at 3 times the standard deviation of the blanks or low concentration samples or as 5% of the error of detecting the analyte when it is not there. The chromatographic LOD can also be determined as the response that gives a signal to noise (S/N) ratio of 3:1. The measurement of the LOD using S/N ratio is strongly recommended as it shows the skills of the analytical chemist to optimize the S/N ratio (Stockl et al., 2009).

2.2.2 Limits of quantitation (LOQ)

The definition of LOQ is based on values of precision, trueness or total error. The limit of quantitation can be determined as the response that gives a signal to noise (S/N) ratio of 10:1 or as a function of Relative Standard Deviation (RSD). According to (Cuadros-Rodreguez, 2001), most researchers calculate LOQ as 10% RSD or simply as 10 times the standard deviation of the error associated with detection of the analyte in the blank sample (De Souza et al., 2007).

2.2.3 Linearity

Linearity defines the ability of the method to obtain test results proportional to the concentration of the analyte. It is recommended to establish calibration curves with five or more calibration points with the use of more than three replicates. The coefficient of regression (*r*) is used to assess the acceptability of a calibration curve. One of the pitfalls of using *r* is its bias towards the range of the data. Visual assessment is an acceptable criterion to define whether a calibration curve is linear or nonlinear (Stephan et al., 2004). Validation of linearity should be done by means of a statistical test using the null hypothesis. Analysis of Variance (ANOVA) should be applied to ensure that calibration curves obtained with each type of calibration are stable in repeatability conditions and hence data from at least three calibration curves should be compared using an F statistic usually at 95% (Stephan et al., 2004).

2.2.4 Verification of linearity

The efficiency of computing the linearity of a calibration curve as calculated by relevant instrumental software often needs to be verified. Verification was described by (Cuadros-Rodreguez, 2001) as the confirmation by examination and provision of objective evidence that specific requirements have been fulfilled (Gaspar & Dudas, 2006).

2.2.5 Calibration Range

In the presence of matrix effects, the range of responses obtained from calibration can be wide, therefore ensuring the linearity over a wide range of concentrations above

and below the added internal standard concentration is paramount for obtaining accurate data (Stephan et al., 2004).

2.2.6 Precision

Precision is an important validation parameter and is specified as a requirement by most validation guidelines and is measured as a function of the true Relative Standard Deviation percentage (%RSD). For a defined number of replicates greater than three, a precision of > 10% is considered good. The precision should be expressed under the same operating conditions over a short interval of time. It is normally recommended that precision be calculated at three different concentrations (Gaspar & Dudas, 2006).

$$S = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(x_i - \bar{x})^2} \quad \text{..... (1)}$$

Where, Mean = X/N; X = Summation of x value N = the count of mean values S = Standard Deviation value x = Mean of the data

$$\% \text{ RSD} = \frac{\text{SD}}{\text{Mean}} \times 100\ \% \quad \text{.....(2)}$$

%RSD = Percent relative standard deviation

2.2.7 Trueness

Trueness is defined as the difference between the average of an infinite number of replicate measured quantity values and a reference quantity value. It is often erroneously confused with accuracy which is the difference between a measured value and the true quantity value of the measured. In HPLC analysis trueness is best

measured through the use of recoveries. Assessing trueness implies estimating separately the proportional bias (in terms of recovery) and the constant bias of the analytical method (Maroto et al., 2001).

2.2.8 Selectivity

Selectivity is the degree to which an extraction technique can separate the analyte from interferences in the original sample. The choice of a sample preparation technique coupled with the analytical instrument of choice has a profound influence on the selectivity of a particular analyte. For efficient selectivity the analytical method needs to be optimized for each particular analyte (Maroto et al., 2001).

2.3. Solid Phase Extraction (SPE)

Solid phase extraction is a sample preparation technique used for extracting semi volatile and non-volatile analytes from their matrices for subsequent chromatographic analysis. It is considered one of the most powerful techniques currently available for isolating trace amounts of organic compounds such as pesticides from water and other environmental samples (Poole et al., 2000; Ferrer & Barceló, 1999). It entails the use of SPE cartridges which are packed with silica bonded to a particular analyte adsorbing phase. SPE can be compared to other extraction techniques like liquid-liquid extraction although it is advantageous in that it provides better selectivity and extraction efficiency (recovery), eliminates problems associated with incomplete phase separation and yields quantitative extractions that are easy to perform.

2.4. Adsorption studies

2.4.1 Activated carbon

Activated carbon (Figure 2.1) is a carbon material that has adsorption performance with a lot of micro pores on its surface. It is widely adopted in the industrial fields related to air, water and sewage clean up in order to remove the odour in the air and contaminants in water by taking advantage of its absorptivity Standing Committee of Analyst (SCA) (2005). Activated carbon with high surface area and pore volumes are produced from a variety of macadamia nutshells. In practice, coal and agriculture by-products or ligno-cellulosic materials are two main sources for the production of commercial activated carbon. Although coal is the most commonly used precursor, in some places agricultural waste is a better choice (Standing Committee of Analyst (SCA), (2005).

There is quite a large number of studies regarding the preparation of activated carbon from agricultural waste which include a number of nutshells and other by-products such as: almond shell, coconut shell, hazelnut shell, olive, peach, apricot and cherry stones, grape seeds and eucalyptus leaves. The previous study in making activated carbons from MNS by physical activation showed that MNS is a good starting raw material for the production of activated carbon with a well-developed structure and high adsorption capacity as well as making CMS (Standing Committee of Analyst (SCA), (2005).

Figure 2.1: Image showing the activated carbon.

Activated carbon is used as a sorbent in wastewater treatment for removing taste and odor from drinking water. It is also known to be capable of removing organic contaminants (Jones et al., 2007; Reungoat et al., 2011; Zhang & Zhou, 2005). Activated carbon removes compounds via sorption both to the surface and within the granules or substrate matrix. The effectiveness of activated carbon is influenced by surface area, porosity, surface pH, and surface charge (Snyder *et al.,* 2006); (Westerhoff et al., 2005). The hydrophobicity of estrogenic compounds determines which and how much of a compound will adsorb on the substrate (Koh et al., 2009). However, some physical factors, such as high levels of organic matter in wastewater, can obstruct efficiency by competing for sorption sites and hindering access to openings within the structure (Fukuhara et al*.,* 2006; Snyder et al., 2006). Inactivation and saturation of binding sites are serious concerns for treatment efficiency with activated carbon because it must be replaced to promote continued removal once these occur.

References

ARDITSOGLOU, A. & VOUTSA, D. (2008). Determination of phenolic and steroid endocrine disrupting compounds in environmental matrices. Environ. Sci. Pollut. Res. 15 (3) 228-236.

ADRASI, N., MOLANAR, B., DOBOS, B., VASANITS-ZSIGRAI, A., ZARAY, G. & MOLANAR-Perl I. (2013). Determination of steroids in the dissolved and in the suspended phases of wastewater and Danube River samples by gas chromatography, tandem mass spectrometry. Talanta, 115, 367.

ALMEIDA, C. & NOGUEIRA, J. M. F. (2006). "Determination of Steroid Sex Hormones in Water and Urine Matrices by Stir Bar Sorptive Extraction and Liquid Chromatography with Diode Array Detection," *J Pharm and Biom Anal*, 41(4) 1303-1311. doi: 10.1016/j.jpba.2006.02.037

ANECK-HAHN, N.H. (2003) Screening for Anti-Oxidant Pollutants and Estrogenicity in Drinking Water in Poverty Stricken Areas of South Africa. Doctorate Technologiae, Technikon Pretoria, Pretoria.

ANECK-HAHN, N.H., BORNMAN, M.S. & DE JAGER, C. (2008). Preliminary assessment of oestrogenic activity in water sources in Rietvlei Nature Reserve, Gauteng, South Africa. Afr. J. Aquat. Sci. 33 (3) 249-254.

ABDOLALI, A., GUO, W.S., NGO, H.H., CHEN, S.S., NGUYEN, N.C. & TUNG, K.L. (2014). Typical lignocellulosic wastes and by-products for biosorption process in water and wastewater treatment: a critical review. Bioresour Technol 160:57–66.

ARNOLD, K.E., BROWN, A.R., ANKLEY, G.T., SUMPTER, J.P. (2014). Medicating the environment: assessing risks of pharmaceuticals to wildlife and ecosystems. Phil. Trans. R. Soc. Lond. B: Biol. Sci. 369. http://rstb.royalsocietypublishing.org

ANECK-HAHN, N.H., DE JAGER, C., BORNMAN, M.S. & DU TOIT, D. (2005). Oestrogenic activity using a recombinant yeast screen assay (RCBA) in South African laboratory water sources. Water SA 31 (2) 253-256. http://www.wrc.org.za/downloads/watersa/2005/Apr05/1784.pdf

ANECK-HAHN, N.H., SCHULENBURG, G.W., BORNMAN, M.S., FARIAS, P. & DE JAGER, C. (2007). Impaired semen quality associated with environmental DDT exposure in young men living in a malaria area in the Limpopo Province, South Africa. J. Androl. 28 (3) 423-434.

BHANOT, D. (2011). Arbo pharmaceuticals, Auriga Research Ltd.

BARONTI, C., CURINI, R., GD'ASECNZO, Di CORCIA, A. & Gentilli A, et al. (2000). Monitoring natural and synthetic estrogens at activated sludge sewage treatment plants and in a receiving river water. Environ Sci Technol 34: 5059-5066.

BURTON, J. & WELLS, M. (2002). The effect of phytoestrogens on the female genital tract. J. Clin. Pathol. 55, 401–407.

CAMPBELL, P.M., FERNANDEZ, M.P., ROYSTON, S., SMITH, J.L., VAN POPPELEN, P., IKONOMOU, M.G. & DEVLIN, R.H. (2006). Male coho salmon (Oncorhynchus kisutch) exposed to a time-course of urban sewage effluent exhibit a sporadic low incidence of sex reversal and intersex. Water Quality Research J of Canada; 41:235–243.

CAMINADA, D., ESCHER, C. & FENT, K. (2006). Aquat Toxicol. ; 79(2), 114-23.

CASEY, F.X., LARSEN, G.L., HAKK, H. & ŠIMMUENK, J. (2003). Fate and transport of 17β-estradiol in soil-water systems. Environ. Sci. Technol. 37, 2400–2409.

COOK-BOTELHO, J.C. & FRENCH, D. (2017). In Mass Spectrometry for the Clinical Laboratory, 205-230.

DAUGHTON, C.H., TERNES T.A. (1999) Special Report: Pharmaceuticals and personal care products in the environment: agents of subtle change? Enviro Health Perspect 107(suppl 6),907–938.

DAUGHTON, C. G. (2001). Illicit drugs in municipal sewage in pharmaceuticals and personal care products in the environment: scientific and regulatory issues. In ACS Symposium Series 791 (eds C. G. Daughton & T. L. Jones-Lepp), 116–139. Washington, DC: The American Chemical Society.

DIAZ-CRUZ, M.S., GARCIA-GALAN, M.L., GUERRA, P., JELIC, A., POSTIGO, C., ELJARRAT, E., FARRE, M., LOPEZ DE ALDA, M.J., PETROVIC, M., BARCELO, D. (2009). Analysis of selected emerging contaminants in sewage sludge. Trends in Anal Chem. 28, 1263-1275.

DAWOOD, S. & SEN, T.K. (2012). Removal of anionic dye Congo red from aqueous solution by raw pine and acid-treated pine cone powder as adsorbent: equilibrium, thermodynamic, kinetics, mechanism and process design. Water Res 46, 1933–1946.

D'ALESSIO, M., VASUDEVAN, D., LICHWA, J., MOHANTY, S.K. & RAY, C., 2014. Fate and transport of selected estrogen compounds in Hawaii soils: effect of soil type and macropores. J. Contam. Hydrol. 166, 1–10.

DUNCAN, L.A., TYNER, J.S., BUCHANAN, J.R., HAWKINS, S.A. & LEE, J. (2015). Fate and transport of 17β-estradiol beneath animal waste holding ponds. J. Environ. Qual. 44, 982–988.

FERRER, I. & BARCELO, D. (1999). Validation of new solid-phase extraction materials for the selective enrichment of organic contaminants from environmental samples. Trends in Anal Chem, 18(3), 181-182.

GOEPPERT, N., DROR, I. & BERKOWITZ, B. (2014). Detection, fate and transport of estrogen family hormones in soil. Chemosphere 95, 336–345.

GOEPPERT, N., DROR, I. & BERKOWITZ, B. (2015). Fate and transport of free conjugated estrogens during soil passage. Environ. Pollut. 206, 80–87.

GUPTA, V.K. & NAYAK, A. (2012). Cadmium removal and recovery from aqueous solutions by novel adsorbents prepared from orange peel and Fe_2O_3 nanoparticles. Chem Eng J 180, 81–90.

GUPTA, V.K., NAYAK, A. & AGARWAL, S. (2015) Bioadsorbents for remediation of heavy metals: current status and their future prospects. Environ Eng Res 20(1), 001–018.

HAIYAN, R., SHULAN, J., UD DIN AHMAD, N., DAO, W. & CHENGWU, C. (2007). Degradation characteristics and metabolic pathway of 17α-ethynylestradiol by Sphingobacterium sp. JCR5. Chemosphere 66, 340–346.

HALLING-SORENSEN, B., SENGELOV, G. & TJORNELUND, J. (2002). Toxicity of tetracyclines and tetracycline degradation products to environmental relevant bacteria including selected tetracycline resistant bacteria. Arch Environ Contam Toxicol; 42,263–71.

HALLGREN, P., NICOLLE, A., HANSSON, L.A., BRONMARK, C., NIKOLERIS, L., HYDER, M. & PERSSON, A. (2014). Synthetic estrogen directly affects fish biomass and may indirectly disrupt aquatic food webs. Environ. Toxicol. Chem. 33, 930–936.

Hill, M., Cibula, D., Havlikova, H., Kancheva, L., Fait, T., Kancheva, R., Parizek, A. & Starka, L. (2007). Circulating levels of pregnanolone isomers during the third trimester of human pregnancy. J. Steroid Biochem. Mol. Biol. 105, 166–175.

HOTCHKISS, A.K., RIDER, C.V., BLYSTONE, C.R., WILSON, V.S., HARTIG, P.C., ANKLEY, G.T., FOSTER, P.M., GRAY, C.L. & GRAY, L.E. (2008). Fifteen years after “wingspread”—environmental endocrine disrupters and human and wildlife health: where we are today and where we need to go. Toxicol. Sci. 105, 235–259.

IFELEBUEGU, A.O., LESTER, J.N., CHURCHLEY, J & CARTMELL, E. (2006). "Removal of an Endocrine Disrupting Chemical (17alpha-Ethinyloestradiol) from Wastewater Effluent by Activated Carbon Adsorption: Effects of Activated Carbon Type and Competitive Adsorption," J. Environ. Tech., 27, 1343-1349. doi: 10.1080/09593332708618748

ISMAIL, I., ENAS, S & Jamal, D. (2010). "Kinetics, Equilibrium and Thermodynamics of the Sorption of Tetrabromobisphenol A on Multiwalled Carbon Nanotubes, "App. Surf. Sci., 256 (23), 7246- 7252. doi: 10.1016/j.apsusc.2010.05.059

JAFARI, A.J., POURKABIREH-ABASABAD, R. & SALEHZADEH, A. (2009). "Endocrine Disrupting Contaminants in Water Resources and Sewage in Hamadan City of Iran, Iran," *J. Environ. Health Sci. & Eng.*, 6(2), 89-96.

JOBLING, S., NOLAN, M., TYLER, C.R., BRIGHTY, G. & SUMPTER, J.P. (1998). Widespread sexual disruption in wild fish. Environ. Sci. Technol. 32, 2498–2506.

JOBLING, S. & SUMPTER, J. P. (1993). Detergent components in sewage effluent are weakly oestrogenic to fish: An in vitro study using rainbow trout (Oncorhynchus my kiss) hepatocytes. Aquatic Toxicol, 27, 361-372.

