UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

FACULTY OF SCIENCE

DETERMINATION OF ESTROGENIC HORMONES IN ENVIRONMENTAL WATER SAMPLES IN VAAL REGION BY ULTRA FAST LIQUID CHROMATOGRAPHY COUPLED TO MASS SPECTROMETRY

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A dissertation submitted to the Faculty of Science, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Science

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DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

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(Signature of candidate)

___30th_____day of___September_____2016__

ABSTRACT

The presence of estrogenic hormones in the environment has been a subject of concern in recent years; they have been classified as “emerging pollutants” and may pose a potential risk for human consumption. Hormones have been detected in ground and surface water at low concentrations. These compounds contaminate the surface and ground water via waste water treatment plants (WWTP) and may elicit endocrine disruption to organisms. Because these compounds are available at low concentration, robust analytical methods are required to quantify these compounds in water and environmental samples.

The common method for the analysis of hormones in water samples is Gas Chromatography (GC) coupled to Mass Spectrometer (MS). The challenge with GC-MS is the required lengthy derivatisation step that involves toxic chemicals.

The first part of this case study was to develop a method to determine trace concentrations of the Estrone (E1), 17α-Estradiol (E2α), 17 β-Estradiol (E2β) and 17α-Ethinylestradiol (EE2) hormones using Ultra-Fast Liquid Chromatography Mass Spectrometry (UFLC-MS-MS). Using the developed method, the second part of the case study was to determine the concentrations of the hormones in raw and potable water samples from the Vaal River catchment area in the South of Johannesburg, South Africa.

Analytes were extracted by solid phase extraction (SPE C_{18} Sorbent, 200 mg/6mℓ cartridges) and subjected to Ultra-Fast Liquid Chromatography coupled to Mass Spectrometer (UFLC-MS-MS) for identification and quantification. Optimum SPE parameters were 1000 mℓ of sample percolated, at flow rate of 10 mℓ/min, sample pH of above 7, 7.5 mℓ of methanol as elution solvent followed by solvent reduction to 250 µℓ.

The limits of quantification were in a range of 0.24 to 0.32 ng/ℓ for all analytes. Accuracy was 95.6, 93.8, 97.6 and 100.9% for 17α-Ethinylestradiol, 17α-Estradiol, 17β-Estradiol and estrone, respectively. In raw water samples taken during the rainy wet season, estrone was detected at concentrations of 0.90 and 4.43 ng/ℓ. However, drinking water samples no presence of hormones with the exception of M-B12 sample point where the estrone amount of 2.88 ng/ℓ was detected. This is potentially due to fact that conventional water treatment plants are able to remove the compounds during water purification process depending on the concentration levels.

DEDICATION

This dissertation is dedicated to my family, my children Sibongumusa, Sinesipho, Siphesihle Mnguni and even more to my dedicated life partner, ally and a friend Sithembile. I thank them because nobody can replace the time I spent on this work while depriving them of my presence in the mist of adversity.

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ABBREVIATIONS AND ACRONYMS

ANOVA	: Analysis of variance
AR	: Androgen receptor
BMR	: Membrane bioreactors
CE	: Collision energies
COD	: Chemical oxygen demand
DAF	: Dissolved air floatation unit
DI	: Direct immersion extraction
DNA	: Deoxyribonucleic acid
DP	: Declustering potentials
E1	: Estrone
E2	: Estradiol
E3	: Estriol
EDC	: Endocrine-disrupting chemicals
EE2	: 17α-ethinylestradiol
EP	: Exit potentials
ER	: Estrogen receptors
ERT	: Estrone replacement therapy
GC	: Gas chromatography
GC-MS	: Gas chromatography mass spectrometry
GC-MS	: Gas chromatograph equipped with mass spectrometer
HF	: Hollow fibre
HPLC	: High performance liquid chromatography
HPLC –MS/MS	: High performance liquid chromatography-tandem Mass Spectrometry (HPLC-MS/MS)
HS	: Headspace extraction
KBluc assay	: Reporter gene assay
Kd	: Adsorption coefficients
LAS	: Linear alkyl sulfonates
LLC	: Liquid-liquid chromatography
LOD	: Limit of detection

LOQ	: Limit of quantification
MIP	: Molecular Imprinted Polymers
MRM	: Multiple Reaction Monitoring modes
MS	: Mass Spectrometry
P&T	: Purge & Trap
PFBE	: Polyfluoro biphenyl ethers (PFBEs)
PPCP	: Pharmaceuticals and personal care products
ppb	: part per billion
ppt	: Part per trillion
PTS	: Primary settling tanks
RAS	: Return Activated Sludge
RTs	: Retention times
SA	: South Africa
SPE	: Solid phase extraction
SPME	: Solid phase micro extraction
SRT	: Solids retention time
SS	: Suspended solids
TDM	: Therapeutic drug monitoring
TIC	: Total ion current
UFLC	: Ultra-fast liquid chromatograph
UP	: University of Pretoria
WWTP	: Waste water treatment plants
YES-assay	: yeast estrogen screen assay

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CHAPTER ONE – INTRODUCTION

1.1 General Introduction

The concerns posed by endocrine disrupting chemicals (EDCs) has been a public health subject for some time now, EDCs are compounds that are available in the environment they interfere with hormonal function, synthesis and metabolism, or mechanisms resulting in the alteration of homeostatic function or reproduction (Maria Huert-Fontel et al,. 2010, Jones-Lepp et al.. 2009, Ncube,.2009, Diamanti-Kandarakis et al,. 2009). Hormones are class of chemical compounds secreted by glands in certain parts of the body that sends out signals that affect cells in other parts of the body. Only a small amount of hormone is required to alter cell metabolism. In summary, a hormone is a chemical compound that transports a message from one cell to another.

According to the studies performed in the first Scientific Statement of The Endocrine Society (Diamanti-Kandarakis E et al. 2009), the effects such as prostate cancer, cancer, breast development, and thyroid, and neuroendocrinology, male and female reproduction just to mention a few are the effects of endocrine disrupters.

The mechanisms of endocrine disrupters in the body system involves more than one pathways such as estrogenic, thyroid, anti-androgenic, peroxisome proliferator-activated receptor, retinoid, and interaction of receptors the nuclear; steroidogenic enzymes; neurotransmitter receptors and systems. Their pathways in human and wildlife can be

investigated in the laboratory using *in vitro* and *in vivo* models (Diamanti-Kandarakis et al,. 2013). The significance of ECDs resulting from epidemiological and toxicological studies to animal and human are currently converged as a public health concern.

The occurrence of these contaminants in water streams possesses environmental concerns and is an issue for public health matters. Endocrine disrupting compounds (EDCs) such as steroid hormones and pharmaceuticals have been classified as "Emerging contaminants" because of their endocrine disrupting potential. Little is known about their availability, fate and toxicity of these compounds in source and drinking water in South Africa.

Additionally, compounds such as organo-chlorine pesticides, plasticisers, fuels, industrial chemicals, brominated flame retardants and other chemicals that might be present in the environment are in use in large quantities in South Africa. Pesticides are the only organic pollutants most studied in the South African environment (Yang et al,. 2010), followed by PCBs and now PAHs (Combalbert et al,. 2012, Sibiya,.2011).

Studies have shown that these compounds result from direct disposal, human and animal excretion and enter water streams via Waste Water Treatment Plants (WWTP), due to inability of WWTPs to remove some of these compounds together with their metabolites in the effluent. EDCs may be found in surface water and in sediments at very low concentrations depending on the location of WWTPs. If the remaining sludge is used for agricultural purpose, EDCs have a potential of leaching through soil hence contaminating the

environment. Therefore, the presence of such EDCs poses a threat to the environment and on the water quality together with unknown toxicological effects (Huert-Fontel et al. 2010).