KIDD, K.A., BLANCHFIELD, P.J., MILLS, K.H., PALACE, V.P., EVANS, R.E., LAZORCHAK, J.M., FLICK, R.W. (2007). Collapse of a fish population after exposure to a synthetic estrogen. Proc. Nat. Acad. Sci. 104, 8897–8901.

LABRIE, F., BELANGER, A., BELANGER, P., BERUBE, R., MARTEL, C., CUSAN, L., GOMEZ, J., CANDAS, B., CHAUSSADE, V., CASTIEL, I., DELOCHE, C. & LECLAIRE, J. (2007). Metabolism of DHEA in postmenopausal women following percutaneous administration. J. Steroid Biochem. Mol. Biol. 103, 178–188.

MA, C., QIN, D., SUN, Q., ZHANG, F., LIU, H. & YU, C.P. (2016). Removal of environmental estrogens by bacterial cell immobilization technique. Chemosphere 144, 607–614.

MAHOMED, S.I., VOYI, K.V.V., ANECK-HAHN, N.H. & DE JAGER, C. (2008). Oestrogenicity and chemical target analysis of water from small-sized industries in Pretoria, South Africa. Water SA 34 (3), 357-363. http://www.wrc.org.za/downloads/watersa/2008/July/1952. pdf.

MITTAL, A., MITTAL, J., MALVIYA, A., KAUR, D. & GUPTA, V.K. (2010). Decoloration treatment of a hazardous triarylmethane dye, light green SF (Yellowish) by waste material adsorbents. J Colloid Interface Sci 342, 518–527.

NAKADA, N., TANISHIMA, T., SHINOKARA, H., KIRI, H. & TAKADA, H. (2006). "Pharmaceutical Chemicals and Endocrine Disrupters in Municipal Wastewater in Tokyo and Their Removal during Activated Sludge Treatment," Water Research, 40 17, 3297-3303. doi: 10.1016/j.watres.2006.06.039.

NGAH, W.S.W. & HANAFIAH, M.A.K.M. (2008). Removal of heavy metal ions from wastewater by chemically modified plant wastes as adsorbents: a review. Bioresour Technol. 99, 3935–3948.

OFOMAJA, A. & HO, Y. (2007) Effect of pH on cadmium bio sorption by coconut copra meal. J Hazard Mater 139:356–362.

PADURARU, C., TOFAN, L., TEODOSIU, C., BUNIA, I., TUDORACHI, N. & TOMA, O. (2015) Biosorption of zinc (II) on rapeseed waste: equilibrium studies and thermogravimetric investigations. Process Saf Environ 94, 18–28.

PHOLOSI, A., OFOMAJA, A. & NAIDOO, E. (2013) Effect of chemical extractants on the biosorptive properties of pine cone powder: influence on lead (II) removal mechanism. J Saudi Chem Soc 17, 77–86.

POOL, E.L., VAN WYK, J.H. & LESLIE, A.K. (2000). Inflammatory activity as an indicator of water quality: the use of human whole blood cultures, J. of Immun. 2(14), 387-399.

RAVEN, P.H. & JOHNSON, G.B. (1999). Biology. 5th Edition; WCB/McGraw- Hill: Boston.

REUNGOAT, J., ESCHER, B. I., MACOVA, M., & KELLER, J. (2011). Biofiltration of wastewater treatment plant effluent: effective removal of pharmaceuticals and personal care products and reduction of toxicity. Water Research, 45(9), 2751–62.

ROIG, B. & TOURAUD, E. (2010). Pharmaceuticals in the environment: current knowledge and need assessment to reduce presence and impact. ed./B. Roig. London: IWA Publishing, 169-190.

ROSE, E., PACZOLT, K.A., JONES, A.G. (2013). The effects of synthetic estrogen exposure on premating and postmating episodes of selection in sex-role-reversed Gulf pipefish. Evol. Apps. 6, 1160–1170.

ROSSNER, A., SNYDER, S.S & KNAPPE, D.R.U. (2009). "Removal of Emerging Contaminants of Concern by Alternative Adsorbent," Water Research, 43 (15), 3787-3796. doi: 10.1016/j.watres.06.009.

SWART, N. & POOL, E. (2007). Rapid detection of selected steroid hormones from sewage effluents using an ELISA in the Kuils River water catchment area, South Africa. J. Imm. Immunochem 28, 395-408.

TETREAULT, G.R., BENNET, C.J., SHIRES, K., KNIGHT, B., SERVOS, M.R., McMASTER, M.E. (2011). Intersex and reproductive impairment of wild fish exposed to multiple municipal wastewater discharges. Aq. Toxicol. 104, 278-290.

VAN DONK, E., PEACOR, S., GROSSER, K., DOMIS, L.N.D.S. & LURLING, M. (2016). Pharmaceuticals may disrupt natural chemical information flows and species interactions in aquatic systems: ideas and perspectives on a hidden global change. In: de Voogt, P. (Ed.), Reviews of Environmental Contamination and Toxicology. Springer, Heildeberg, Germany, 91–105.

WESTERHOFF, P., YOON, Y., SNYDER, S. & WERT, E. (2005) Fate of endocrine-disruptor, pharmaceutical, and personal care product chemicals during simulated drinking water treatment processes., Environmental Science and Technology, 39(17), 6649–6663.

Ying, G.G., Kookana, R.S. & Ruy, J. (2002). Occurrence and fate of hormone steroid in the environment, Environ. Internal. 28, 545-551.

ZHANG, Y. & ZHOU, J. L. (2005). Removal of estrone and 17beta-estradiol from water by adsorption. Water Research, 39(16), 3991–4003.

CHAPTER 3

3. EXPERIMENTAL

3.1 Apparatus and reagents used for the study

All solvents and chemicals were purchased from Sigma-Aldrich. Ultrapure water was used for all dilutions and standard preparations. Table 3.1 lists the equipment used while Table 3.2 shows chemicals and materials used.

3.1.1 Apparatus

Table 3.1: Apparatus

HPLC	Agilent 1200 Series
Column	Eclipse XDB-C18 [4.6mmx 250mm id (5 µm) 80Å]
SPE Cartridges	C18
SPE Vacuum Manifolds	Sulpelco Visiprep
Vials	2ml capacity
Beakers	Various (Grade A)
Volumetric flasks	Various (Grade A)
Filters	4.5 µm PSF syringe filter, Quartz filter and 20x25cm whatmann

3.1.2 Reagents

Table 3.2: Reagents used

Component	Purity	Manufacturer
Acetonitrile	99.9%	Sigma-Aldrich
Methanol	99%	Sigma-Aldrich
Acetone	99%	Sigma-Aldrich
Estrone	N/A	Sigma-Aldrichβ
β-estradiol	N/A	Sigma-Aldrich
MAC	N/A	Filtertech

3.2. Sampling

The sampling of water from Vaal, Barrage and Klip River (Figure 3.1) started from May 2014. Samples were taken twice a month at about the same time at each site in the morning. Samples were taken using either a 1-L stainless steel bucket (for sampling from river bank) or 5-L container attached to an aluminium pole (for sampling from the bank) depending on the sampling location. The bottles were filled with the aid of a stainless steel funnel; both the funnel and the bucket were rinsed thoroughly with river water/sewage effluent from each site prior to the first sample being collected. All sample bottles were sanitized prior to use as previous work has shown that steroids adsorb to un-sanitized glassware resulting in poor recovery. Sanitization of glassware was carried out after they had been cleaned using a proprietary cleaning agent, rinsed with deionized water, and deactivated using dichloromethane. Upon aqueous sample

collection, preservatives, such as formaldehyde (1% v/v) and MeOH, were added to halt microbial activity.

A = Vaal River S26° 45.168' E027° 48.837' **B = Barrage River** 26.7647° S**,** 27.6917° E **and C = Klip River** 26° 40' 15" S, 27° 57' 15" E

Figure 3.1: Map showing the sampling points around Vaal.

3.3. Method development

Samples were prepared by spiking distilled water with a known amount of steroid compounds. The standard stock solution of estrone (E1) and β-estradiol (E2) (100 mg/L) were prepared by dissolving both compounds in methanol in a volumetric flask respectively. Working standard solutions (0.5 - 10 mg/L) were prepared by taking aliquots from the stock solution and transferred into different volumetric flasks and filled

to the mark using methanol. Three solvents namely, acetonitrile, methanol and acetone were used respectively to ascertain which among them will extract sample of interest from the sorbent better with high recoveries. The optimization and adjustment of conditions procedure were carried out at only one concentration level. Retention time, detection limit, quantification limit, accuracy, specificity, selectivity, repeatability were determined.

3.4 Solid phase extraction

3.4.1 SPE Vacuum Manifold

Samples and cartridges were arranged in corresponding visible vacuum manifold which enables analysts using solid phase extraction tubes to simultaneously prepare up to 24 samples. The manifold consists of a chemical-resistant cover, gasket, and base, a glass basin, a vacuum gauge and vacuum bleed valve, 24 flow control valves, 24 replaceable solvent guide needles, and a rack for sample collection vessels (base 5 support plates, and support rods, retaining clips).

Figure 3.2: Showing the vacuum manifold Source: Supelco bulletin (1998)

3.4.2 SPE cartridges used

The following 3 mL reverse solid phase extraction columns were used: Strata X – 200 mg. C18 (Isolute) is produced from trifunctional octadecyl silane sorbent with enhanced secondary silanol interactions which can be very useful for example in the extraction of basic compounds from aqueous solution. It has the average particle size of 50 µm with irregular shape particles and the porosity of 60Å. It is applied to aqueous analytes and has a strong non-polar (hydrophobic) phase (International Sorbent Technology, 1997) the functional group is displayed below in figure 3.3.

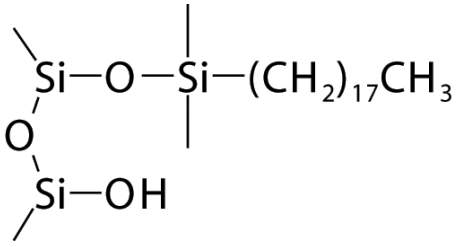


Figure 3.3: Showing the structure of C-18 sorbent

3.4.3. Conditioning of cartridge

In this study, SPE using bonded silica C18 was optimized with regard to sample pH, sample concentration, sample flow rate, elution solvent, washing solvent, sample volume, elution volume and sorbent type.

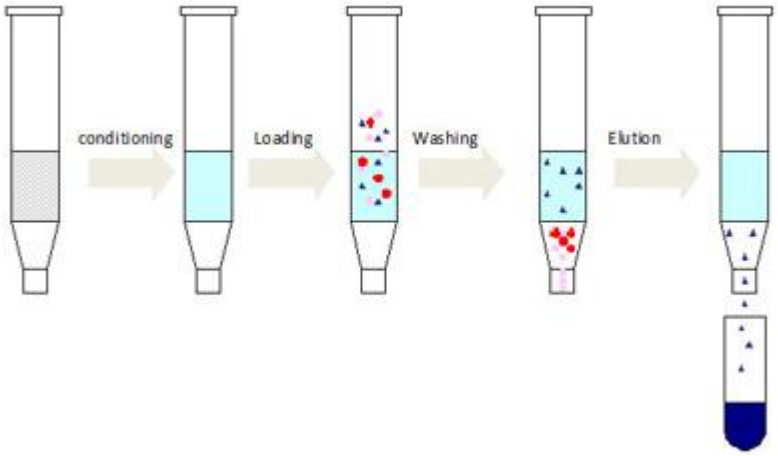


Figure 3.4: SPE conditioning until collection of analyte of interest.

Steroids residues were concentrated on Strata C18-E (1g/20 mL) SPE cartridges. The SPE cartridges were conditioned with 15 mL methanol, equilibrated with 20 mL HPLC grade water, loaded with 770 mL filtrated sample, washed with 20 mL HPLC grade water and eluted with 15 mL methanol. After evaporation to dryness (rotary evaporator, 40 °C) extracts were reconstituted in 750 µL methanol. The 750 µL methanol was concentrated to dryness (Eppendorf Concentrator Plus at 30 °C, V-AL mode) and reconstituted in 50 µL methanol. The extracts were then analyzed with HPLC-UV.

3.4.5 Efficiency of the Eluting and Volume Solvent

The capacity and the effectiveness of elution solvent from the C_{18} cartridge were tested by using the selected solvents, namely, acetonitrile, methanol and acetone. In separate experiments, solvents were respectively spiked with known amounts of selected steroids and were passed by gravity through a column that was preconditioned. Later, seven aliquots of 2 mL were collected and then analyzed separately to know total recovery and volume necessary for the elution step.

3.5. Limit of detection and quantification

Standards ranging between (0.05-1 mg/L) were prepared in 100ml volumetric flask by taking aliquots from the stock solution in order to get lowest concentration that instrument can detect.

3.6 Repeatability

Standards ranging between (0.05-1 mg/L) were prepared from stock solution and ran in ten replicates per concentration from the instrument in order to get the peak and to check the consistency.

3.7 Linearity

The linearity of peak area response versus concentration for analytes was studied between ranges of (0.1-10 mg/L). Calibration curve was constructed and evaluated by its correlation coefficient.

3.8 Precision and recovery

Recovery is the efficiency of determining analytes from sample matrix by spiking samples with analytes at various concentrations. Ten replicates of each sample were analysed and the mean, standard deviation, %Recovery and %RSD were calculated. The samples were then spiked with standard solutions at different concentrations and % recoveries were calculated using the equation below:

$$\% \text{ Recovery} = \frac{Cs - C}{S} \times 100\,\% \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(3)$$

where C_s = spiked sample concentration, C = sample concentration, S = concentration equivalent to the analyte added in the sample.

The %RSD should be less or equals to 10 and % Accuracy should be between 80-120%.

The repeatability precision for the two selected steroids was determined from ten replicate analyses of the Vaal river sample spiked with 1mg/l standard of each steroid respectively. The spike samples were extracted with LC18 using methanol as an elution solvent.

3.9. High Performance Liquid Chromatography

HPLC is a technique for separation, identification and quantification of components in a mixture. It is especially suitable for compounds which are not easily volatilised, thermally unstable and have high molecular weights. The liquid phase is pumped at a constant rate to the column packed with the stationary phase. Before entering the column, the analysis sample is injected into the carrier stream. On reaching the column

the sample components are selectively retained on the basis of physico-chemical interactions between the analyte molecules and the stationary phase. The mobile phase moving at a steady rate elutes the components based on the operating conditions. Detection techniques are employed for detection and quantification of the eluted components.

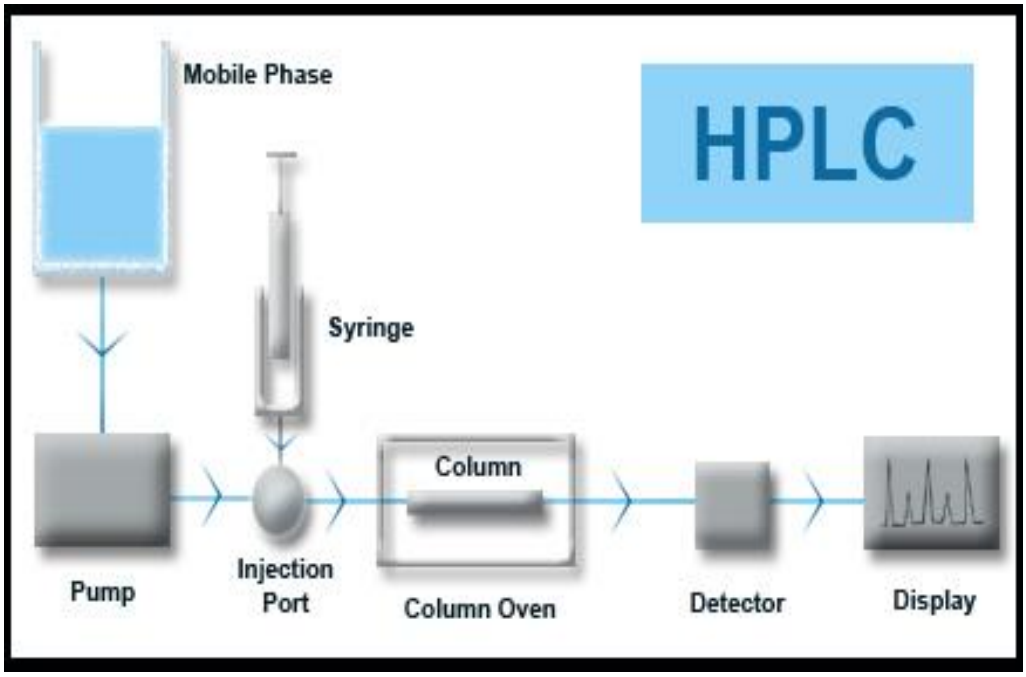


Figure 3.5: Showing the schematic HPLC diagram

3.9.1 Mobile Phase

Mobile phase serves to transport the sample to the system. Essential criteria of mobile phase are inertness to the sample components. Pure solvents or buffer combinations are commonly used. The mobile phase should be free of particulate impurities and degassed before use. Bhanot (2011).

3.9.2 Mobile Phase Reservoirs

These are inert containers for mobile phase storage and transport. Generally transparent glass bottles are used as to facilitate visual inspection of mobile phase level inside the container. Stainless steel particulate filters are provided inside for removal of particulate impurities in the mobile phase if any Bhanot (2011).

3.9.3 Pumps

Variations in flow rates of the mobile phase effect elution time of sample components and result in errors. Pumps provide constant flow of mobile phase to the column under constant pressure Bhanot (2011).

3.9.4 Injectors

Injectors are used to provide constant volume injection of sample into the mobile phase stream. Inertness and reproducibility of injection are necessary to maintain high level of accuracy Bhanot (2011)

3.9.5 Column

A column is a stainless steel tube packed with stationary phase. It is a vital component and should be maintained properly as per supplier instructions for getting reproducibility separation efficiency run after run Bhanot (2011).

3.9.6 Column Oven

Variation of temperature during the analytical run can result in changes of retention time of the separated eluting components. A column oven maintains constant column

temperature using air circulation. This ensures a constant flow rate of the mobile phase through the column Arbo Pharmaceuticals (2011).

3.9.7 Detector

A detector gives specific response for the components separated by the column and also provides the required sensitivity. It has to be independent of any changes in mobile phase composition. Majority of the applications require UV-VIS detection though detectors based on other detection technique are also popular these days Arbo Pharmaceuticals (2011).

3.9.8 Data Acquisition & Control

Modern HPLC systems are computer based and software controls operational parameters such as mobile phase composition, temperature, flow rate, injection volume and sequence and also acquisition and treatment of output Arbo Pharmaceuticals (2011).

3.9.9 Preparation of standards

Stock solution of estrone and β-estradiol was prepared in a 100 mL volumetric flask and the aliquots were taken from the stock solution into 50 mL volumetric flasks to prepare the standard solutions of different concentrations (1 – 10 mg/L), and the stock solution for estrone and β-estradiol was prepared in a 1000 mL volumetric flaks separately to prepare the 1000 mg/L, aliquots were taken into 100 mL volumetric flasks to prepare standards of different concentrations (1- 10 mg/L) and methanol as solvent.

The stated preparations were done to establish the retention time, repeatability, limit of detection and limit of quantification of the instrument.

3.9.10 Sample analysis

Steroids were analysed with Agilent 1200 Series HPLC system with a programmable wavelength diode array detector, and a UV detector. Separation of the selected steroids was performed using a column Eclipse XDB-C18 4.6mm x 250mm ID containing 5 µm. Baseline separations of the steroids were achieved within 10 min by isocratic method. Acetonitrile and ultra-pure water were used as the mobile phase. All data for quantification of the steroids were obtained by applying the isocratic elution program shown in Table 3.3 at a flow rate of 1 ml/min and a controlled oven temperature of 20°C.

Table 3.3: Instrument operating conditions.

Mobile Phase	Water (A) Methanol (B)
Isocatic method	A% B% 50 50
Detector	DAD, UV on 254nm
Column	Eclipse XDB-C18 [4.6mmx 250mm id (5 µm) 80Å]
Column Temperature	Ambient (20°C)
Flow Rate	2 ml/min
Sample Volume Run Time	20µl 10 Min

3.10 Adsorption studies

3.10.1 Characterization of Macademia nutshell.

The elemental composition of the raw Macadamia nutshells was performed with a Thermo Flash 2000 series CHNS/O Organic Elemental Analyzer. The morphological evaluation of raw and modified Macadamia nutshells was done with a scanning electron microscope and energy-dispersive X-Ray spectrometer (SEM–EDS) from FEI

Nova NanoLab FIB/SEM (Milpitas, CA). Thermogravimetric analysis (TGA) was performed with a PerkinElmer STA 600 Simultaneous Thermal Analyzer (Waltham, USA). The infrared absorption spectra were obtained with a PerkinElmer Spectrum 400 FT-IR/FT-NIR spectrometer (Waltham, USA). Batch adsorptions of E1 and E2 onto adsorbents were performed on a Multichannel stirrer MS-53 M model Jeio Tech (Seoul & Korea, 2008)

3.10.2. Thermal gravimetric analysis (TGA)

Thermal gravimetric analysis was performed to identify the thermal behavior of macadamia nutshell activated carbon, phosphoric acid impregnated shells and phosphoric acid impregnated chars. In TGA analysis, Shimadzu DTG-60H simultaneous DTA-TG apparatus in VUT Chemistry Department was used. TGA experiments were conducted at the same experimental conditions with respect to carbonization and activation temperature, N_2 flow rate and heating rate.

3.10.3. Fourier Transform Infrared Spectroscopy (FTIR) analysis

In this study, commercial MAC was modified using chemical treatment. Hence, changes in the functional group should be compared. Both the commercial and modified activated carbon was analyzed by FT-IR Spectroscope (FTIR-100, Perkinelmer) to detect the surface functional group. A small amount of dry MAC was crushed into powder form and tested. The spectra were recorded from 4000 to 400

cm^{-1} (Shaarani & Hameed, 2011). The FT-IR spectra of powder macadamia activated carbon were measured by a Perkin- Elmer FT-IR system, Spectrum GX. The spectra were recorded from 4000 to 500 cm^{-1} with a resolution of 4 cm.

3.10.4. Scanning Electron Microscopy (SEM) analysis

SEM is an instrument which applies a narrow electron beam to scan over the surface of the specimen which is coated with a thin layer of metal. Secondary electron will be collected by a detector and produce a three-dimensional image on television screen. By using SEM, the morphology, pore structure, and structural changes of activated carbon can be observed (Jiachuann et.al., 2017) AC was prepared in granular form and placed at the sample placement. After the AC was coated, SEM was run to determine its characteristics.

3.10.5. Evaluation by a batch adsorption method

Adsorption experiments were carried out in batch mode using multistirrer in a series of Erlenmeyer flasks of 50 ml capacity covered to prevent contamination. The effect of time, temperature, concentration and solution pH were studied. Adsorption isotherms were obtained by fitting the data obtained during the effect of concentration study into Langmuir and Freudlich isotherm. An equal amount of absorbent was added separately into each individual flask and the other parameters time, temperature, concentration and pH were set different and the flasks were agitated. After prescribed contact times, the solutions were centrifuged and the concentrations of steroids in the

supernatant were determined by HPLC methods as described later. The absorption capacity for steroids uptake, q_e (mg/g) and the amount removed were determined as follows respectivel.

$$q_e = \frac{(C_e - C_O)V}{W} \quad \text{.....(4)}$$

$$\%E = \frac{(C_e - C_o)100}{C_e} \quad \text{.....(5)}$$

Where, C_o and C_e are initial and equilibrium steroids concentrations (mg/L) respectively, V is the volume of solution (L) and W is the weight of adsorbent (g).

- **Effect of initial concentration and contact time**

About 0.1g sample of activated carbon was added to each 50 mL Erlenmeyer volumetric flask containing estrone and β-estradiol solution with initial concentrations of 1, 2, 6, 8 and 10 mg/L and the experiments were carried out at different temperature for 90 min.

- **Effect of temperature**

The effect of temperature on adsorption was carried out in flasks sealed with Teflon lined caps. About 0.1g sample of activated carbon was added to each 50 mL volume of 2 mg/L estrone and β-estradiol solution. The experiments were carried out at 20 ºC, 25ºC, 30 °C and 35 °C for 90 min.

- **Effect of pH**

The pH of the solution was varied from 2.0 to 10.0, while the amount of adsorbent (0.1g), volume of solution (50 mL), initial concentration of solution (2 mg/L), temperature (25°C) and shaker speed (250 rpm) were kept constant. The solution pH was adjusted by using the diluted 0.1 M HCl and 0.1 N NaOH solutions.

CHAPTER 4

4. RESULTS AND DISCUSION

4.1 HPLC method validation

The HPLC method validation was aimed at establishing if the method was fit for the purpose. The validation parameters tested were repeatability, linearity, detection limit, sensitivity, precision, accuracy, specificity, selectivity and recovery. The efficiency of the methods was evaluated by spiking ultrapure water samples with selected steroids at 10 different levels of concentrations of estrone and β-estradiol steroids respectively. The recoveries were independent ($P > 0.05$) of added mass of target analytes with a repeatability lower than 6.5% for estrone and 12.1% for β-estradiol. The recovery factor (coefficient of variation, CV) was higher than 66% for estrone (CV < 3.8%) and >55% for β-estradiol (CV < 5.2%).

4.1.1 Limit of detection

The 1 mg/L was prepared and ran into the instrument to check the lowest concentration that it can be detected so the standards have been detected in less than 3 minutes' time (see figures next page).

4.1.2 Repeatability

Repeatability precision gives an idea of the sort of variability to be expected when a method is performed by a single analyst over a short time scale. Repeatability was evaluated using standards and real samples. Ten replicates of each samples were

analysed and the mean, SD and %RSD were calculated. The %RSD should be ≤to 10 and % Accuracy (where standards are used). Should be between 80-120%

$$\% \text{ RSD} = \frac{\text{SD}}{\text{Mean}} \times 100 \%$$

Where SD is the standard deviation of the test results and mean is the mean of the test results obtained.

$$\% \text{ Accuracy} = \frac{\text{X}}{\mu} \times 100\% \text{ ....(5)}$$

where x is the Mean of test results obtained for reference sample, μ = “true”/certified value given for reference sample.

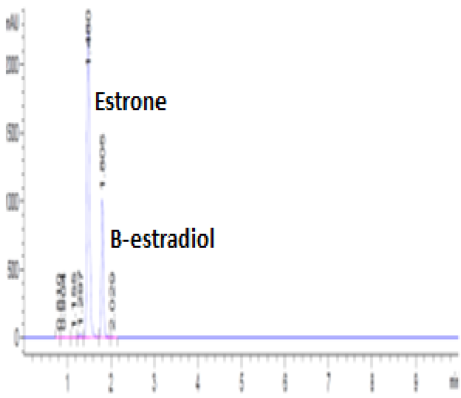


Figure 4.1.

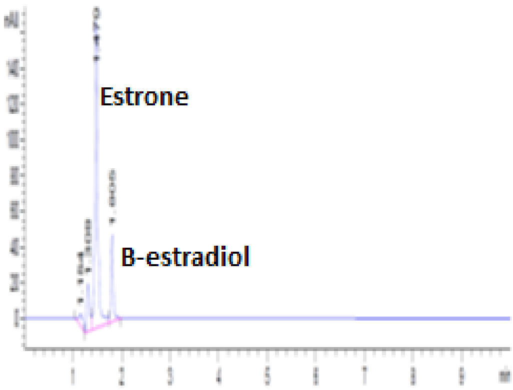
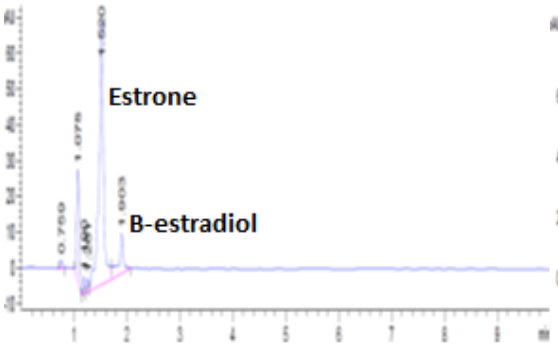


Figure 4.2.



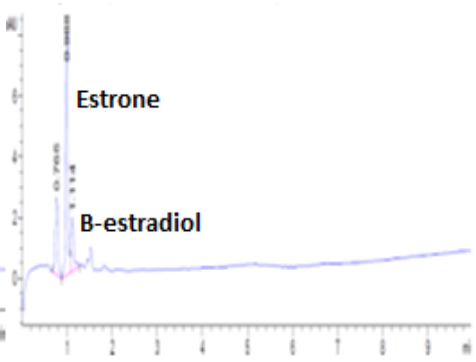


Figure 4.3. Figure 4.4.

Figures 4.1-4.4: Showing the HPLC chromatograms of the 1 mg/L estrone and β-estradiol for repeatability.

Table 4.1*:* The statistics data of β-estradiol

Concentration	5 ppm		50 ppm		80 ppm	
	Standard	Accuracy	Standard	Accuracy	Standard	Accuracy
Mean	5.32	106.48	52.69	105,39	83.17	103,97
SD	0.05	1.03	0.03	0.06	0.38	0.48
%RSD	0.97%	0.97%	0.06%	0.06%	0.46%	0.46%

Table 4.2: The statistics data of Estrone

Concentration	5 ppm		50 ppm		80 ppm	
	Standard	Accuracy	Standard	Accuracy	Standard	Accuracy
Mean	5.21	104.24	51.94	103.88	85.16	106.45
SD	0.03	0.57	0.14	0.27	0.28	0.34
%RSD	0.55%	0.55%	0.27%	0.26%	0.32%	0.32%

4.1.3 Linearity, Limit of detection (LOD) and limit of quantification (LOQ)

The linearity of the proposed spiked range was also evaluated plotting the observed response or absorbance, after the treatment of the sample, against that of the different concentrations. The correlation coefficients (r^2) were 0.899 and 0.996 for estrone (Figure 4.2) and β-estradiol (Figure 4.3) respectively ($P < 0.05$).

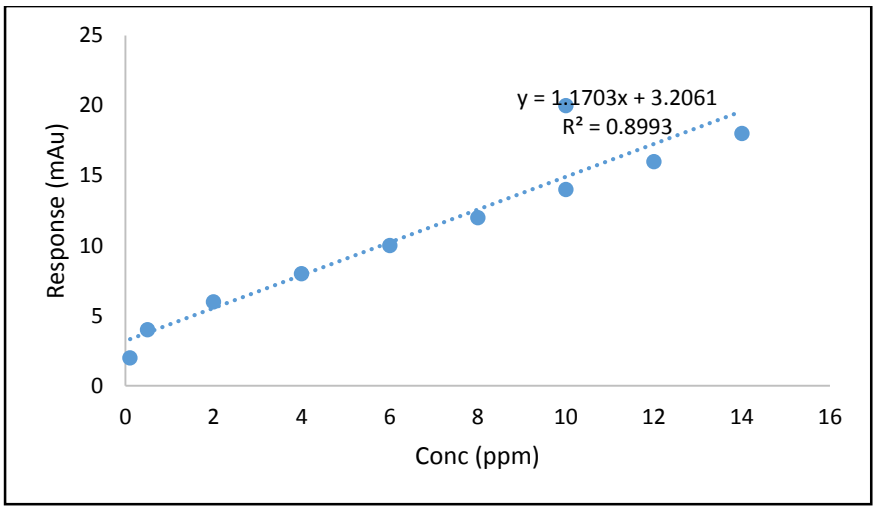


Figure 4.5: Linear curve for Response versus Concentration for estrone

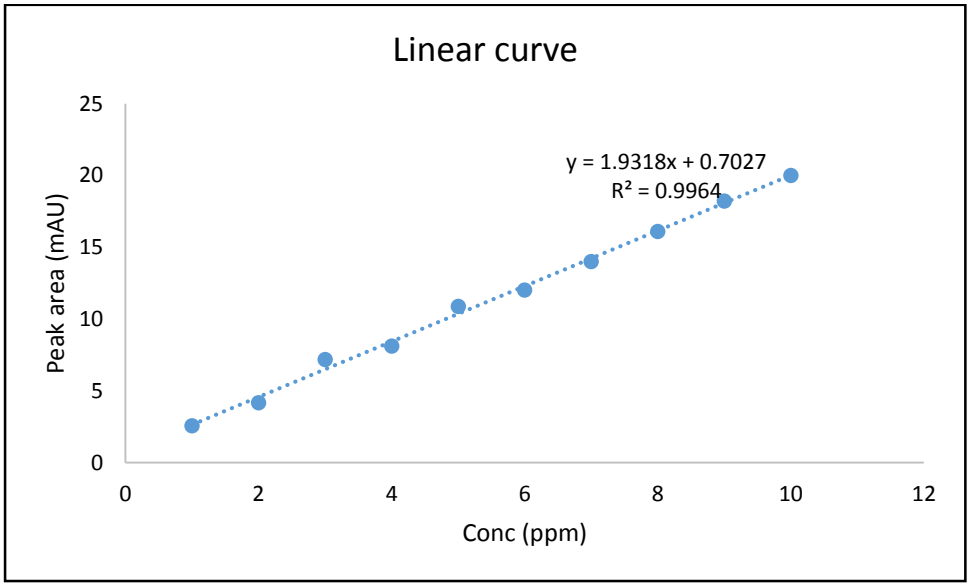


Figure 4.6: Linear curve for Peak area versus Concentration for β-estradiol.