The public health effect caused by emerging organic contaminants has attracted regional and international concerns when released into the environment. Organic pollutants that are disposed into the environment include agricultural waste, industrial pollution and other hazardous pharmaceuticals and personal care products that are not biodegradable. It has been widely reported that most organic emerging contaminants such as dyes, detergents, paint substance, agricultural waste, additives to plastics, paint components and PPCPs also possess endocrine disrupting effects (Huert-Fontel et al. 2010, Theres Koal et al. 2011, Mu¨llera et al. 2002, Ncube, (2009) and Diamanti-Kandarakis E et al. 2009).

Ncube (2009) revealed that the human risk of long-term exposure to the low levels of most organic contaminants, especially emerging contaminants such as hormones are not yet known throughout the world. Thus, there is a need to monitor these emerging contaminant in South African water resources.

Little is known of the availability or absence of hormones and PPCPs in raw waters that is treated for drinking water. The need for further studies on detection of these emerging pollutants should be of utmost importance to ensure the health and safety of consumers. Several research articles have been written that discuss the fate and behaviour of these emerging pollutants in waste water effluents (Huerta-Fontela

et al., 2011), but few articles have been written on their detection and fate in drinking water in South Africa (Huerta-Fontela et al., 2011). The lack of systematic monitoring programmes in South Africa and fluctuating concentrations of hormones with inadequate analytical method limits of detection may possibly exacerbate the situation.

In other studies, the availability and effects of hormones, pharmaceuticals and their transformation by-products in drinking waters have been reviewed (Jones et al,. 2005, Mompelat et al,. 2009). Until recently, some studies have found the availability of these contaminants in European and United State of American (USA) drinking waters at low concentrations (Huerta-Fontela et al., 2011).

However, there is a challenge in formulating appropriate standards for organic substances in water due to the complexity of this group of chemicals, their ability to degrade into other organic substances, the difficulty in analysing these substances at the required ultra-low concentration levels, the high cost of analyses and the extensive resources and skills required to perform such analyses. There is also insufficient knowledge on the probable health implications of many of these substances hence the reason for taking a cautious approach resulting in the near impossible detection limits required for many of these organic constituents.

The presence of endocrine disrupting compounds (EDCs), pharmaceutical and personal care products in waste and surface waters has attracted lots of attention and focus in the recent years (Richardson et al., 2009). If these compounds together with their metabolites are not removed from waste water treatment plants, they

may enter rivers and streams which can have adverse impact in the aquatic life and the quality of potable water for consumption. (Maria Huerta-Fontela et al., 2011).

Low concentrations result in effects such as feminization. Because these compounds are available at low concentration, robust analytical methods are required to quantify these compounds in water and environmental samples. (Huerta-Fontela et al., 2011; Izumi et al., 2009). The common method for the analysis of hormones in water samples is gas chromatography (GC) coupled to a mass spectrometer (MS). The challenge with GC-MS is the required lengthy derivatisation step that involves toxic chemicals (Huerta-Fontela et al., 2010; Huert-Fontel et al., 2011). To take advantage of the weaknesses of immunoassays methods in the determination of hormones, high performance liquid chromatography coupled to tandem mass spectrometry is a good alternative. HPLC-MS-MS is capable of simultaneous determination various organic pollutants in environmental samples while producing good accuracy, sensitivity and precision. HPLC –MS-MS has also been applied in toxicological diagnostics and therapeutic drug monitoring in the past decade (Koal et al., 2012).

Like all hyphenated techniques, UFLC-MS-MS requires several technical modifications before it can be applied to routine analysis. Proper instrument optimisation is necessary to increase efficiency, accuracy, precision and to decrease bias and uncertainty (Müller et al., 2003; Izumi et al., 2009; Huerta-Fontela et al., 2011).

Although immunoassays have been used in diagnostics of pollutants because they are fast and thus have high sample throughput, they have some drawbacks. The disadvantages of bioassays in the analysis of hormones are as follows (Sabine Mu¨llera et al. 2002):

- It is not suitable for multi-compound determination but often the sample contains a number of similar compounds.
- Low ability of selectivity and specificity because some metabolites have similar structural orientations.
- Less sensitive especially in testosterone analysis
- Linear dynamic range is limited because of interferences associated with complex matrices
- The kits that are used in immunoassay methods vary significantly and their standardisation are not always traceable.

In this study, method for the simultaneous identification and quantification of hormones in water matrices has been developed and validated using a highly advanced UFLC coupled to MS-MS. This method was applied to the determination of hormones in raw and treated potable water samples in the south region of Johannesburg, South Africa.

1.2 Statement of a problem

Despite the low concentrations of EDCs, the fate of hormones in the environment together, with their persistent biological activities explains the concern over this specific group of water contaminants. First reports dealing with the availability of EDCs/ pharmaceutical and personal care products in environmental water samples were performed by using gas chromatography (GC) following derivatisation.

Various studies conducted in water analysis still shows apparent existence of PPCPs in the environment water samples (Chung Chow Chan et al. 2004). Because of the ecotoxic effect of both natural and synthetic hormones such as E1, E2s and EE2 are of concern even at low concentration levels. A concentration of approximately 1 ng/ℓ is enough to cause feminisation which reflects the extent of endocrine disruption potential of these contaminants (Ali Khan, et al, 2011). The status of these compounds in South African water bodies is not known because very little studies have been reported.

As a result of very low concentrations expected in the water bodies, a more robust analytical methodology is required for the accurate identification and quantification of these compounds in environmental samples. Estrogen residues in water and sediments have commonly been determined using GC/MS; the main challenge with this technique is that it requires samples to be derivatised which involve time consuming and tedious steps rendering it unsuitable where the turnaround time and sample throughput is expected to be high.

This study investigates the power of LC/MS/MS for the analysis of four hormones using Solid Phase Extraction (SPE) approach of potable water and raw water source samples. Liquid chromatography coupled to mass spectrometry (MS; MS/MS) is the preferred technique for multi-analyte determinations of polar compounds such as hormones.

CHAPTER TWO – LITERATURE

2.1 Occurrence of hormones

Recent research work has been directed towards the occurrence of estrogenic hormones in the environment (Komori et al,.2004). In general, steroid hormones are biological compounds that are synthesised from cholesterol. Natural hormones various human and animal organs including ovaries and adrenalin to mention but a few (Guang-Guo Ying et al., 2002)

The group of compounds in this study is primarily produced in the female body and is essential for female characteristics such as keeping reproductive cells healthy. These types of hormones can also be synthesised commercially. Hormones such as estrone and 17β-estradiol are naturally excreted by females (both human and animals) in the quantities of 2–12 μg/person/day and 3–20 μg/person/day, respectively (Belfroid A.C et al., 1999; Guang-Guo Ying et al., 2002). Several drugs are used in medicine to influence the endocrine hormonal system. One well known example is the contraceptives utilized as ingredients of birth control pills. A synthetic steroid hormone of concern that is studied here is ethinylestradiol which is used as a contraceptive.

Like all endocrine disruptors, hormones exert their action by passing through the plasma membrane and binding to intracellular receptors. They may interfere with the normal functioning of endocrine system resulting in the negative impact in the reproduction and development in aquatic life.

Many effects observed in the aquatic environment concerning the reproductive system, like the feminisation of male fish within WWTP effluents are attributed to the presence of endocrine disrupters but the specific individual compounds or group of compounds attributed to this effect are still not known as a matter of fact

(Ternes et al., 1999). Other suspected endocrine disrupting compounds such as nonylphenols, phthalic esters, polychlorinated biphenyls, dioxins, phytoestrogens and human estrogens are suspected to influence the hormonal system. Recent studies have hypothesised that the statistically derived decrease in sperm counts over the last decades, increasing incidents of testicular cancer and other disorders regarding male infertility may be caused by the intake of estrogens via food or drinking water (Diamanti-Kandarakis et al, .2009).

Some of the main synthetic sources of larger part of environmental pollutants are pharmaceutical production, wastewater treatment plants, agricultural activities and fish farming (Boxall et al, .2012). Conventional drinking water treatment processes such as coagulation/flocculation, sedimentation and chlorination are not primarily designed to remove completely these organic pollutants unless assisted by granular activated carbon filtration or membrane filtration (Wang et al., 2012; Kim et al., 2007). Kim et al., (2007) further reported that in wastewater treatment processes, membrane bioreactors (MBR) achieves limited target compound removal, but are effective at eliminating hormones and some pharmaceuticals.

2.2 Occurrence of Pharmaceuticals and Personal Care Products (PPCP)

In general, Pharmaceuticals and Personal Care Products (PPCP) and other organic compounds which are used around the globe in big quantities are found in Municipal wastewaters (Ramirez et al,. 2009, Kasprzyk-Hordern et al 2008, Chenxi et al,. 2014).

Drugs that are used for human consumption are subjected to metabolic processes; significant portion of these drugs leaves human or animal bodies un-metabolised via excretion and therefore emitted into raw sewage (Carballa et al,. 2004). Furthermore, some of the excreted metabolites can be transformed back into their original form (Carballa et al,. 2004).

Some of PPCPs such as fragrances, galaxolide naproxen which is an anti-inflammatory, ibuprofen, 17 β-estradiol, iopromide, sulfamethoxazole which is an antibiotic, estrone and, diclofenac, roxi-thromycin, 17 α-ethinylestradiol, diazepam, and carbamazepine have been detected in the work carried out by Carballa et al (2004) in the wastewaters in north west Spain.

The availability of pharmaceuticals and personal care products in sewage water can be used as an indicator of organic pollution of municipal waste waters generated by possibly hospitals, cities, informal settlements and agricultural activities around the population of the study area such as Vaal region (Johannesburg, south) in Gauteng, a province in South Africa.

Most work reported in the literature deals with the availability of these organic pollutants in waste water treatment plants followed by their removal, little or no information is available for assessment of the fate of these compounds in the environment especially in the receiving and drinking water bodies. The study is thus very important for South Africa as very few studies have been reported of these compounds in the environment.

2.3 Environmental and Public Health Impact of EDCs

Estrogens are known as hormones that regulate reproductive functions in females, they also play a role in bone protective tissues, brain and the cardiovascular system.

The major hormone of concern when it comes to binding capacity to the estrogen receptor is estradiol. It can further be metabolised to form estrone and consequently estriol through further oxidation processes (Reina et al,. 2004). Estradiol is used as precursor towards the synthesis of man-made estrogens such as ethinylestradiol (EE2), mestranol and the valerate of estradiol which are used in human hormone therapy treatments, like the contraceptive pill as stated above. Before they are excreted by the body system they are first metabolised to inactive biological state and soluble(water) conjugates of glucuronides or sulphate esters, however a bacteria can deconjugate them and make them biodegradable in activated sludge process with the synthetic estrogens of greater recalcitrance (Bevan et al,. 2012).

The hormones are synthesized in both males and females along with estrogen receptors (ERs). ERs act as ligand-activated transcription factors (Reina et al 2004). These hormones interact with intracellular ERs and activate the expression of gene encoding proteins, which in turn control the important biological functions in animals and humans (Klinge et al,.2008). Those receptors have higher affinity for 17β-E2 than the other estrogens; thus, 17β-E2 binds more vividly to the receptors and it is recognized as the predominant endogenous activator and initiator for a number of downstream ER-mediated cellular events (Maria Marino et al,. 2006). In contrast, 17α-E2 is generally considered to be a less active and feminising estrogen (Nguyen (2010) Thesis)

Estrogens are eliminated from the body by conjugation to hormonally inactive water-soluble metabolites, mainly the sulphates and glucuronides. Estrone sulphate is the most abundant circulating estrogen at a concentration of 10 times

higher than that of unconjugated estrone (Nguyen, .2010). This finding affirms the belief that sulphated steroid hormones serve an important role as steroid hormone precursors, and sulphation/desulphation processes of estrogens may represent a vital endogenous system in regulation of biologically-active steroid hormones in target tissues (Nguyen,.2010). A current hypothesis is noted, that inactive estrone sulphate is transported to target tissues via the circulatory system and taken into target cells, most likely by anion transporters (Notelovitz et al 2007). After arrival in target cells, estrone sulphate is enzymatically hydrolysed to E1 by intracellular membrane-bound sulphatases, and then, catalytically oxidized to active 17β-E2 by 17β-hydroxysteroid dehydrogenases (Nguyen, .2010). The transport of estrogen conjugates into target cells is not well understood, but it is believed that the process occurs by virtue of the high affinity of anion transporters for both estrogen sulphates and glucuronides. Estrogen glucuronides have received less attention as steroid hormone precursors, primarily because of their less abundance and more ready excretion from the body (Notelovitz et al 2007).

17α-ethynylestradiol (EE2) for an example is a known synthetic hormone, derived from the natural hormone, estradiol (E2). It has been used widely as medication in livestock as well as in humans however it creates challenges in the environment due to its high resistance to degradation, absorbs organic matter, accumulates in sediment, and concentrates in biota (Jones-Lepp et al,. 2009 : Yinga et al,. 2002: Belfroid et al,. 1998 , Guang-Guo et al,. 2008 : Horbart,. et al ,1988). EE2 is used widely as a contraceptive in females and studies have reported the ability of EE2 to alter sex determination, delay sexual maturity, and decrease the secondary sexual characteristics of exposed organisms even at a low concentration (ng/*l*) by mimicking its natural analogue, 17β-estradiol (E2) (Aris et al,. 2014).

Moreover, it has been demonstrated that estrogens that are used in hormone therapy can prevent and decrease the incidence of stroke-related mortality in postmenopausal women (Yinga et al,. 2002). It has also been demonstrated that in pre- and post-ischemic 17β-estradiol (17β-E2) and its less bio-active metabolite,

estrone (E1), have been the endogenous estrogens of greatest interest (Horbart,. et al ,1988, Ncube,. 2009, Nguyen ,.2010, Belfroid et al,. 1999).

The study conducted by Drake et al. (2000) found a positive association between circulating estradiol and delayed verbal memory, but a negative association between circulating estradiol and immediate and delayed visual memory in healthy postmenopausal women not on estrone replacement therapy (ERT).

Another study conducted by Yaffe et al., (2000) found that women with high total E2 and bioavailable E2 were less likely to demonstrate cognitive decline over a 6-year follow up period, even after adjusting for current ERT use. Other studies have found that verbal skills are enhanced during the estrogen-dominant phase of the menstrual cycle (Benotti et al,. 2009, Nguyen,.2010).

The release of estrogens into the environment possesses endocrine disruptive potential even at low concentration levels (Maria Huert-Fontel et al,. 2010, Yoshihiro Izumi, et al,. 2009). This is related to various other conditions linked to reproductive abnormalities, decreased fertility and feminization and related to various types of cancer, prominently ovarian and breast cancer (Aris et al,. 2014). Figure 2 shows the mechanism of cancer formation from estrogens.

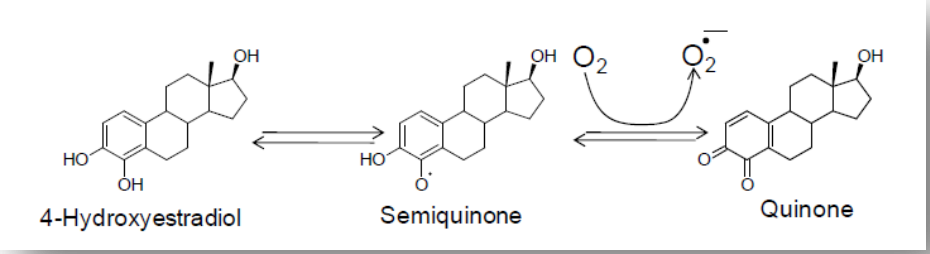


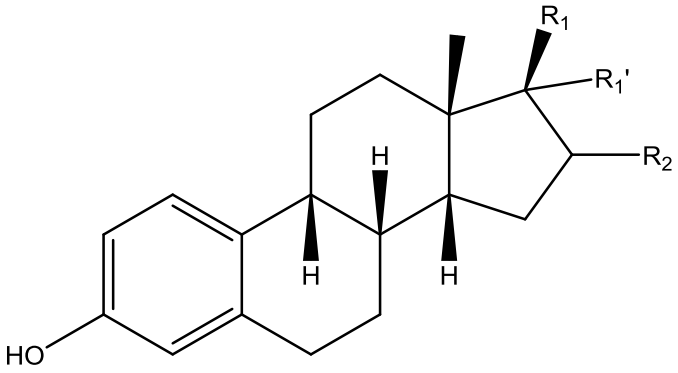
Figure 2: Metabolic redox cycling of 4-hydroxyestradiol (Nguyen,.2010)

In Figure 2, in the first stage target tissues or cells are subjected to excessive mitogenic (a chemical process that encourage a cell to divide) stimulation by estrogens which increases cell production. In the second stage, 4-hydroxyestradiol

undergoes metabolic redox cycling to produce free radicals of semiquinone/quinone. These metabolic intermediates may damage DNA, proteins, lipids, induce cell transformation, and cause tumorigenesis.