Limits of detection (LOD) and quantification (LOQ) were determined. The LODs were 0.112 mg/L and 0.100 mg/L for E1 and E2, respectively, and LOQ values for E1 and E2 were 2.16 mg/L and 1.89 mg/L, respectively (Table 4.3).

Table 4.3: Linearity data

Steroids	Linearity range (ppm)	Linear equation	Linear regression (r^2)	LOD (ppm)	LOQ (ppm)
Estrone	1-10 ppm	Y=1.1703x3.2061	0.8993	0.112	2.16
β-estradiol	1-10 ppm	Y=1.9318x-0.7027	0.9964	0.100	1.89

Linearity is the ability of a test method to obtain the test results proportional to the concentration of the analyte within a given working range. A method is linear if Correlation Coefficient is ≥0.995. The Correlation Coefficient for β-estradiol was found to be 0.996, and that of estrone 0.8993 respectively. Therefore, the method is linear for β-estradiol.

4.1.4 Sensitivity of the Method

Sensitivity is the capability of a method to discriminate between small differences of a concentration of an analyte. From the regression equation for a straight line, y = mx + c, a method is calibration sensitive if the slope of the graph is ≠ 0. From the experimental results above, the slopes of graph for β-estradiol and estrone were not equal to zero (Table 4.3) and therefore, the method is calibration sensitive.

4.2. Recoveries using SPE

The optimized method of extraction in the solid phase was applied to spiked water samples with different concentrations of the selected steroids in order to know the behavior of recovery regarding the amount of selected steroids present in the sample. Each spiked level was performed in duplicate and the recovery was calculated by comparing the response in the tested sample with the dissolution of the reference standard as shown by the equation below.

$$\% \text{ Recovery} = \frac{Cs - C}{S} \times 100\ \%$$

Where C_s = spiked sample concentration, C = sample concentration, S = concentration equivalent to the analyte added in the sample.

High recoveries were observed at concentration 12.5 and 25 mg/L, (90.1% and 91.2%) for β-estradiol and 25 and 50 mg/L for estrone (89.1% and 89.5%). This suggest that there is no much impact of the concentration between 12.5 and 25 mg/L with regards to β-estradiol since there is no significant difference in terms of the recoveries. With regards to the estrone, a significant difference is seen between the concentration 12.5 ppm and the subsequent concentrations, namely, 25 mg/L and 50 mg/L. The extraction efficiencies (Table 4.4) were independent of the loaded mass of analyte (ANOVA test $P > 0.05$). The recoveries of the selected steroids were found to be lower than those reported by López de Alda & Barceló (2000). Higher recoveries were

observed at concentrations of 12.5 and 25 mg/L for β-estradiol and 25 and 50 mg/L for estrone respectively.

Table 4.4: Percentage recoveries of estrone and β-estradiol using SPE

Concentration (ppm)	**Estrone %R (CV)**	**β-estradiol %R (CV)**
12.5	82.0 ± 4.1	90.1 ± 4.6
25	89.1 ± 0.8	91.2 ± 1.0
50	89.5 ± 6.5	80.1 ± 4.3
R^2	0.8993	0.9964
M ±SD	0.969 ± 0.010	0.832 ± 0.032

%R: recovery percentage, CV: coefficient of variation (*n* = 2), R^2: correlation coefficient, m: slope or recovery factor, sd: standard deviation

4.2.1 Accuracy results

Accuracy depends on two factors, trueness and precision.

4.2.2 Trueness results

To evaluate the trueness, the calculated amounts of the standards were compared to the measured amounts by reprocessing the calibration sample data as samples. The results obtained are given in Table 4.5.

Table 4.5*:* Trueness data

Steroids	True value(mg/L)	Experimental mean (mg/L)	Standard deviation	%RSD
Estrone	1.0	0.9211	0.0164	1.78
β-estradiol	1.0	0.9834	0.0051	0.52

As a quality control procedure, % RSD of less than 5% is considered to be valid. Accordingly, all analytes tested showed a percentage RSD of less than 5%.

4.2.3 Precision

This parameter for quality of the method was represented by the coefficient of variation (CV) and it was evaluated as repeatability for each level tested and as reproducibility throughout the levels (*n* = 10). For the selected steroids the precision limit was 0.0852 (Estrone) and 0.0619 (β-estradiol) respectively (Table 4.6).

Table 4.6: Precision data

Steroids	Mean	Standard deviation	%RSD	t-value	Precision limit
Estrone	0.7940	0.0258	3.25	2.18	0.0852
β-estradiol	0.8533	0.0405	3.57	2.18	0.0619

Both analytes tested showed a percentage RSD of less than 5%. These values of validation parameters indicated that the analysis using the instrument method is repeatable.

4.2.4 Specificity and Selectivity results

The identification of peaks was confirmed by injecting the steroids separately and noting the retention times. The method performs very well on these compounds and there is no evidence of co-elution.

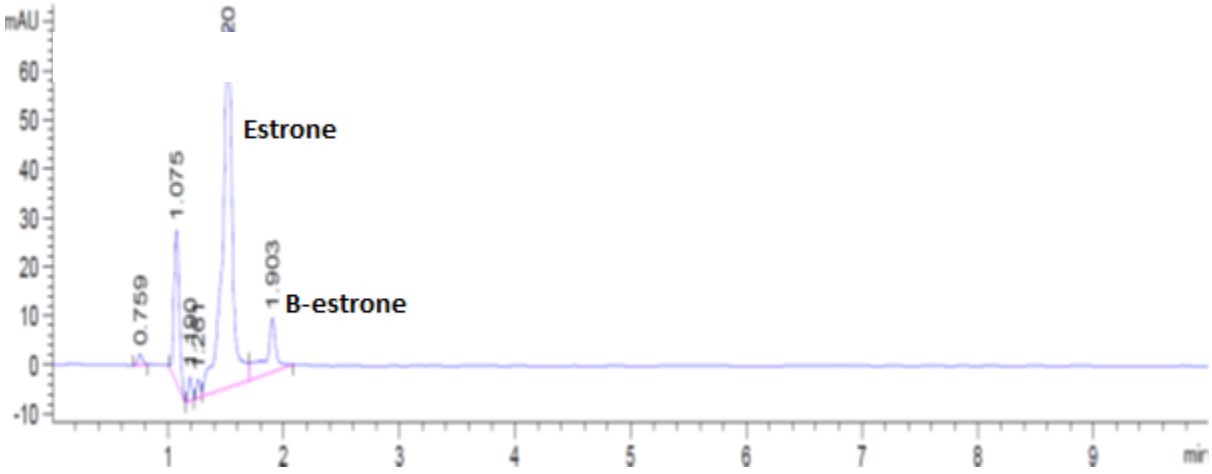


Figure 4.7: Showing chromatogram of distilled water spiked with 1 mg/l steroids.

4.3 Activated carbon

4.3.1 Characterization of activated carbon by FTIR spectroscopy, SEM and TGA.

The adsorption capacity of activated carbon depends upon porosity as well as the chemical reactivity of functional groups at the surface. Knowledge on surface functional groups gives insight to the adsorption capability of the produced activated

carbon. FTIR spectra were obtained for qualitative characterization of surface functional groups of macademia nutshell activated carbon (Figure 4.8).

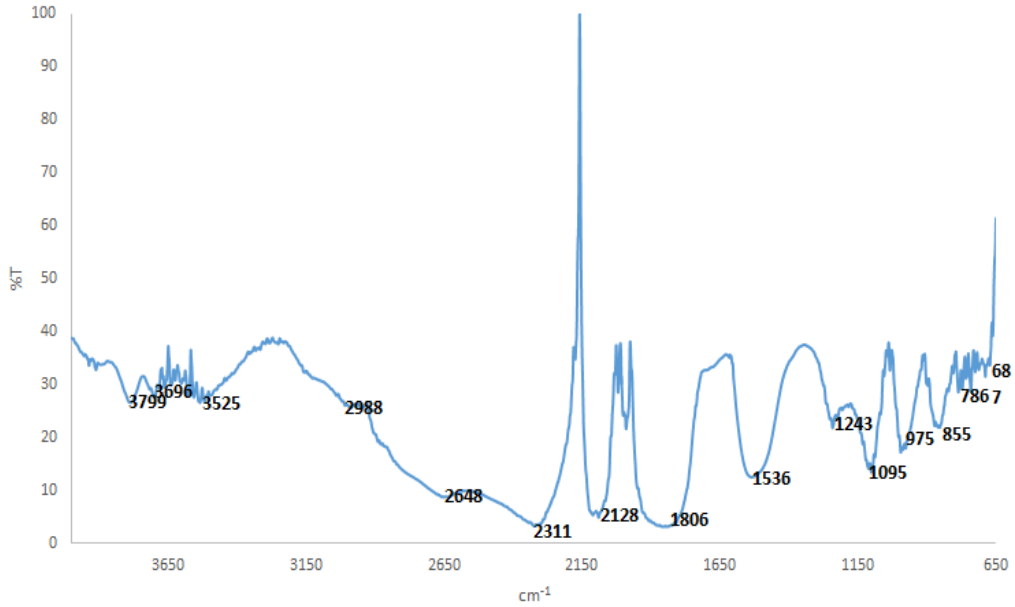


Figure 4.8: Showing spectra of characterized Macadamia Activated Carbon by Fourier Transform Infrared (FTIR) spectra before adsorption.

Figure 4.8 displays the FTIR spectra of macademia activated charcoal (MAC) before adsorption of E1 and E2. The notable differences were observed at the following wavenumbers, 3525, 2988, 2648, 2128, 1536, 1243 and 1095 cm^{-1}. The spectrum from activated carbon at 3525 cm^{-1} indicated the presence of the –OH group (Zhao et al., 2013). The methylene group is detected by –CH stretching at a wavenumber of 2988 cm^{-1}. The aldehyde group of $–O–CH_3$ is found around 2648 cm^{-1}. Strong bands at 1805 cm^{-1} indicate C–O stretching of carboxyl or carbonyl groups and 1536 cm^{-1} presence of C-C stretch ring aromatics (Yang et al., 2007). Methyl or amine groups are shown by a peak around 1243 cm^{-1}. The band from 1200 to 1000 cm^{-1} is the fingerprint

of syringyl units. Aldehyde and derivatives of benzene are detected by peaks at 855 and 795 cm^{-1} (Aik & Jia, 1998).

4.3.2. SEM

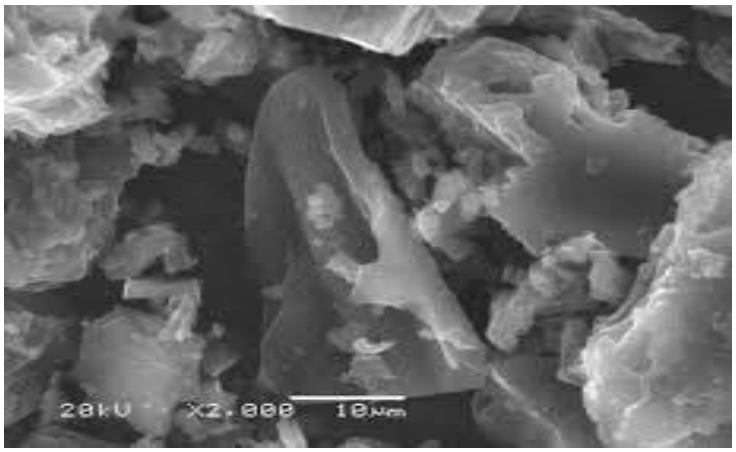


Figure 4.9: Scanning Electron Microscope (SEM) for MAC.

The morphology and structure of MAC was characterized with scanning electron microscope (SEM) (Figure 4.9). The SEM images revealed that the material consisted of flaky long fold-like structure and cavities.

4.3.3. TGA

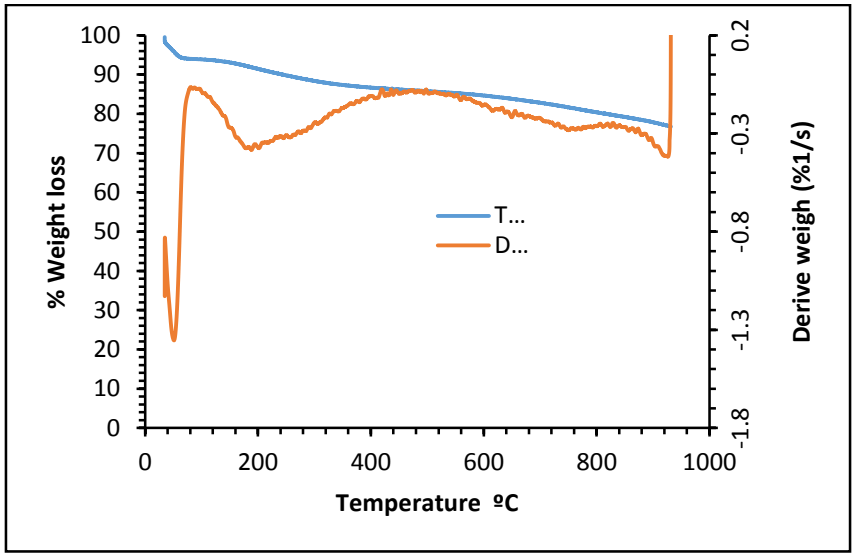


Figure 4.10: Percentage weight loss and derivative weight loss curve for MAC.

Thermogravimetric analysis (TGA) of the activated macademia nutshell was conducted to evaluate the change in percentage weight as a function of temperature (Fig. 4.10). Thermal degradation of plant biomass can be categorized into four successive individual stages, namely moisture evolution, and the decomposition of hemicelluloses, cellulose and lignin (Paduraru et al., 2015). Figure 4.11 exhibits the weight loss curve (blue) from 25 to 1000 ºC. The weight loss at the beginning (< 150 ºC) was most likely due to the loss of moisture in *Macadamia* activated carbon. The amount of adsorbed water in plant material is a measure of the hydrophilic components in its structure. The hydroxyl groups of the hemicellulose can associate with water molecules via hydrogen bonding, thereby leading to higher water content (Azwa & Yousif, 2013). No weight lost was observed around 280 – 450 ºC due to stability of mac. After that, a slight decrease of weight loss was still observed until 900 ºC. DTG

curve corresponded with the energy absorption while weight loss was reduced as shown in Fig.4.11 In this stage, the main compound cellulose and hemicelluloses and partial lignin were loss from *Macadamia* activated carbon. Finally, the weight loss was slowly decreased in between 450 – 800 ºC. It was mostly associated the phase structure stability when compared with weight loss.