2.4 Chemical, Physical and Structural properties of the Compounds of interest in this study

Natural estrogens, consisting of estrone (E1), estriol (E3), 17α- and 17β-estradiol (17α- and 17β-E2), are steroid hormones with the general skeleton as shown in Figure 3, generated from cholesterol via testosterone and androstenedione in ovaries, brain, and body fat deposits.



Estrone (E1)	R_1=O; R_2:H
Estradiol (E2)	R_1: OH: R_1':H R_2:H
Ethinyl Estradiol (EE2)	R:OH; R_1':CH≡CH; R_2:H

Figure 3: Structure of estrogen hormones (Huerta-Fontela et al,.2011)

EE2 (19-nor-17α-pregna-1, 3, 5(10)-trien-20-yne-3, 17-diol) is a derivative from the natural hormone, estradiol (E2) (Aris et al,.2014). EE2 is used in almost all modern formulations of combined oral contraceptive pills and is one of the most commonly used medications. It is worth noting that EE2 is sparingly soluble in ethanol (1 part in 6 parts of ethanol), but has relatively low solubility in water (4.8 mg/l at 20 °C) compared to natural estrogenic steroid (Table 1).

Table 1: Physical and chemical properties of estrogens (Lai et al, 2000, Carpinteiro et al, 2004 and Lewis and Archer, 1979)

Estrogen	**Molecular weight**	**Vapour pressure** (Pa)	**Water solubility** (mg/l)	**Log Kow** (ESC)	**Log Kow**
Estradiol	272.39	3E-08	13	3.94	4.01/3.10
Estrone	270.37	3E-08	13	3.43	3.13
Estriol	288.39	9E-13	13	2.81	2.60
EE2	296.40	6E-09	4.8	4.15	3.67
Mestranol	310.42	1E-07	0.3	4.67	4.10

Estrogen metabolism is presented in Figure 4. Estradiol and estrone are synthesized from the precursor of cholesterol through the formation of testosterone and androstenedione. Once formed, these three estrogens can be interconverted between each other or hydroxylated to estriol (Aris et al,. 2014). All native estrogens can also be conjugated by sulphation or glucuronidation and vice versa (Nguyen,. 2010)

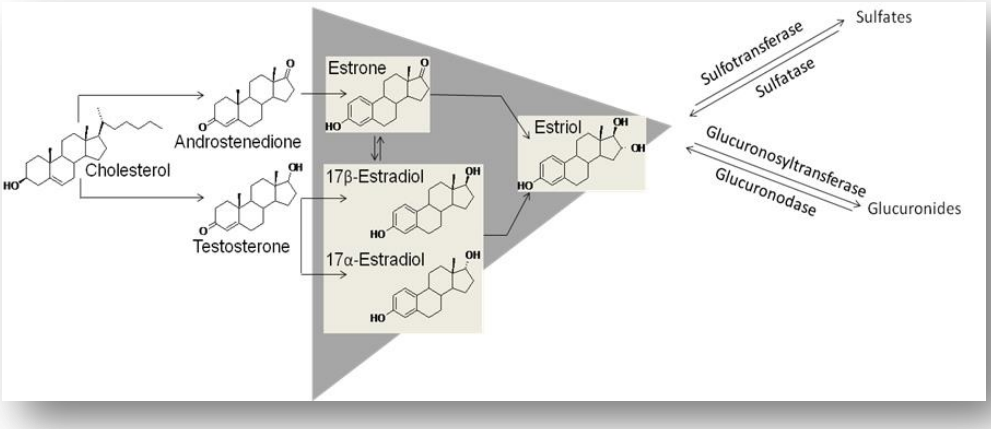


Figure 4: Estrogen metabolism (Nguyen,. 2010)

2.5 Review of Common Extraction Techniques

Sample preparation and handling, "a heart of measurement analysis", plays a very important role in the measurement of the analytical data. Wrong sampling and sample preparation procedures will lead to invalid and misleading interpretation of results.

Most samples are not ready for direct introduction into instruments; mostly analytes must be in a suitable phase or state to be amenable for specific instrumentation. For example, in the analysis of estrogens in water sample, it is not possible to analyse the water directly into the GC-MS. The analytes have to be extracted into a solution and derivatised, before they can be analysed by an instrument.
There might be several processes within sample preparation itself depending on the nature of the sample. Sample extraction choice can be helped by classification of the analyte whether it is volatile or semi volatile.

Liquid-liquid extraction

Liquid-liquid extraction is a solvent based extraction technique whereby non-polar organic solvents which are immiscible in an aqueous media are used to dissolve analyte of interest from water matrix based on the differences between the solubility of analytes in the organic and aqueous phases. Target compound can be extracted selectively by using a solvent whose polarity is similar to that of the analyte of interest. However, in practice, other matrices with similar polarity as the analyte are extracted along making the method less attractive.

Semi-volatile organic compounds in water samples are normally extracted using organic solvents such as dichloromethane, hexane, ether, benzene and ethyl acetate. When non-polar compounds such as aliphatic hydrocarbons are to be extracted, hexane is normally a suitable solvent for their extraction. Benzene is normally preferred for the analysis of aromatic compounds whereas ether and

ethyl acetate are normally used for the extraction of polar compounds containing oxygen. Dichloromethane has high extraction efficiency for a wide range of non-polar to polar compounds.

Dichloromethane is suitable for simultaneous analysis because of the following advantages: it has a low boiling point and easy to re-concentrate after extraction, it has a higher specific gravity which makes it easy to separate from water, and it is non-flammable.

However, these organic solvents (hexane, benzene and others) are known carcinogens, and recently the call towards minimising the use of these solvents during sample preparation has been encouraged. It is sometimes possible to selectively extract semi-volatile compounds from water by changing the character of samples, not changing solvents. For example, by changing the pH of samples, only acidic or basic substances can be extracted. When the pH of water is less than 2, basic compounds become fully ionised and are not extracted by the solvent, allowing selective extraction of acidic and neutral compounds (Ram Chandra,. 2015).

In the extraction water soluble compounds, salting-out techniques are used to enhance or aid the rate of extraction. Addition of a salting agent in to the aliquot of a water sample reduces the solvation power of the solution and the solubility of target compounds. This is also useful in solid phase extraction and headspace extraction techniques.

Extraction is normally performed in a separating funnel by vigorously mixing the aqueous phase sample with a suitable organic solvent. Sometimes the formation of emulsion prevents the proper extraction to occur. If ever the formation of emulsion occurs, addition of anhydrous sodium sulphate, ethanol followed by sonication can break down the emulsion making the extraction more effective and efficient (Skoog et al, 1998). Because of the lengthy extraction time by continuous liquid- liquid extraction techniques, thermally unstable compounds cannot be

extracted with high level of efficiency even though the technique itself is regarded efficient (Tölgyessy et al, .2004, Skoog et al, 1998).

Solid-Phase Extraction (SPE)

SPE is modern and robust; it is an alternative extraction technique for liquid-liquid extraction (LLE).
Solid phase extraction principle is based on adsorption and desorption. Analytes of interest are adsorbed into a suitable sorbent in a cartridge (usually C_{18}).