4.4 *M*AC Optimization

4.4.1 Selection of the best elution solvent for *MAC*.

Three solvents acetone, methanol and acetonitrile were tested for best elution using activated carbon. The deionised water was spiked with standard solution containing 1mg/L steroids, then sorbent was soaked for 10 min with elution solution before each elution. The eluant was collected and analysed on HPLC. The results in Table 4.7 below shows the recoveries from *MAC* using different solvents. High recovery values were observed when methanol was used. However, there was no significant difference in terms of recoveries between estrone and β-estradiol since the recoveries were 52% and 49.7% respectively. Methanol is a protic solvent which means it solvate anions strongly via hydrogen bonding whilst acetonitrile and acetone are aprotic which tend to have large dipole moments (separation of partial positive and partial negative charges within the same molecule) and solvate positively charged species via their negative dipole. The disadvantage of methanol is that its pressure increases when mixed with water. From the results, it can be deduced that although methanol was

better than the other two extractants, the results indicates that *MAC* was not as effective as SPE in terms of holding the two selected steroids.

Table 4.7. The recovery values in % of *MAC* using different solvents**.**

Steroids	Methanol	Acetone	Acetonitrile
Estrone	52%	32%	42%
β-estradiol	49.7%	28%	46%

4.5 Effect of selected parameters on adsorption of the steroids on *MAC*

Adsorption experiments were carried out in batch mode using multistirrer in a series of Erlenmeyer flasks of 50 mL capacity covered to prevent contamination. The effect of time, temperature, concentration and solution pH were studied. Adsorption isotherms were obtained by fitting the data obtained during the effect of concentration study into Langmuir and Freudlich isotherm. An equal amount of absorbent was added separately into each individual flask and the other parameters time, temperature, concentration and pH were different and the flasks were agitated. After prescribed contact times, the solutions were centrifuged and the concentrations of steroids in the supernatant were determined by HPLC methods as described in the experimental section above.

4.5.1 Effects of pH

The effect of pH on the adsorption of E1and E2 was determined by batch adsorption experiments at varying pH values of 2, 4, 6, 7, 8 and 10, with all other experimental

variables kept constant (2 mg/L E1 and E2; temperature 25˚C; adsorbent dose 0.1 g). The effect of pH on E1 and E2 adsorption is illustrated in Figure 4.11. It was observed that the pH values had effects on the adsorption process. The percentage extraction efficiency (%E) was better in the acidic and neutral pH, and lowest in the alkaline pH. The pH values affect the surfaces charges on activated carbon. Percentage of E1 and E2 adsorbed at varying pH, at lower and neutral pH (4, 6 and 7) both E1 and E2 exist in non-ionic molecular form and can easily be adsorbed onto the MAC. At higher pH (8 and 10) the surface carbon particles tend to go negatively charged and hence reduce the sorption process by electrostatic repulsion (Chakraborty et al., 2011). It is also known that an increase in hydroxyl ion at the higher pH results in the production of aqua-complexes (Kumar et al., 2009.), which reduces the adsorption capacities of the activated carbon. So pH 7 gave the optimum results and was accepted as optimum pH in succeeding experiments.

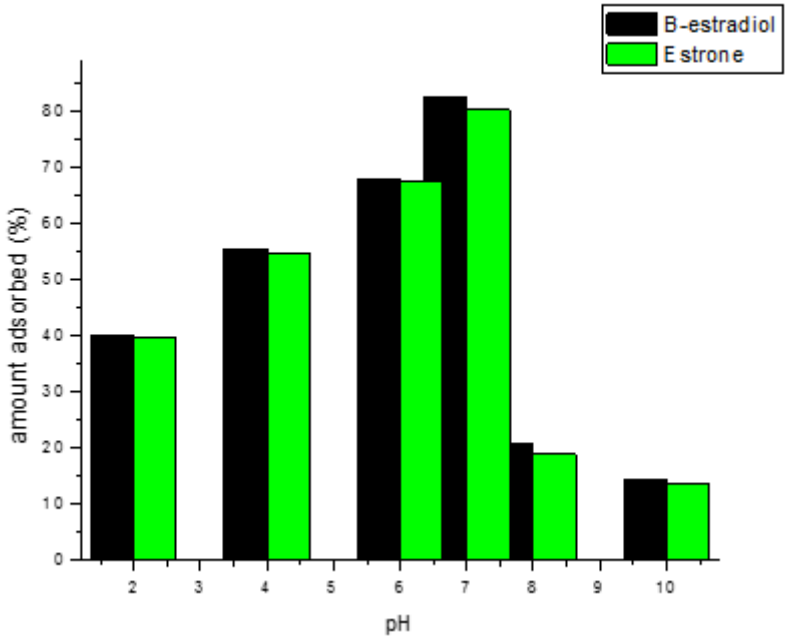


Figure 4.11: Percentage of estrone and β–estradiol adsorbed at varying pH. (Conditions: pH 2 to 10, concentration 2 mg/L, Adsorbent dose 0.1g, temperature 25 ˚C, time 120 min and solution volume 25 mL)

4.5.2. Effect of contact time.

AC dosage of 0.1 g was added to 50 ml of 2 mg/L steroids solution. Experiments were conducted at a temperature of 25˚C for 10-360 min and pH 7 to test the effect of contact time on the adsorption process. The results (Figure 4.12) indicated that the adsorption of E1 and E2 onto MAC was very rapid in the first 25 min hence the %E reached 60%. Then the adsorption of E1 and E2 increased gradually during the following 45 min until reached equilibrium at about 90 min. The results showed that the uptake of steroids by MAC depends on contact time. This may be due to the time required for the steroids to encounter the boundary layer effect, then diffuse to the surface of MAC and finally

diffuse to the porous structure of the adsorbent (Lata et al., 2007). To ensure complete equilibrium of the data, adsorption samples were collected at 360 min.

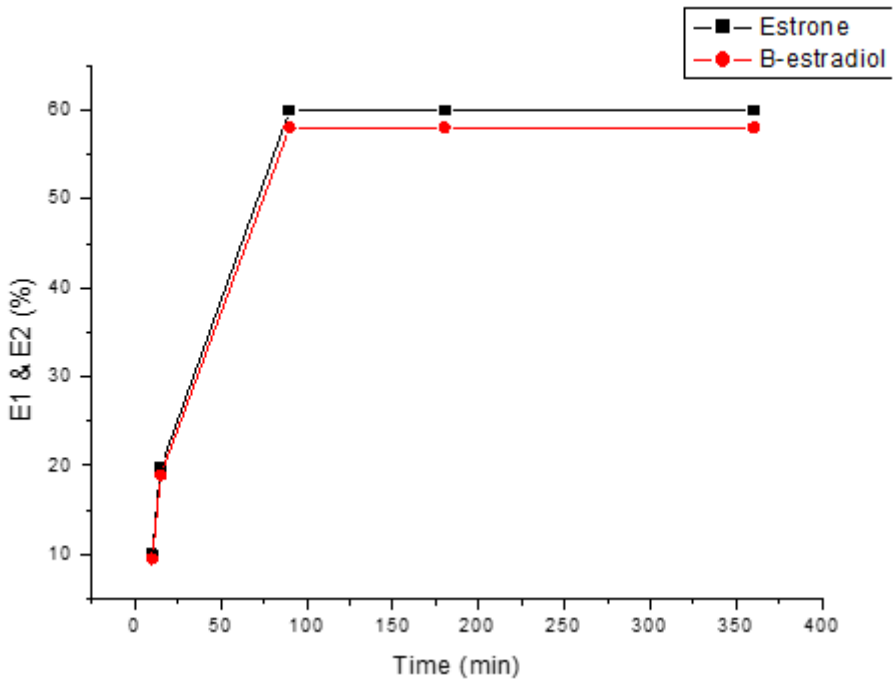


Figure 4.12: Percentage of estrone and β-estradiol adsorbed at varying time. (Conditions: pH 7, concentration 2 mg/L, adsorbent dose 0.1, temperature 25 ˚C, time 10 to 360 min and solution volume 25 mL).

4.5.3. Effect of Adsorbent Dose

At constant steroid concentration (2 mg/L), different amounts of MAC (0.1, 0.4, 1, 1.2, 1.6 g/L) were added to steroids solutions (50 mL) to study the effect of MAC amount on E1 and E2 adsorption. Results in (Figure 4.13) shows that the adsorption capacity in the first stage increased rapidly with the increase in the adsorbent dose then increased slowly until when equilibrium was reached with a further increase in the adsorbent dose (Lata et al., 2007). It can be seen that, at 1.0 g of the adsorbent dose,

the % removal of E1 and E2 reached the most at 83%. Then an increase in the dose of MAC from 1.0 to 2.0 g/L resulted only in about 3% more to reach 85%. Thus 1.0 g/L of AC was chosen as the optimum dose and used in the further experiments. The increase in % removal of E1 and E2 with the increase in the amount of MAC up to 1.0 g can be assigned to the increase in both the surface area and the adsorption sites to E1 and E2. However, the adsorption rate was not enhanced effectively by increasing the amount of the adsorbent from 1.0 to 2.0 g/L, this may be due to increase the overlapping and/or aggregation of adsorbent sites at high dose.

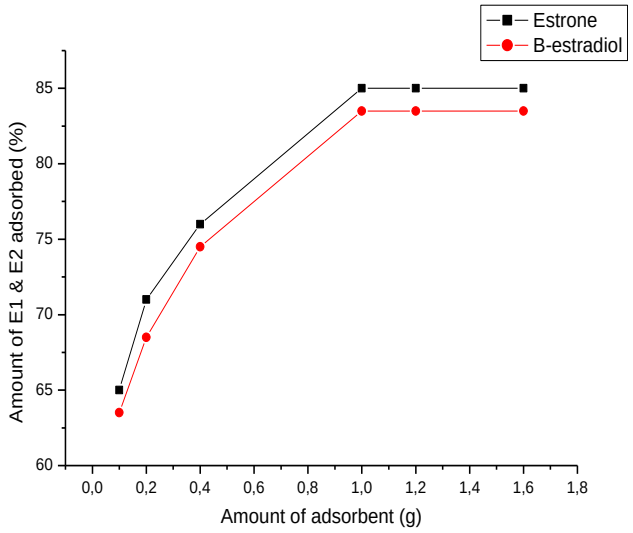


Figure 4.13: Percentage of E1 and E2 adsorbed with varying adsorbent mass. (Conditions: pH 7, concentration 2 mg/L, Adsorbent dose 0.1 to 2 g, temperature 25 ˚C, time 120 min and solution volume 25 mL).

4.5.4. Effect of concentration

Adsorption was done at the following varying concentrations 1-10 mg/L and all other parameters temperature, pH at neutral or acidic medium time were set constant (Figure 4.14). The calculations revealed that almost 85% E1 and E2 removal was achieved at low concentrations (0.1–0.6 mg/L) for *Macadamia*. When the concentration was increased from 0.6 to 1.4 mg/L, the percent removal of E1 and E2 decreased to about 75 % for all materials, while adsorption capacities increased with increase in concentration. The decrease in sorption of E1 and E2 at high concentration was due to the saturation of active binding sites (Basal et al., 2009; Gupta & Rastogi., 2009). Equilibrium concentration and adsorption capacity results were fitted in different sorption isotherms

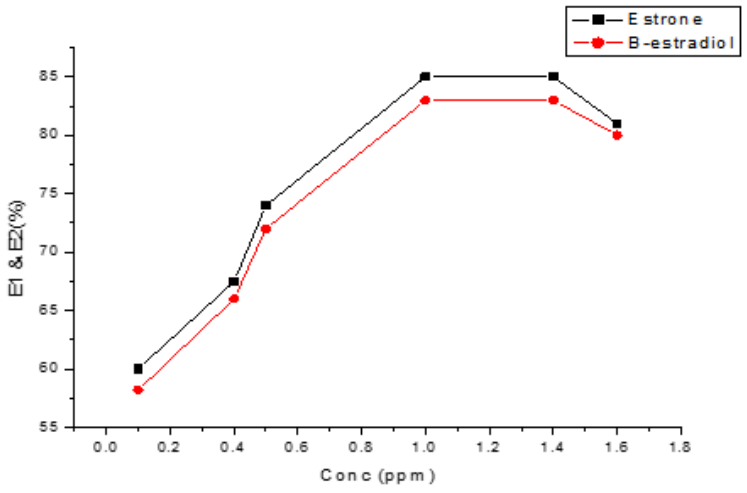


Figure 4.14: Percentage of E1 and E2 adsorbed with varying concentration. (Conditions: pH 7, concentration 1 to 2 mg/L, Adsorbent dose 0.1 to 2 g, temperature 25 ˚C, time 120 min and solution volume 25 mL).

4.5.5 Effect of temperature

Adsorption was done at the following varying concentrations 1-10 mg/L and all other parameters temperature, pH and time were set constant (Figure 4.15). The calculations revealed that almost 65 % E1 and E2 removal was achieved at low temperature (15–20 ºC) for *Macadamia.* The equilibrium sorption decrease from 65 to 58 % with increase in temperature. This is because room temperature is suitable for binding of steroids on surface adsorbent. This may be due to a tendency of steroids molecules to escape from solid phase to bulk phase with an increase in temperature of the solution. Extent of adsorption varies inversely as temperature that is lower the temperature higher is the adsorption. From this it can be concluded that heat must be liberated during adsorption.

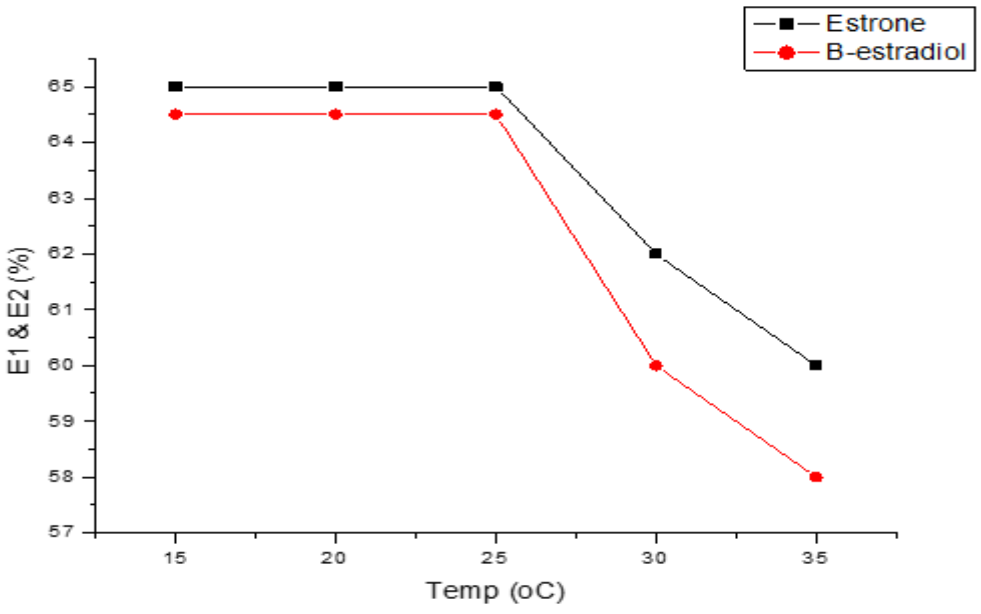


Figure 4.15: Percentage of E1 and E2 adsorbed with varying temperatures. (Conditions: pH 7, concentration 1 to 2 mg/L, Adsorbent dose 1 g, temperature 15 to 35 ˚C, time 120 min and solution volume 25 mL).

Under optimum condition as shown in Table 4.8, recoveries improved from 52% to 85% for estrone and from 49.9% to 74.4% for β-estradiol. These compared well with SPE recoveries (Table 4.3) which are 89.5% for estrone and 91.2% for *β*-estradiol. These conditions were used to determine the concentration of the selected steroids from real samples from the rivers (Figure 3.1). The adsorption decreases with the increase in concentration, the decrease in sorption of E1 and E2 at high concentration was due to the saturation of active binding sites (Basal et al., 2009; Gupta & Rastogi, 2009).