The SPE can remove matrix interferences in complex samples by passing through the suitable cartridge or by elution with the solvent and then desorbing the target compounds with a suitable solvent selective to the group of target compounds. SPE uses less amounts of organic solvent when compared to liquid-liquid extraction and can be automated easily. Common solid phases used as packing materials are reversed phase and polymeric sorbents. The recovery analysis can be determined where known quantities of standard are added into the known sample matrix volume, subjecting the samples through solid phase extraction column, eluting the target analytes using the suitable solvent and subsequently determining the amount of analyte recovered in order to get better results.

There are commercially available cartridges which are pre-packed with known amounts of adsorbents; they can simply be conditioned before use in the laboratory. Just like any one technique, SPE has advantages and disadvantages as given below:

The advantages of SPE technique:

- ✓ Can easily be automated
- ✓ Use small volume of organic solvent.
- ✓ Simultaneous extraction is easily performed thus giving high sample throughput.
- ✓ Large number of sorbents to choose from some which are selective.

- ✓ High concentration factors can be obtained.

The disadvantages to solid-phase extraction technique:

- ✓ The solvent flow rate can affect the recovery rate of analytes.
- ✓ Suspended solid composition must be separated from the water sample.
- ✓ It is possible to get analyte breakthrough in highly contaminated matrices
- ✓ It is important to optimise the method by testing various factors that affect recovery and reproducibility in order to improve method capability

Henriques et al,. (2010) reported that estriol, 17-α-ethinylestradiol, diethylstilbestrol, Mestranol, estrone and 17-β-estradiol were not detected in all tested Lisbon drinking waters using solid phase extraction. Some raw waters (43 samples) in Portugal showed trace amounts of other endocrine disrupting compounds such as bisphenol A, progesterone, 4-tert-octylphenol, and 4-n-nonylphenol. The quantities of endocrine disruptive compounds in the samples where were in the region of 2.9 - 4.8 ng/ℓ for bisphenol A, 27 - 29 ng/ℓ for 4-tert-octylphenol, 0.45 - 1.8 ng/ℓ for progesterone and 2.7 - 5.7 for 4-n-nonylphenol (Henriques et al,. 2010).

Hollow fibre liquid phase micro-extraction (HF-LPME)

In HF-LPME the extracting phase is placed inside the lumen of a porous polypropylene hollow fiber in which the extraction solvent is protected and stabilized (Sibiya, .2012).

The most common fiber has a porosity of 70%; a pore size of 0.2 μm, a wall thickness of 200 μm and an internal diameter of 600 μm. A supported liquid

membrane (SLM) is formed easily by dipping the hollow fiber into the organic solvent for a few seconds. The solvent penetrated the pores of the hollow fiber and is bound by capillary forces to the polypropylene network comprising the fiber wall. The inside of the fibre with filled organic solvent acts as the trapping media.

In HF-LPME, analytes are extracted from an aqueous sample, into the organic solvent immobilized as a SLM, and into the acceptor solution placed inside the lumen of the hollow fiber.
This acceptor solution may be an organic solvent (same as used for the SLM) resulting in a two phase extraction system, or the acceptor solution may be aqueous that is immiscible with SLM solvent providing a three-phase extraction system (Sarafraz-Yazdi et al,. 2010).

Subsequently, the acceptor solution is removed by a micro-syringe and transferred to final chemical analysis. Two-phase HF-LPME could be done in two ways: direct immersion extraction (DI) and headspace extraction (HS) (Sibiya, .2012, Sarafraz-Yazdi et al,. 2010). The mechanisms such as pH gradient, carrier transport and electro membrane extraction can be used to facilitate the three phased mode of HF-LPME (Ghambarian et al, 2012).

Hollow fibre-protected liquid-phase micro-extraction (HF-LPME) was used by different researchers. Padrón et al,. (2014) discovered that after optimising the LPME method, very clean extracts could be obtained, avoiding signal suppression during the LC-MS/MS analysis of the analytes; the limits of quantification in their developed method ranged from 0.5 to 42 ng/ℓ. For estrogens (estrone, 17β-estradiol, estriol, ethynylestradiol, diethylstilbestrol), the limit of detection ranged from 2.7–11.7 ng/ℓ when IT-SPME was used in Spain (Padrón et al,. 2014).

Selective sorbents based on Molecular Imprinted Polymers (MIPs)

Molecular Imprinting Technology (MIT) is a novel synthetic approach to develop robust molecular recognition materials that are able to mimic natural recognition of compounds such as antibodies. The synthesised polymers, which are able to mimic and achieve recognition, found in nature, have become an emerging and active area of research today. They are also known as plastic antibodies and operate on the basis of lock and key principle.

MIPs provide specific molecular recognition ability for selective retention (Bartsch et al., 1999). In the traditional way of preparing MIPs, the target molecule is used as a template. After polymerization, the template is removed, and the specific recognition site created selectively adsorbs the template molecule which is the target compound in the sample.

MIPs have been applied in different fields of analytical separations including LC columns as stationary phases (Sellergren at al., 2000). It is very difficult to completely remove the template molecules from the prepared MIPs, even by washing with some organic solvents repeatedly. In microanalysis, leakage of the residual template molecules, which are the same as the target molecules, prevents the accurate determination of the target compound. This can be a serious problem for quantitative microanalyses of chemical substances such as E2 at the ng/ℓ level (Kubo et al., 2012). As a result, a structurally-related analog, which can be separated from the target molecule in the subsequent chromatographic process, is employed as the template molecule instead (Watabe et al,. 2006).

In the study conducted in China, the recoveries from the MIP–SPE method were in the range of 87.5–97.3% when analysing for diethylstilbestrol (DES) in fish samples. The detection limit (LOD) was obtained from the signal-to-noise ratio (S/N) and calibration curve. In this work, the noise of the baseline was measured from chromatogram of blank fish sample. Three times of the noise as the signal

value (S/N = 3) was used to calculate the LOD in the calibration curve. The LOD obtained was 60 ng/mℓ (Jiang et al,.2008).

In the separate study done in Czech Republic, the LOD of estrogens, bisphenol A, and alkylphenols in their water and sediments samples ranged in between the region of 0.70 – 1.90 ng/ℓ and 0.30 – 0.60 ng/g respectively. The use of MIPs in their extraction contributed greatly in the sample clean-up stage, its efficiency was increased even when larger samples quantities were used. (Matějíček et al,.2013).

The concentration levels of 18.5 to 34.5 ng/ℓ for bisphenol A and 7.2 to 8.4 ng/ℓ for nonylphenol were found in four samples. There were three samples where estrone was detected and quantified, it was found in the concentration region of 3.4 – 5.1 ng/ℓ.

Ten samples were collected from Svratka for analysis. Bisphenol A and nonylphenol were detected in all ten samples in the region of 14.6 to 83.2 ng/ℓ for bisphenol A and 6.7 to 9.4 ng/ℓ for nonylphenol. Estrone was detected in 70% of samples collected in the region of 7.2 – 9.2 ng/ℓ concentration levels. Estradiol was detected only in three water samples. Of the sediments samples collected in the Svratka River, only estrone and nonylphenol were detected (Matějíček et al,.2013).The use of MIP can therefore be a good alternative to SPE due to its adequate limits of detection at low ng/ℓ concentration levels.

2.6 Review of Common Analytical Methods of Analysis

This section describes a few techniques that are available for the determination of estrogenic hormones in the water and environmental samples.

2.6.1 Immunoassays

Estrogens are have been widely analysed by biological assays (Rubing et al,.2011). Use of bioassays makes sense since endocrine disruptors or endocrine disrupting chemicals are exogenous substances or mixture that alters function(s) of the endocrine system (hormonal system) by binding to hormone receptors and then causes endocrine disruptive effects in an organism or its offspring.

The basic principle of bioassay is based on the comparison between the sample with the international standard of the same type in order to determine the how much substance is required to produce similar biological effect, as produced by the standard. They represent the fixed units of activity (definite weight of preparation) for drugs (Ramesh,. 2008).