Table 4.8. Shows the adsorbed amount of E1 and E2 in percentage on MAC calculated from equation (2). (Conditions: pH 7, concentration 1 to 2 mg/L, Adsorbent dose 1 g, temperature 15 ˚C, time 120 min and solution volume 25 mL)

Concentration	**% R (CV)**	**% R (CV)**
Mg/L	**E_1**	**E_2**
0.1	52.1	49.9
0.2	65	60.5
1	74	64.3
1.6	85	74.4

4.6 Determination of steroids concentrations in water samples.

HPLC chromatogram of two (2) compounds of the steroids standard mixture is shown Figure 4.15. The analytical method development was successful as shown by relatively narrow and well separated peaks of individual steroid compounds. Deionized water samples were extracted using SPE.

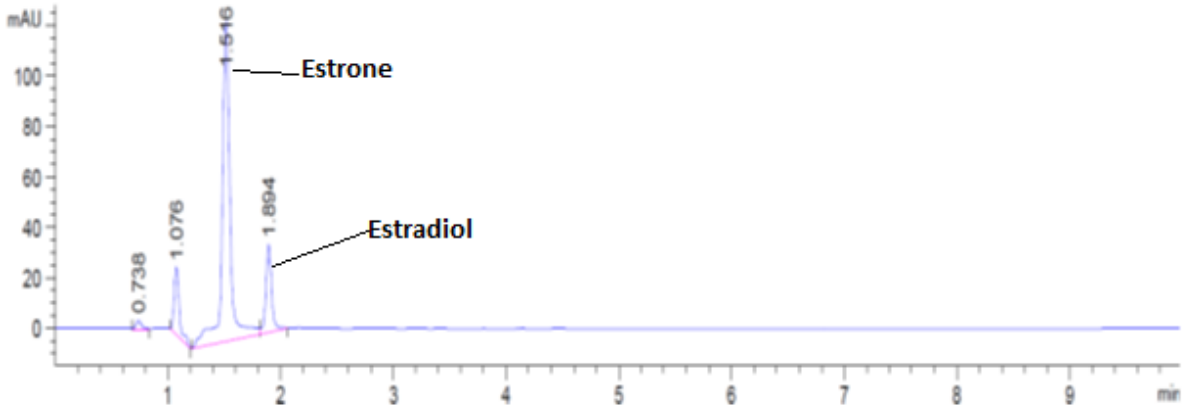


Figure 4.16: Showing chromatogram of E1 and E2 after extraction by SPE.

4.7. Adsorption Kinetics

4.7.1 Pseudo First Order (PFO) and Pseudo Second order (PSO) Kinetics

In adsorption system design, adsorption kinetics is the most important factor of consideration. It helps to investigate the potential rate controlling mechanism and helps in selecting optimum operating conditions in designing and optimizing full scale applications. In this study, the mechanism of adsorption was investigated by using PFO and PSO kinetic models based on aqueous phase concentrations of E1 and E2.

The pseudo-first-order equation was first represented by Lagergren for the sorption of oxalic acid and malonic acid onto charcoal (Lagergren, 1898). The model is generally expressed as:

$$\text{Log } (qe - q) = \log q_e - k_1 t/2.303 \quad \ldots\ldots (6)$$

$$t/q_t = 1/k_2 q^2 + 1/qt \quad \ldots\ldots (7)$$

Where t is the contact time (min), k_1 = pseudo-first-order adsorption rate constant (min^{-1}), qe and q are the amount of the adsorbate at equilibrium time and time t (mg/g) respectively.

Pseudo first order plot of ln ($q_e - q$) against t should give a linear relationship from which k_1 in (min^{-1}) can be calculated from the slope obtained from the graph. A plot of t/q_e against t will give a rate constant k_2 ($L \cdot mg^{-1} \cdot min^{-1}$) for pseudo second-order adsorption kinetics. Table 4.9 depicts the results obtained for the pseudo first-order and pseudo second-order kinetic model for E1 and E2. Table 4.9 also presents constant values and correlation coefficient R^2 of both pseudo first-order and pseudo second order kinetic models for adsorption of E1 and E2 onto the AC. The kinetic plots showed a good fit of sorption equilibrium data with respect to the pseudo second order kinetic model. Although the pseudo first order showed a fairly good fit, the second order was more superior with respect to the correlation coefficient. This suggests that chemisorption is the rate controlling step as expressed (Ho et al., 2000).

4.7.2. Intra-Particles Diffusion Kinetics

One of the models to express the mechanism of solute adsorption onto an adsorbent is the intra particle diffusion kinetics in which the linear equation is expressed as (Ho et al., 2000):

$q_t = K_{diff} t^{1/2} + C$.....(8)

Where K_{diff} is the intra-particle diffusion rate constant.

A plot of q_t (mg/g) versus the square root of the contact time, $t_{1/2}$ (min1/2) should be linear with the straight line passing through the origin, if the sorption process obeys the intra-particle model. It can then be assumed that the mechanism involves the diffusion of the species and the slope of the linear curve is the rate constant of the intra particle transport (K_{diff}). Figures 4.17 and 4.18 show the intra-particle diffusion plot, which showed poor fit (R^2 = 0.9959 and 0.771) and a multi-linearity profile. None of the plots for E1 and E2 passed through the origin. There are two sections on the plot which show some fit (the initial and the last sections). The initial part of the diffusion profile may be considered as the faster adsorption stage and hence can be attributed to the boundary layer diffusion of E1 and E2 on the external surface of the activated carbon. The last stage is where intra particle diffusion kinetics starts to slow down as a result of the lower adsorbate concentration in the aqueous solution. The middle stage (curved part) is where the intra-particle diffusion kinetics is controlled.

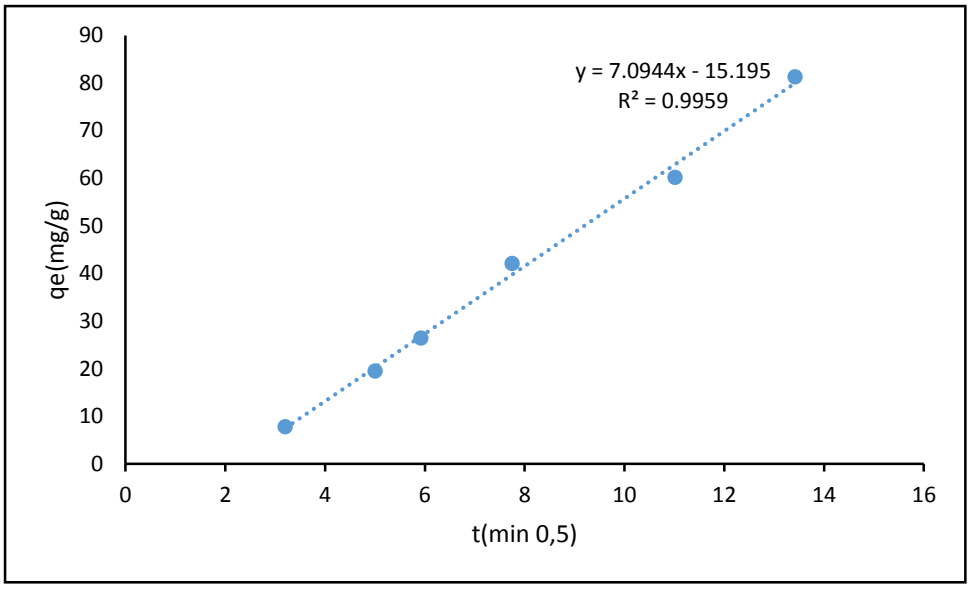


Figure. 4.17: Shows Intra-Particles Diffusion Kinetics **(**IPD) profiles for the adsorption of E1 and E2 onto MAC

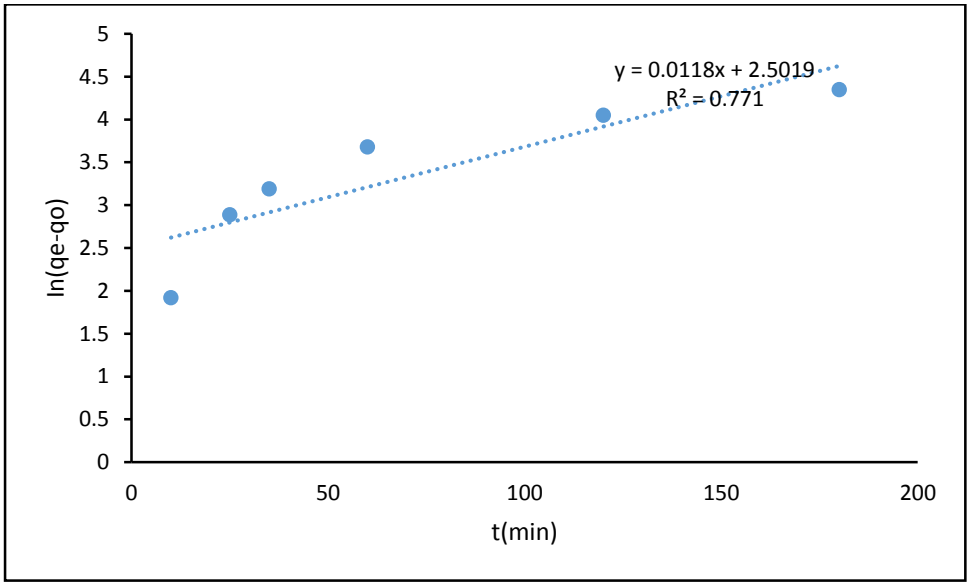


Figure 4.18: Shows Pseudo First Order kinetic (PFO) profiles for the adsorption of E1 and E2 onto MAC

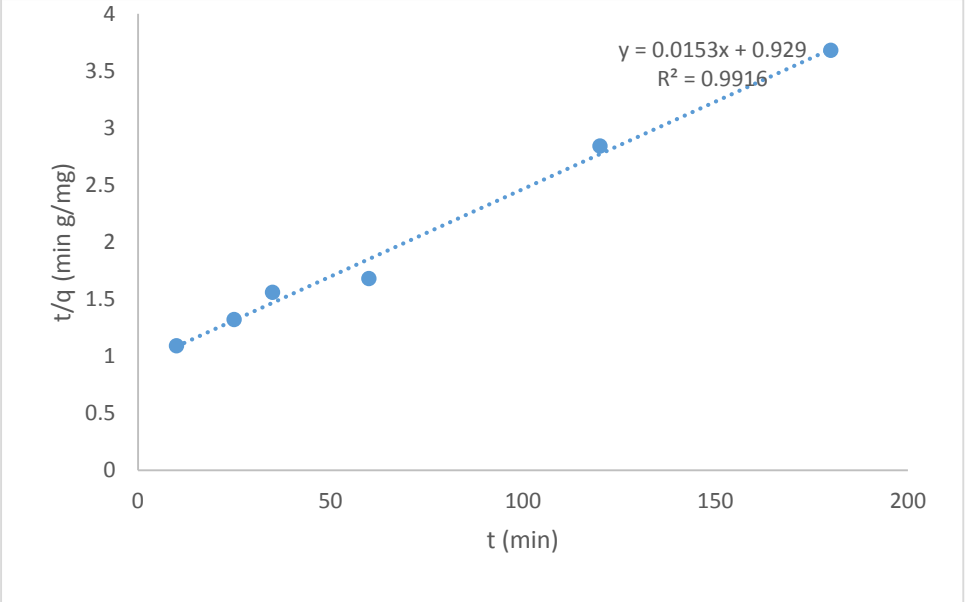


Figure 4.19: Showing Pseudo Second order kinetitic (PSO) profiles for the adsorption of E1 and E2 onto MAC

Table 4.9: Pseudo first and second order parameters for E1 and E2 sorption onto MAC.

Analyte	**Equation**	**K_2(gmg^{1}min^1)**	**R^2**	**K_1(g mg^{-1}min^{-1})**
E_1	Y=0.0151x+0.8122	0.00930	0.9872	0.0151
E_2	Y=0.0111x+0.9265	0.00599	0.9927	0.0111
E_1	Y=0.0066x+1.0846	0.00304	0.9767	0.0066
E_2	Y=0.0153x+0.9291	0.00584	0.9916	0.0153

The kinetics of steroids sorption on MAC was studied at different initial concentrations. Using equation (1), t/q_e vs t was plotted (Figure 4.19) which shows that the pseudo-second order model was applicable to steroids sorption for various initial concentrations as the plots are linear and correlation coefficient, R^2 values are very close to one. Which clearly indicates that the sorption of steroids onto sorbent can be better described by pseudo-second order model. The values of second order rate constant (k_2) and q_e determined from the plots and the values along with correlation coefficient, R^2 Table 4.8 showed that k_2 decreases with the increasing initial steroids concentration.

4.8 Thermodynamic study

Adsorption involves attracting molecules of adsorbate on surface of the adsorbent. Due to this, energy is released and thus heat of adsorption is negative i.e. adsorption is always exothermic. Further physical adsorption involves weak forces of attraction, heat evolved is less whereas chemical adsorption involves strong forces of attraction, and heat evolved is much higher.

4.8.1. Free Energy Change during Adsorption:

For adsorption free energy ΔH is negative. The molecules of the adsorbate are held on surface of the solid adsorbent due to this entropy decreases i.e. ΔS is also negative.

$$\Delta G=\Delta H-T\Delta S \quad \text{..... (9)}$$

$$\Delta G=RT\ln K_L \quad \text{..... (10)}$$

Therefore, adsorption will occur only when ΔG is negative and this is possible only if $\Delta H > T\Delta S$.

Initially this condition is met but as adsorption process continues ΔH value decreases whereas $T\Delta S$ increases and finally ΔH becomes equal to $T\Delta S$ so that $\Delta G = 0$. This is when we say the state of adsorption equilibrium has been achieved.

Table 4.10: Tabulated data for the plot of C_e/q_e versus C_e.

Initial concentration Ci (mg/l)	**Equilibrium concentration Ce(mg/l)**	**Amount adsorbed at equilibrium Qe(mg/g)**	**Ce/Qe(l/g)**
1	0.4	90	0.0044
3	2.5	75	0.033
6	4.7	195	0.024
8	6.4	240	0.027
10	8.6	210	0.041

Figure 4.20 shows the linear plots of C_e/q_e versus C_e and is used to determine the value of q_{max} (mg/g) and b (L/mg). The data obtained are listed in Table 4.10 for all adsorbents. The Langmuir constants q_{max} and b are related to the adsorption capacity (amount of adsorbate adsorbed per unit mass of the adsorbent to complete monolayer

coverage) and energy of adsorption, respectively. The essential characteristics of the Langmuir isotherm may be expressed in terms of a dimensionless constant separation factor or equilibrium parameter.

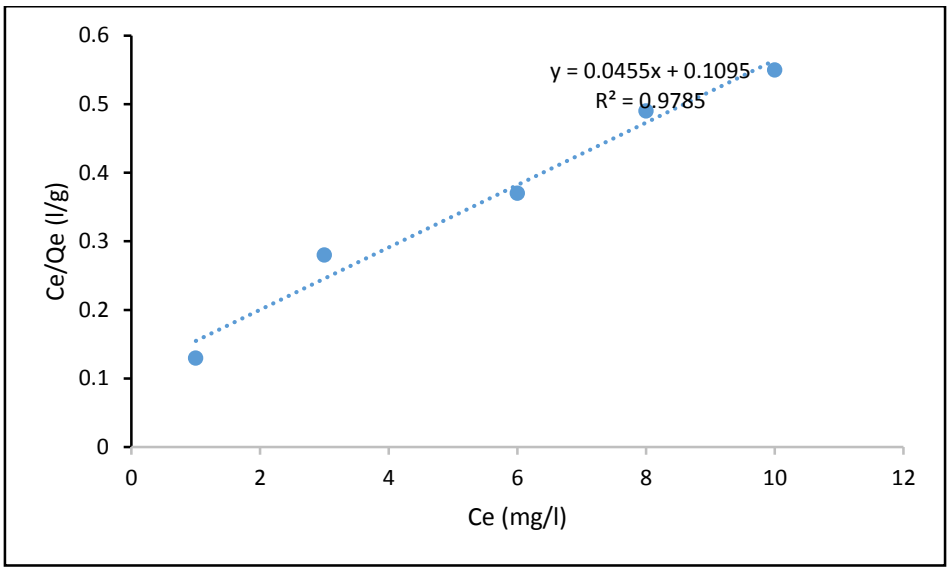


Figure 4.20: Langmuir isotherm plots for the adsorption of E1 and E2 onto MAC at variable temperatures.