An agonist may produce graded response or quantal response. Graded response means that the response is proportional to the dose and response may lie between no response and the maximum response. Quantal response is form of "all or none", i.e. either no response or maximum response. The drugs producing quantal effect can be bioassayed by end point method. The drugs producing graded responses can be bioassayed by matching or bracketing method or graphical method (Ramesh, 2008).

The identification and quantification of bioassays

Types of bioassays

(i) **On a basis of intent**: On the basis of intent, bioassays can be classified in one of two main groups, - absolute or comparative. **Absolute assays:** Absolute assays involve an attempt to obtain some quantitative measurement that can be expressed in absolute terms, such as a Minimal Lethal Dose (MLD) or Median Effective Dose (ED 50, LD50, etc.). Such attempts are based on the assumption or belief that some such absolute value exists and that universally it can be determined with adequate precision. However, the absolute potency of substance X for 'the cat" typically depends on just which cat is used and, unfortunately, cats invariably do differ. Laudable though the goals and objectives may be, absolute assays of biologically active substances, with few (if any) exceptions, have little useful quantitative meaning (Wells et al,. 1997, Batson., 1963, Ramesh, 2008).

Comparative assays: Although absolute assays seldom if ever yield adequately reproducible results, it generally is possible to achieve experimental quantitation of many biologically active substances through assessment of the substance of interest (unknown) in direct comparison with a reference substance (standard) qualitatively identical or, at least, similar in terms of the response evoked in the test subject of choice (Wells et al,. 1997, Batson., 1963). While the absolute potency of either may never be known, the comparative or relative activity of the two may be assessed and the biological activity of the unknown expressed in relation to that of the standard in terms of relative potency, - whether expressed in proportions, percentages or in arbitrarily defined units.

By using a common reference or standard substance, various investigators may obtain quantitative results with a degree of comparability adequate for

their needs. Such relative potency estimates are subject to uncertainty (experimental error), of course, but ideally this may be kept within manageable proportions. It is this innate element of uncertainty that makes bioassay a candidate for statistical consideration (Batson.,1963).

(ii) **On the basis of response**: On the basis of the response evoked in the test subjects of choice, most bioassays may be categorized into one of the following types: Direct assays: In these the response in the individual test subject is absolute (live, die; response, non-response; etc.) and critical (thresh-hold) levels of the assayed material are determinate, at least within reasonable limits (Wells et al,. 1997, Batson., 1963). Computations mainly involve calculation of means and ratios, and estimation of standard errors or confidence limits of such statistics. Example: the cat assay of digitalis. Graded response-parallel line assays: In these, the response in the individual is proportional to the dose of test substance administered and the degree of response is experimentally determinable (Batson., 1963, Wells et al,. 1997). Typically, the degree of response is a linear function of log-dose and the dosage-response regression lines of "Unknown" and "Standard" will be parallel denoting identity or similarity of action. Statistical analysis involves mainly regression analysis and analysis of variance. With proper design (balanced or partially balanced factorial assays), analysis can be simplified greatly through the use of coefficients. Example: assay of insulin in the rabbit. Slope-ratio assays: These include mainly the microbiological assays, a group of rather limited general interest in which the degree of measurable response in the individual probably is absolute, but since masses of test subjects (microorganisms) are dealt with, the total response measured, as density, acid formation, etc., approaches a continuous function. Statistical analysis involves multiple regressions and relative potency is estimated from the ratio of the partial regression coefficients. Example: microbiological assay of riboflavin. Quantal response assays: In these, response in the individual test subject is absolute (frequently, live or die) but the critical dose of test material

necessary to evoke the response is not directly determinable. Quantitation is achieved through the use of groups of test subjects and determination of the proportion responding to various dosage levels of "Unknown" and "Standard" test products. Following suitable transformation of the data (probits, angles, etc.) response typically is a linear function of log dose and statistical analysis is essentially similar to that employed with the graded response-parallel line bioassays. Examples: mouse-protective potency assays of typhoid, pertussis and rabies vaccines (Batson., 1963, Wells et al,. 1997, Ramesh, 2008)

The androgen receptor (AR) is involved in the development of male sexual characteristics and the estrogen receptor (ER) is involved in female maturation and reproductive function. Since estrogens are the most powerful and common endocrine disruptors, estrogenic bioassays which identify compounds (chemicals) that can interact with the human estrogen receptor are used to assess the presence of endocrine disrupting chemicals (Chimento et al 2014).

The estrogenic potential is performed by the application of recombinant yeast estrogen screen assay (YES assay) and the reporter gene assay (KBluc assay). The two assays are selected based on their different sensitivities and end points. The yeast cells contain only one estrogen receptor (ERα), whereas the T47D-KBluc cells contain two receptors (both the endogenous ERα and Erß), making the KBluc assay more sensitive than the YES assay (Nguyen,. 2010)

There is no significant difference between the sample preparation for bioassays and for chemical analysis. For samples preparation procedures that involves sample clean up step, has a low risk of positive bias in their analysis. Suspended solids must be removed from sample matrices as are adsorbed on to antibodies. If good sample preparation and clean-up was performed properly in bioassays. The target analytes can be identified without the need for chromatographic separation. Some of the hyphenated tandem mass spectrometry GC may require less clean-up

procedures because of their selective detection nature of the instrument configuration (Chan et al,. 2004).

Table 2: A comparison of chemical and in vitro bioassays applications for the analysis of estrogens in water samples (Leusch et al. 2010)

Chemical applications	**Bioassays applications**
Both natural and synthetic hormones are vigorous; they can cause high endocrine effects even at concentration levels below the detection limits.	Because of the sensitive nature of bioassays, they have the capability of detecting biologically active chemicals even at very low concentrations.
Since the quantification of analytes is based on calibration with known structural configuration, only targeted analysis can be identified and quantified. Other bioactive compounds will not be detected unless the proper qualitative scan has been performed.	The response of bioassays is based on physiological effects in vitro instead of the structural orientation of the compound. This means that bioassays can detect any estrogen independence of their chemical behaviour.
Chemical techniques requires continuous improvements in order to measure contaminants of major concerns, because of the complex nature of chemical behaviours of these compounds	The bioassays respond to modes of action. Hence the effects of chemicals can be integrated in a mixture.
Chemical methods provides no biological information (such as bioavailability) to specify toxicological effects which can be used for risk assessment.	Bioassays are the preferred methods for the determination of emerging pollutants and their methods need not be continuously modified to detect unknown bioactive contaminants.

Chemical analysis does not provide toxicity determinations or biological changes between the different compounds in the matrix.	Bioassays can inform the exposure assessment.
The nature of potential emerging compounds is wide and extensive, given that there is a number (thousand) of registered chemicals with hundreds of them being EDCs.	Bioassays provide extensive biological information which can be used in risk assessments and other epidemiological studies.
Can analyse multiple analytes in one single analysis	Only one targeted analyte can be analysed at a given time.

Some chemical and bioassay analysis have been performed worldwide. In the Tiaoxi River, China, only estrone was found, other estrogenic hormones were not detected with the HPLC–MS –MS system. The concentration levels of these compounds in environmental water samples were in the region of 0 to 17.25 ng/ℓ (Tang et al, 2012). In the same study, the estrogenic activity in most of the environmental water samples tested positive using the yeast estrogen screen. The 17β-estradiol equivalent was present in all tested water samples, it was found to be 17.60 ng/ℓ particularly in the intake of Taihu Lake.

In a separate work done by Tremblay et al,.(2013) in Hamilton City , estrogenic activity was found in the Taupo Gates and Ohaaki Bridge samples at levels close to the detection limit of the bioassay and an order of magnitude below the PNEC of 2 ng/ℓ estimated for 17β-estradiol (Caldwell et al. 2012).

2.6.2 Gas chromatography- mass spectrometry

In gas chromatography (GC) analytes under investigation are subjected into an injector port, vaporised and eluted through the capillary n by means of an inert carrier gas into the detector. GC is the preferred method of choice when compounds that are thermally stable and volatile are to be separated and quantified.

The most preferred form of gas chromatography is partition chromatography; the Gas-liquid chromatography (GLC) is a form of partition chromatography where the separation is based on the partitioning of the components of a chemical mixture between a mobile carrier gas and stationary liquid phase supported by the solid. The solid sorbent is used as the stationary phase. There is a wide range of selective and sensitive detectors with the possibility of hyphenating the gas chromatography to mass spectrometer or other detectors to get as much information on a sample as possible. (Jager et al,. 2011).

Gas chromatography – mass spectrometry is still the most widely known and preferred technique of all hyphenated configurations for GC, and the "marriage" of a potent analytical technique with the high level of molecular and structural recognition given by the mass spectrometry (MS) has made GC-MS the ideal for the analysis of trace organic compounds. This combination allows the use of an automated separation on the GC with structural elucidation on the MS in order to get the advantages of both. The most common mass analyser is the quadrupole mass ion trap or mass filter that allow high scanning speeds up to a transmission range of m/z equal 2000 (Perry at al,. 2008).

For structural elucidation, GC can be hyphenated to tandem mass spectrometry (a combination of two mass analysers). Tandem mass spectrometry (MS-MS) is an instrumentation set up that uses least two stages of mass analysis either in conjugation with a dissociation process or a chemical reaction that causes a change in the mass or charge of a molecular ion produced. In the most common

MS-MS a first mass analyser is used to analyse a precursor ion, which then undergoes a fragmentation, either spontaneously or by some activation, to produce product ions and neutral fragments. A second mass analyser analyses the product ions (Chan et al, 2011)

Criteria for characterisation must be developed if the single gas chromatography mass spectrometer is used especially in sophisticated matrices such as highly polluted, treated of untreated waste water samples. These criteria should include fragmentation ion, matching of the retention times, presence of at least two additional qualifier ions and matching of fragmentation ion ratios within a reasonable percentile for other ion for qualitative identification.

A most preferred version of the tandem mass configuration is the ion-trap MS/MS. An ion trap itself is like a quadrupole bent on itself in order to form a closed loop. A large number of molecules are trapped to induce ionisation hence increasing the instrument sensitivity.

The aspect that determines the differentiation between single MS and MS-MS is in the selectivity of the analysis. Matrix interferences are the major drawbacks in the single MS. This challenge is common for ethynylestradiol, it found that concentrations, sometimes are higher than anticipated suggesting that this difference may be due to interference by natural organic matter (Grebe et al,. 2011).

Since steroids are not readily volatile, the analysis is performed following the derivatisation step for GC-MS analysis. The process of derivatisation is the major disadvantage in the analysis of estrogen hormones when GC is used, it is not only tedious to perform but it is laborious and time consuming as well.

In Romanian drinking water, E1 and EE2 were found at an average of 0.40 and 0.35 ng/ℓ, respectively; α-E2 could only be determined in one sample at 0.30 ng/ℓ, while β-E2 was found at an average of 0.70 ng/ℓ. The steroids were found in the

lower ng/ℓ range with mean concentrations of 2.5 ng/ℓ E1, 1 ng/ℓ E2 and E2, and 1.5 ng/ℓ EE2 (Briciu et al,. 2009).

Hua Huang et al,.(2000), using GC-MS following derivatisation, have reported the concentrations of estrogenic hormones 17β-estradiol and 17α-ethinyl-estradiol discharged by conventional wastewater treatment plants range from approx. 0.2 to 4.1 ng/ℓ in Califomia, United State of America.

Recently in Poland, the results of water samples obtained from Brda River (Człuchów) showed that estrone, 17β-estradiol, and ethynylestradiol were detected (GC-MS analysis following derivatisation) in concentration of 2.2 ± 1.0 ng/ℓ, 1.8 ± 0.8 ng/ℓ, and 3.6 ± 1.7 ng/ℓ (Woźniak et al,.2014).

2.6.3 Liquid chromatography- mass spectrometry

Unlike gas chromatography where mobile phase is a gas, liquid chromatography uses a liquid as a mobile phase. When the separation involves predominantly a simple partitioning between two immiscible liquid phases, one stationary and the other mobile, the process is called liquid-liquid chromatography (LLC). When physical surfaces forces are mainly involved in the retentive ability of the stationary phase, the process is denoted liquid-solid (or adsorption) chromatography. Liquid chromatography where stationary phase is solid is less popular.

In liquid chromatography analytes are introduced into the mobile phase via a loop valve and are carried into an analytical column containing distributed stationary phase. The species undergo repeated interactions based on the affinity of solutes to the stationary phase. The species are gradually separated into bands in the mobile phase. At the end of the process, separated components emerge in order of increasing interaction with the stationary phase. The least retained component emerges first while the most strongly retained components elute last (Jager et al,. 2011).

There are different types of LLC used in the analysis of environmental samples, a reverse phase chromatography a normal phase chromatography. In normal phase HPLC analytes are separated based on polarity and the interactions between the analyte and the stationary phase is purely adsorptive. This type uses polar stationary phases (amino, cyano or silica) and a non-polar mobile phase (e.g. dichloromethane). It is used when an analyte of interest is relatively polar. Equilibration between mobile phase and stationary phase takes long time and therefore cannot perform gradient elution, while reverse phase chromatography consists of a non-polar stationary phase (C_{18}, C_8 etc) and a polar mobile phase (methanol – water mixture). It is used when an analyte of interest is non-polar. Reverse phase operates on the principle of hydrophobic interactions which results from the repulsive forces between a relatively polar solvent, the relatively non polar analyte, and the non-polar stationary phase. Reverse phase chromatography has been widely used in analytical techniques and is the most popular among the liquid chromatographic techniques.

The main advantage of liquid chromatographic techniques in the analysis of environmental samples for estrogens is that sulphuric and glucuronic metabolites can be determined without the need for derivatisation as it is the case in gas chromatographic systems. Most common derivatisation in LC is intended to enhance detectability by ultraviolet absorption, fluorescence, or electrochemistry (Chan et al,. 2011, (Jager et al,. 2011). In some publications, the use of triethylamine or ammonium-acetate for buffering the mobile phase is widely documented. A pH-adjustment of the eluent sometimes react with the silica in based columns unless the post-column addition of ammonia or trimethylamine is used (Ingerslev et al, .2003).

In Mess River during the floodwater season in Luxembourg (11 floods, 66 samples), the following highest concentrations were reported;- ibuprofen (9–2382 ng/ℓ), E1 (4–27 ng/ℓ) and diclofenac (3–20 ng/ℓ). The river concentration of E2 was relatively high (35 ngL^{-1}), especially when taking into account its presumed

endocrine disrupting effect on animals at this level (Pailler et al, 2009). The conditions of SPE followed by LC-MSMS were used to obtain the results.

Vulliet et al,.(2011) reported estrone to be detected in 20% (Out of 21 samples) of waters at concentrations generally in the concentration region between 0.1 and 1 ng/ℓ. Only three out of 21 samples exceeded 1 ng/ℓ of estrone. The presence of estrone is common in most hormones analysis because it is the most abundant natural estrogen excreted by cycling women, It is also the by-product of biodegradation of estradiol (Huerta-Fontela et al,. 2010).

Ethinylestradiol, 17α- and 17β-estradiol were quantified in only few samples. The concentrations of ethinylestradiol ranged from 0.5 to 2.6 ng/ℓ, that of 17a-estradiol from 0.2 to 1.6 ng/ℓ and that of 17b-estradiol from 0.03 to 1.3 ng/ℓ in Barcelona, Spain (Vulliet et al,.2011).

2.7 Concentration levels of EDCs in water bodies

Estrogens have been detected in drinking waters in Germany (up to 2.1 ng/ℓ), France (0.2–26.4 ng/ℓ) and Spain (Kuch and Ballschmiter, 2001; Vulliet et al., 2011; Kuster et al., 2008).

The estrogen such as 17β-Estradiol was detected in groundwater in Austria, in the range 0.10 – 0.80 ng/ℓ (Hohenblum et al., 2004), and 11 different hormones in France, in the range of 0.30 – 4.0 ng/ℓ even though the fate and impact of these compounds is not clearly known on public health.