Table 4.17 presents the Gibbs free energy (ΔG) for the sorption of steroids calculated from equation (6). The Gibbs energy is a fundamental criterion of spontaneity. ΔG value of -11.72 kJ/mol was negative indicating that the sorption process was spontaneous. The value obtained for ΔG was also less than -20 kJ/mol suggesting electrostatic interaction between the E1 and E2 and the MAC which supported physisorption mechanism.

The plot of lnK vs 1/T from equation (7) was linear as presented in Figure 4.18, with the slope and intercept equal to - ΔH/R and ΔS/R, respectively. The value of enthalpy change (ΔH) calculated from slope was -11.72 kJ/mol. Negative ΔH suggest that

sorption process proceeded favourably at lower temperature and the sorption mechanism was exothermic. The value of the entropy change computed from intercept was 37.34 J/Kmol and was presented in Table 4.11. A positive ΔS suggest that the freedom of the adsorbed steroids is not restricted in AC, indicating that physisorption mechanism predominates.

Table 4.11: Gibbs free energy change parameters of E1 and E2 on MAC adsorption system.

Temp (K)	**ΔS(J/ Kmol)**	**ΔH (kJ mol^{-l})**	**ΔG (kJ mol^{-1})**
288	37.34	-11.72	-1.223
298			-1.266
303			-1.287
308			-1.308

Thermodynamic studies have been used to assess the spontaneity of the adsorptive process. The value of thermodynamic parameters for the sorption of steroids onto AC at various temperatures were calculated and listed in Table 4.11 The Gibbs free energy was change (ΔH) calculated using equation (9) while the values of ΔH° and ΔS° have been calculated from the slope and intercept of the plot lnK and 1/T Figure 4.21, which gives a straight line with acceptable coefficient determination (R^2=0.9014). The negative values of ΔG° at lower temperature indicates that sorption of steroids on AC

is a spontaneous process. The negative value of ΔH^o and ΔS^o indicates that sorption is exothermic and suggest probable occurrence of favourable adsorption.

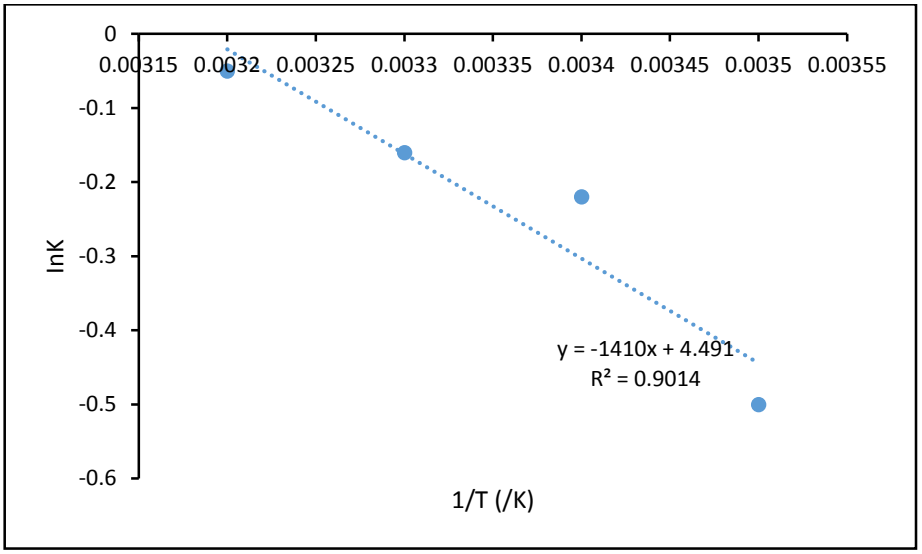


Figure 4.21: Plot of ln Kc versus 1/T for E1 and E2 adsorption onto MAC.

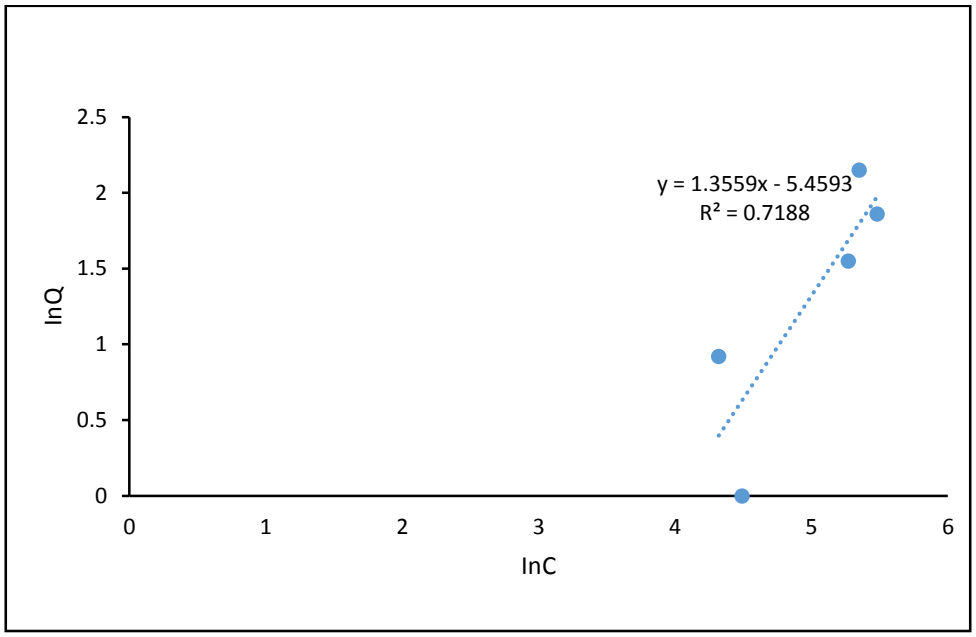


Figure 4.22: Freundlich isotherm plots for the adsorption of E1 and E2 onto MAC at variable temperatures.

4.8.2 Langmuir isotherm model

The basic assumption of Langmuir model is that the formation of monolayer takes place on the surface of the adsorbent, indicating that only one steroids molecule could be adsorbed on one adsorption site and the intermolecular forces decrease with the distance. It is also assumed that the adsorbent surface is homogeneous in character and possesses identical and energetically equivalent adsorption sites. It was presented as Eq (11):

$$q_e = q_m K_L C_e / 1+K_L Ce \ldots\ldots\ldots\ldots\ldots\ldots\ldots (11)$$

A linear Langmuir adsorption isotherm is presented in Figure 4.17. The values of q_m and K_L of linear expression of Langmuir adsorption isotherm were calculated from the slopes and intercept of the linear plot of C_e/q_e versus C_e in Figure.8 and table 1 according to Eq (12).

$$1/q_e = 1/q_m + (1/K_L q_m)*(1/C_e) \ldots\ldots\ldots\ldots\ldots\ldots\ldots (12)$$

The isotherm was found to be linear over the entire concentration range studied with a good linear correlation coefficient (R^2 = 0.9785) (see table 4.11), showing that Langmuir equation represents the best fit of experimental data than the other isotherm equation.

4.8.3. Freundlich isotherm model

While Langmuir isotherm assumes that enthalpy of adsorption is independent of the amount adsorbed, the empirical Freundlich equation, based on sorption on heterogeneous surface, can be derived assuming a logarithmic decrease in the enthalpy of adsorption with the increase in the fraction of occupied sites. The Freundlich equation is purely empirical based on sorption on heterogeneous surface and is given by Eq. (13):

$$q_e = k_f C_e 1/n \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (13)$$

Eq. (14) can be rearranged to obtain a linear form by taking logarithms Eq. (14):

$$\ln q_e = \ln k_f + n\ 1\ \ln C_e \ldots\ldots\ldots\ldots\ldots\ldots\ldots (14)$$

The slope and the intercept correspond to (1/n) and k_f, respectively. It was revealed that the plot of $\ln q_e$ and $\ln C_e$ yields a straight line Figure.4.22. The results are indicated in Table 4.11. The favourable adsorption of this model can be characterized such that if a value for n is above unity, adsorption is favourable and a physical process. In the present study the value of n (n= 0.74) is close to the unit, indicating that the adsorption process is favourable. And the value of correlation coefficient (R^2 = 0.7188) is slightly lower than the Langmuir isotherm value. The results of Langmuir and Freundlich implies that the adsorption of E1 and E2 onto AC show a complex mechanism involving both monolayer and heterogeneous surface condition.

Table 4.11: Showing Langmuir and Freundlich isotherm parameters.

Langmuir Isotherm			**Freundlich Isotherm**		
a	b	R^2	K_f	n	R^2
22	0.42	0.9785	2879	0.74	0.7188

4.9. Application of the developed method

Preliminary studies involved trying the optimum condition for the MAC and testing mobile phase compositions and using different chromatographic parameters for the separation of the two studied steroids. A C18 column (4.6mm length, 250 mm inner diameter, and 5 µm) was used as a stationary phase for separation. As a mobile phase, a mixture of water and methanol was used (50% methanol and 50% water, v/v). Isocratic elution was performed for analysis using a flow rate of 2 ml/min, and UV detection at a wavelength of 220 nm. Injection volume was set to be 20 µL for all samples and standards. Figure 23 shows chromatogram of E1 and E2 separated using the current developed method.

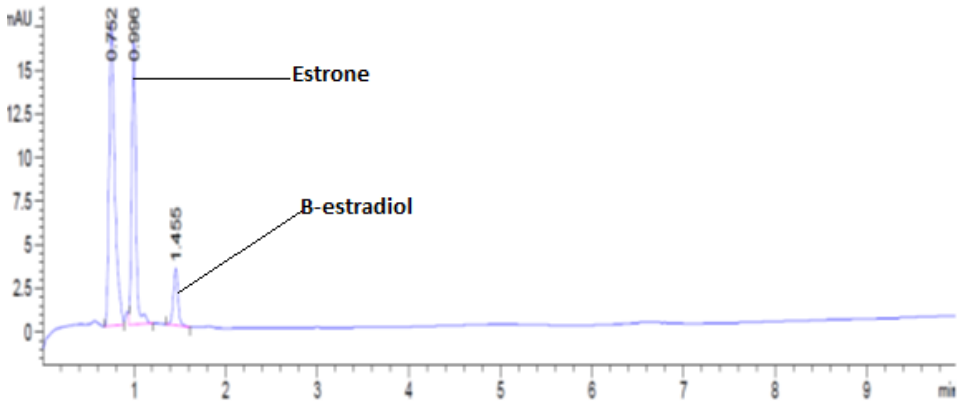


Figure 4.23: Showing Vaal river sample spiked with 0.1mg/L steroids standard**.**

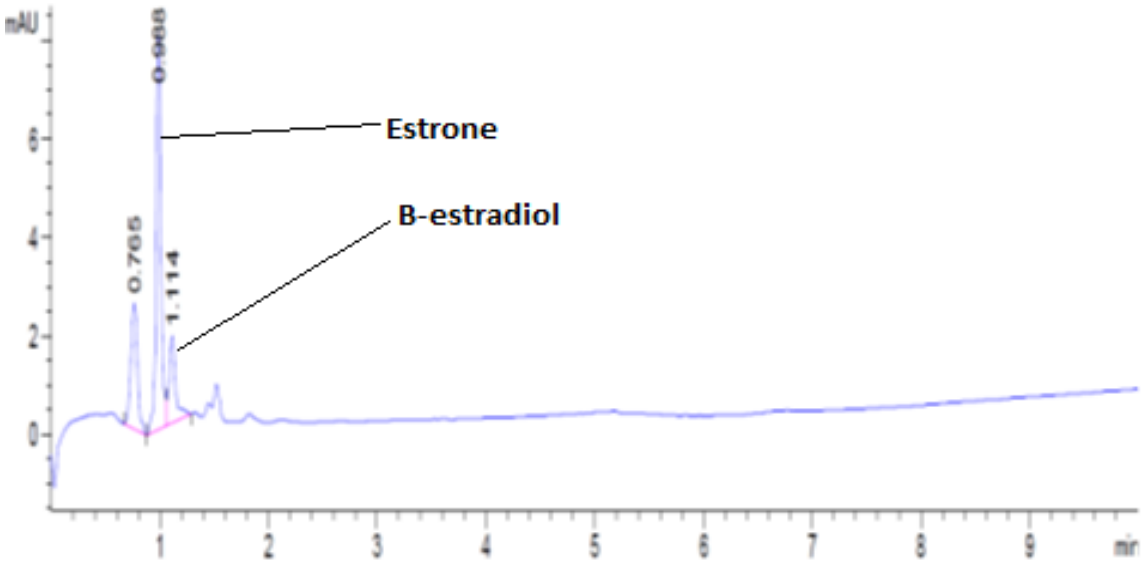


Figure 4.24: Showing Barrage river sample spiked with 0.1 mg/L steroids standard

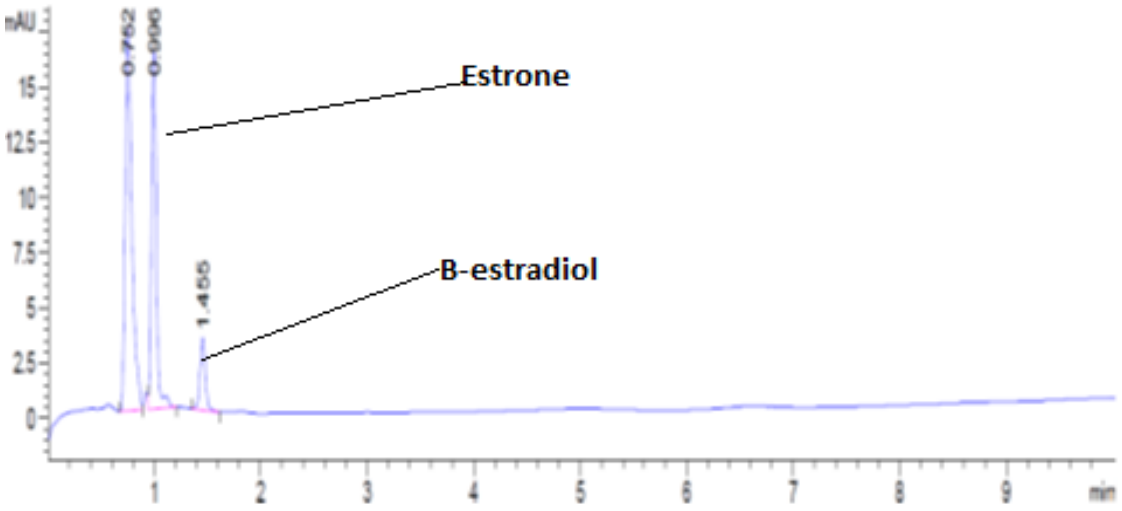


Figure 4.25: Showing Klip River sample spiked with 0.1 mg/L steroids standard.

Chromatograms obtained with optimised method HPLC mode spiked with 0.1 mg/L of standard steroids solution after MAC adsorption from real samples. Comparison of these 3 chromatograms and analysis of water samples collected between 01 June to 30 September 2015 shown that E1 and E2 are not present at detectable levels in Vaal,

Barrage and Klip River Water (Table 4.12). The reason might be that the concentration levels were below detection limit or they were not detected because the method developed was not sensitive enough.

Table 4.12: Table showing concentration of steroids from the three rivers

Barrage River				
Steroid	June 2015	July 2015	August 2015	September 2015
Estrone (ppm)	ND	ND	ND	ND
β-estradiol (ppm)	ND	ND	ND	ND
Vaal River				
Steroid	June 2015	July 2015	August 2015	September 2015
Estrone (ppm)	ND	ND	ND	ND
β-estradiol (ppm)	ND	ND	ND	ND
Klip River				
Steroid	June 2015	July 2015	August 2015	September 2015
Estrone (ppm)	ND	ND	ND	ND
β-estradiol (ppm)	ND	ND	ND	ND

ND = Not detected

From the results, all the three rivers studied showed the absence of the two steroids studied.

CHAPTER 5

5.1 Conclusion and recommendations

In conclusion, it was established that activated *macademia* nutshell charcoal can replace the usage of SPE since its adsorption capacity came close to that of SPE after optimization. The experimental results show that steroids are not present in water within the working range of concentrations 0.01-0.25 mg/l. In comparison with the result from the experiments obtained from spiked water samples and that of standard samples, the results support the conclusion that the method is suitable for the determination of steroids in water using HPLC with a UV-detector. The maximum uptake of Steroids onto MAC was found to be 80 %. t=250 min, pH=7, Co=2mg/l, T= 25 ^{o}C and m= 0.1 g/l were the optimum condition for Steroids-MAC system. The kinetics studies confirmed Steroids-MAC adsorption system can be described by pseudo- second-order kinetics model. Over all analysis of equilibrium model analysis indicates the fitness of Langmuir isotherm model to Steroids-MAC adsorption system, suggesting a monolayer adsorption of Steroids on the surface of MAC. Steroids adsorption capacity of MAC was found to be decreasing with increase in temperature suggesting that the adsorption process was exothermic in nature, which was further supported by the negative values of change in enthalpy. The negative values of Gibb's free energy suggested that adsorption of steroids onto MAC was a spontaneous process. Characterization of MAC confirmed highly carbonaceous nature and a higher effective surface area.

REFERENCES

ALUM, A., YOON, Y., WESTERHOFF, P. & ABBASZADEGAN, M. (2004). Oxidation of bisphenol A, 17beta-estradiol, and 17alpha-ethynyl estradiol and by-product estrogenicity, Environ. Toxicol. 19 (3), 257-264.

AIK, C. L. & JIA, G. (1998) "Preparation and Characterization of Chars from Oil Palm Waste" *Carbon*. 36. 1663-1670.

BENIJTS, T., DAMS, R., GUNTHER, W., LAMBERT, W. & LEENHEER, D.A. (2002). Analysis of estrogenic contaminants in river water using liquid chromatography coupled to ion trap based mass spectrometry, Rapid Communications in Mass Spectrom. 16, 1358-1364.

BHANOT, D. (2011). Arbo pharmaceuticals, Auriga Research Ltd.

BARONTI, C., CURINI, R., D'ASCENZO, G., Di CORCIA, A., GENTITI, A. & SAMPERI, R. (2000). Monitoring natural and synthetic estrogens at activated sludge sewage treatment plants and in a receiving river water. Environ. Sci. Technol. 34, 5059–5066.

BONEFELD-JORGENSON, E.C. (2004). The human health effect programme in Greenland, a review. Sci. Total Environ. 331, 215–31.

CARGOUET, M., PERDIZ, D., MOUATASSIM-SOUALI, A., TAMISIER-KAROLAK, S. & LEVI, Y. (2004). Assessment of river contamination by estrogenic compounds in Paris area (France). Sci. Total Environ. 324, 55–66.

CHAKRABORTY, S., CHOWBURY, S. & SAHA, P.D. (2011). Adsorption of Crystal Violet from aqueous solution onto NaOH- modified rice husk, Carbohydr. Polym. 86, 1533–1541.

DESBROW, C., ROUTLEDGE, E.J., BRIGHTY, G.C., SUMPTER, J.P. & WALDOCK, M.J. (1998). Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening. Environ. Sci. Technol. 32, 1549–1558.

7. Jobling, S., Nolan, M., Tyler, C.R., Brighty, G., Sumpter, J.P., 1998. Widespread sexual disruption in wild fish. Environ. Sci. Technol. 32, 2498–2506.

DIAZ-CRUZ, M.S., LOPEZ DE ALDA, M.J., LOPEZ, R. & BARCELO, D. (2003). Determination of estrogens and progestogens by mass spectrometric techniques (GC/MS, LC/MS and LC/MS/MS), J. Mass Spectrum. 38, 917–923.

DIAL, S.L., MOLAND, C.L., & SHEEHAN, D.M. (2001). Structure–activity relationships for a large diverse set of natural, synthetic and environmental estrogens. Chem.

DE SOUZA, S.V.C., PINTO, C.T. & JUNQUEIRA, R.G. (2007). In-house method validation: application in arsenic analysis, J Food Comp and Anal. 20, 241-244.

DIETZ, C., SANZ, J. & CAMARA, C. J. (2006). Chromatogr. A 1103, 183.

DURBECK, H.W, BUKER, I., SCHEULEN, B. & TELIN, B. (1978). Gas chromatographic and capillary column gas chromatographicmass spectrometric determination of synthetic anabolic steroids; J. Chromatogr. 167, 117–124

DULKA, J.G., STACEY, N.E., SORENSEN, P.W. & VAN DER KRAAK, G.J. (1987). A steroid sex pheromone synchronises male-female spawning readiness in goldfish. Nature 325, 251–253.

DZIEWECZYNSKI, T.L., EKLUND, A.C. & ROWLAND, W.J., (2006). Male 11-ketotestosterone levels change as a result of being watched in Siamese fighting fish, Betta splendens. Gen. Comp. Endocrinol. 147, 184–189.

EARLEY, R.L., EDWARDS, J.T., ASEEM, O., FELTON, K., BLUMER, L.S., KAROM, M. & GROBER, M.S. (2006). Social interactions tune aggression and stress responsiveness in a territorial cichlid fish (Archocentrus nigrofasciatus). Physiol. Behav. 88, 353–363.

ELLIS, T., JAMES, J.D. & SCOTT, A.P. (2005). Branchial release of free cortisol and melatonin by rainbow trout. J. Fish Biol. 67, 535–540.

FANG, H., TONG, W., SHI, L.M., BLAIR, R., PERKINS, R., BRANHAM, W., HASS, B.S., XIE, Q., FUKUHARA, T., IWASAKI, S., KAWASHIMA, M., SHINOHARA, O. & ABE, I.(2006).Absorbability of estrone and 17beta-estradiol in water onto activated carbon. *Water Research*, *40*(2), 241–8.

GIBSON, R., TYLER, C.R. & HILL, E.M. (2005). Analytical methodology for the identification of estrogenic contaminants in fish bile. J. Chromatogr. A 1066, 33–40.

GHULAM, A., KOUACH, M., RACADOT, A., BOERSMA, A., VANTYGHEM, M.C. & BRIAND, G. (1999). Quantitative analysis of human serum corticosterone by high-performance liquid chromatography coupled to electrospray ionization mass spectrometry. J. Chromatogr. B Biomed. Sci. Appl. 727, 227–233.

GEOLOGICAL SURVEY (USGS). From (1999 to 2000) steroid.

GOMES, R.L., SCRIMSHAW, M. D., CARTMELL, E. & LESTER, J. N. (2011). "The Fate of Steroid Estrogens: Partitioning during Wastewater Treatment and onto River Sediments," Environmental Monitoring and Assessment. 175(1), 431-441.

HANSELMAN, T.A., GRAETZ, D.A. & WILKIE, A.C. (2003). Manure-borne estrogens as potential environmental contaminants: a review. Environ. Sci. Technol. 37, 5471–5478.

HILL, M., CIBULA, D., HAVLIKOVA, H., KANCHEVA, L., FAIT, T., KANCHEVA, R., PARIZEK, A. & STARKA, L. (2007). Circulating levels of pregnanolone isomers during the third trimester of human pregnancy. J. Steroid Biochem. Mol. Biol. 105; 166–175.

JONES, O.H., VOULVOULIS, N. & LESTER, J. N. (2007). The occurrence and removal of selected pharmaceutical compounds in a sewage treatment works utilising activated sludge treatment. *Environ Pollut*, *145*(3), 738–44.

KANNAN, N. & INBARJA, R. (2006). Studies in kinetic removal of metal ions by orange peel. IJEP. 26(1): 39-46.

KICHA, A.A., IVANCHINA, N.V., KALINOVSKY, A.I., DMITRENOK, P.S. & STONIK, V.A. (2005).Structures of new polar steroids from the Far-Eastern Starfish Ctenodiscus crispatus*, Russian Chem. Bull. Int. Ed. 54, 1266–1271.

KELLY, T., GRAY, T.R. & HUESTIS, M.A. (2008). Development and validation of a liquid chromatography-atmospheric pressure chemical ionisation-tandem mass spectrometry method for simultaneous analysis of 10 amphetamine, methamphetamine- and 3, 4 methylenedioxymethamphetamine-related (MDMA) analytes in human meconium, J of Chrom B. 867, 197-198.

KOPLIN, D.W., FURLONG, ET, MEYER, M.T., THURMAN, E.M., ZAUGG, S.D., BARBER, L.B. & BUXTON, H.T., (2002). Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999–2000: a national reconnaissance. Environ.Sci. Technol*. 36, 1202–1211.*

KOSANAN, H., PRAKASH, P.K.S., YATES, C.R., MILLER, D.D. & RAMAGIRI, S. (2007) Rapid screening of doping agents in human urine by vacuum MALDI-linear trap mass spectrometry, Anal. Chem. 79, 6020–6026.

LABADIE, P. & BUDZINSKI, H. (2005). Determination of steroidal hormone profiles along the Jalle D'Eysines River (near Bordeaux, France), Environ Sci and Techno. 39, 5113-5120.

LABRIE, F., BELANGER, A., BELANGER, P., BERUBE, R., MARTEL, C., CUSAN, L., GOMEZ, J., CANDAS, B., CHAUSSADE, V., CASTIEL, I., DELOCHE, C., LECLAIRE, J. (2007). Metabolism of DHEA in postmenopausal women following percutaneous administration. J. Steroid Biochem. Mol. Biol. 103, 178–188.

LANDIS, M.S. (2007). The use of mixed mode ion exchange solid phase extraction to characterize pharmaceutical drug degradation, J of Pharml and Biom Analysis. 44, 1029-1020.

LAI, K.M., SCRIMSHAW, M.D. & LESTER, J.N. (2002). Biotransformation and bioconcentration of steroid estrogens by Chlorella vulgaris. Appl. Environ. Microbiol. 68, 859–864.

LATA, H., GARG, .V.K. & GUPTA, .R.K. (2007). Removal of a basic dye from aqueous solution by adsorption using Parthenium hysterophorus: An agricultural waste, Dyes Pigm. 74, 653–658.

LEGLER, J., DENNEKAMP, M., VETHAAK, A.D. & BROUWER, A. (2002). Detection of estrogenic activity in sediment-associated compounds using in vitro reporter gene assays. Sci. Total Environ. 293, 69–83.

LEE, F.P., IDEN, C. R. & McELROY, A.E. (2001). Determination of steroid estrogens in wastewater by immunoanity extraction coupled with HPLC-Electrospray-MS J, Anal Chem. 73, 3890–3895.

LOPEZ DE ALDA, M.J., GIL, A., PAZ, E. & BARCELO, D. (2002). Occurrence and analysis of estrogens and progestogens in river sediments by liquid chromatography–electrospray–mass spectrometry. Analyst 127, 1299–1304.

MAROTO, A., BOQUE, R., RIU, J. & RIUS, F.X. (2001). Measurement uncertainty in analytical methods in which trueness is assessed from recovery assays, *Anal Chimica Acta.* 440:171-172.

NENTWIG. (2004). ASTM (American Society for Testing and Materials) (1995). Standard test methods for measuring the toxicity of sediment-associated contaminations with freshwater invertebrates, E 1706-95a, *In*: Annual Book of ASTM Standards. 11(5), 1201-1285. ASTM, Philadelphia.

ROUTLEDGE, E.J. & SUMPTER, J.P. (1996) Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. Environ Toxicol Chem; *15, 241–8.*

SEKI, M., YOKOTA, H., MAEDA, M., TADOKORO, H. & KOBAYASHI, K. (2003) Effects of 4-nonylphenol and 4-tert-octylphenol on sex differentiation and vitellogenin induction in medaka (Oryzias latipes). Environ Toxicol Chem; 22, 1507–16.

SNYDER, S.A., KEITH, T.L., VERBRUGGE, D.A., SNYDER, E.M., GROSS, T.S., KANNAN, K., GIESY, J.P. (1999). Analytical methods for detection of selected estrogenic compounds in aquatic mixture. Environ. Sci. Technol. 33, 2814–2820.

SNYDER, S.A., ADHAM, S., REDDING, A.M., CANNON, F.S., DECAROLIS, J., OPPENHEIMER, J. & WERT, E. C. (2006). Role of membranes and activated carbon in the removal of endocrine disruptors and pharmaceuticals. Desalination, *202*, 156–181.

STANDING COMMITTEE OF ANALYST (SCA). (2005). "The Determination of Steroid Estrogens in Environmental and Waste-water Using Chromatography and Mass Spectrometry", Environ Agency, Bristol.

STEPHAN, R., HAJSLOVA, J., KOUCOUREK, V. & TICHA, J. 2004). Uncertainties of gas chromatographic measurement of troublesome pesticide residues in apples employing conventional and mass spectrometric detectors, Anal Chimica *Act.* 520, 245-247.

STOCK, L. D., D'HONDT, H. & THIENPONT, L.M. (2009). Method validation across the disciplines- Critical investigation of major validation criteria and associated experimental protocols, *J. of Chromatography B.* 877, 2180-2190.

SUMPTER, J.P. (2005). Endocrine disruptors in aquatic environment: an overview. Acta Hydrochem. Hydrobiol. 33, 9–16. Res. Toxicol. 14, 280–294.

SUMPTER, J.P. & JOBLING, S. (1995). Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. Environ. Health Persp. 103 (Suppl.), 173–179.

SUPELCO (1998), Bulletin 910 guide to solid phase extraction. Sigma-aldrich co.

TABATA, A., KASHIWA, S., OHNISHI, Y., ISHIKAWA, H., MIYAMOTO, N., ITOH, M., MAGARA, Y. (2001). Estrogenic influence of estradiol-17b, p-nonylphenol and bisphenol A on Japanese Medaka (Oryzias iatipes) at detected environmental concentrations. Water Sci. Technol. 43, 109–116.

TERNES, T.A., ANDERSON, H., GILBERG, D. & BONERZ, M. (2002). Determination of estrogens in sludge and sediments by liquid extraction and GC/MS/MS. Anal. Chem. 74, 3498–3504

TERNES, T.A., STUMPF, M., MUELLER, J., HABERER, K., WILKEN, R.D. & SERVOS, M. (1999a). Behavior and occurrence of estrogens in municipal sewage treatment plants— I. Investigations in Germany, Canada and Brazil. Sci. Total Environ. 225, 81– 90.

TERNES, T.A., KRECKEL, P. & MUELLER, J. (1999b). Behavior and occurrence of estrogens in municipal sewage treatment plants – II. Aerobic batch experiments with activated sludge, The Sci of Total Environ. 225, 91-99.

TAN, B.L.L., HAWKER, D.W., MULLER, J.F., LEUSCH, F.D.L., TREMBLAY, L.A. & CHAPMAN, H.F. (2007). Comprehensive study of endocrine disrupting compounds using grab and passive sampling at selected wastewater treatment plants in South East Queensland, Australia, and Environ Int. 33:654-669.

ZHANG, Z.L. & ZHOU, J.L. (2007). Simultaneous determination of various pharmaceutical compounds in water by solid-phase extraction-liquid chromatography-tandem mass spectrometry, Journal of Chromatography A. 1154, 205-213.

ZHANG, Y., ZHOU, J.L (2008). Occurrence and removal of endocrine disrupting chemicals in wastewater. Chemosphere 73, 848- 853.

ZHA, J., SUN, Y., ZHOU, Y., SPEAR, P. A., MA, M. & WANG, Z. (2008). Toxicol. Appl. Pharm. 22,62-98.

PRABHU, S.V., VARSOLONA, R.J., WEHNER T.A., Egan, R.S. & Tway, P.C. (1992) Rapid and Sensitive HPLC-Method for the Quantitation of Abamectin and its Delta 8, 9-Isomer.J. Agr. Food Chem. 40 (4), 622-925.