According to a study performed by Pool et al. (2012), estrone levels above 5 ng/ℓ can adversely affect reproduction of aquatic animals. Estrogens are female sex hormones which are responsible for the development, reproductive system and secondary sex characteristics in females (Martini,. 2006).

Estrone is also the main metabolite of 17-β-estradiol (a natural estrogen) and reaches the environment via the sewer system or animal excretion (Ying et al., 2002; Wenzel et al., 2003). The study conducted in The Netherlands also shows that generally, concentrations of hormones in surface water range between 1 and 5 ng/ℓ with estrone being the most frequent one (Ghambarian et al,. 2012)

2.8 Conclusion statement

In the literature demonstrates that the availability of certain endocrine disrupters in surface water is mainly due to/lack of the following.

- ✓ The quantities of some pharmaceuticals in sediments or in the solid phase can be expected to be quite high, and therefore an effort should be made to quantify this load, either by developing analytical techniques to measure pharmaceuticals concentrations in sludge samples or by the determination of adsorption coefficients (Kd) to calculate indirectly those concentrations.

- ✓ Although there have been reports of the analysis of hormones in the water bodies, the literature is still limited and in many developing countries, nothing has been reported. Thus the status of these compounds in not known in such countries.

CHAPTER THREE – OBJECTIVES OF THE STUDY

3.1 Hypothesis of the study

Estrogenic hormones and their metabolites have polluted Vaal river catchment area due to environmental pollution such as industrial pollution and human excretion. Some of the indicators of possible pollution by these compounds in the Vaal River are increasing number of illegal informal settlements within the catchment area and due to inability of waste water treatment plants to cope with the influent load of these compounds and to effectively remove these organic contaminants during sewage treatment, hence polluting the source water.

3.2 Aims and the objectives of the Study

The objectives of this study were to extract, screen available hormones in source and potable water supplies, and to develop methodology to separate and quantify the amounts of these compounds present in environmental water samples using UFLC-MS.

This project has three aims listed as follows;-

- To develop a method suitable for the determination of estrogen hormones using UFLC-MS following SPE of water samples from Vaal river catchment area, Johannesburg south, South Africa.

- To determine the availability of identified hormones in Drinking water samples and in Vaal river catchment area of South Africa.

- To compare the concentrations of these compounds to literature values and to any available water standards.

3.3 Novelty of the study

The study is novel in that although these compounds have been reported in literature, not much has been reported in South Africa. There are few results that have been reported in literature using UFLC-MS which is the state of the art instrumentation. Thus the results are critical in giving an idea of the extent of the problem from these compounds.

CHAPTER FOUR – RESEARCH METHODOLOGY

4.1 Sampling

Samples were collected in 1000 mℓ glass amber bottles and transported into the laboratory. Samples were extracted immediately to avoid biological degradation. A total number of 28 samples of source, surface and drinking water samples were collected in 2013 and 2014 periods for hormones analysis. Figure 5 below illustrates the map of Vaal dam and Vaal River Catchment area where samples were taken for analytical determination. The direction of the flow is from top to bottom, Rand water abstract the raw water for purification directly from the Vaal dam (Sampling point V1). Table 3 shows the sampling points while Table 4 indicates the names of hormones of interest in the study.

A volume of 1000 mℓ of each sample and laboratory prepared spiked standards were extracted using solid phase extraction technique and subjected in to the pre-optimised UFLC – MS-MS. Some sample aliquots were taken to hydrobiology lab at the University of Pretoria for estrogenic activity and mutagenicity analysis, see results in Table 7.

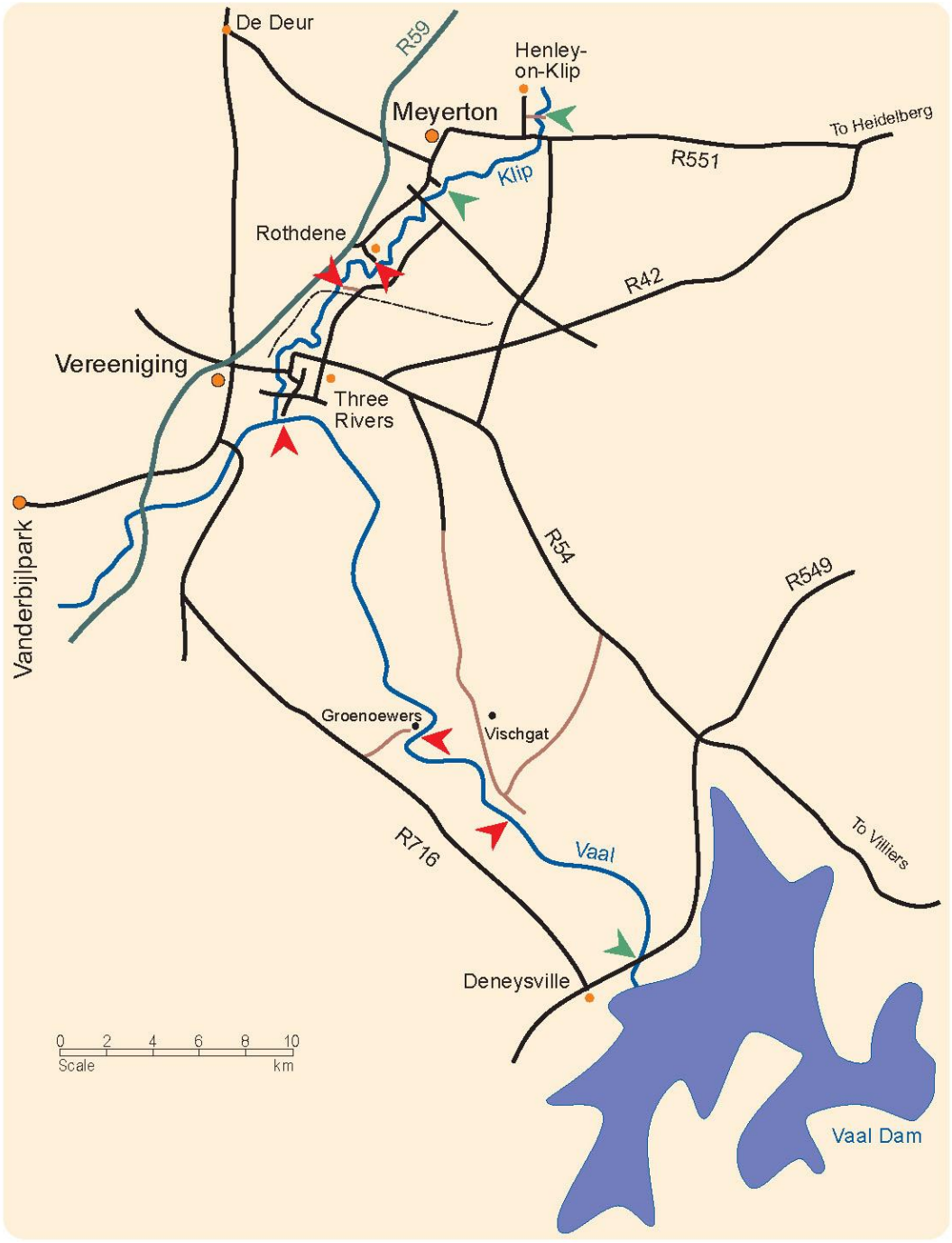


Figure 5: The Map of Vaal Dam and Vaal River Catchment

Table 3: Sampling points and site description of the collected samples within the Vaal catchment areas.

Sample point	Description
M-A18	Sample point information withheld
M-A8	Sample point information withheld
D-DA8	Sample point information withheld
D-PAL-B4	B4 at Palmiet before chloramination
M-Canal Raw	Canal source water at Zuikerbosch
M-B12	Sample point information withheld
D-DB8	DB8 – 5km after chlorination at Zuikerbosch
D-MAP_S1	S1 at Mapleton after chloramination
B-RAW	Barrage source water
B-DOM	Barrage domestic water
C-Vaalkop Raw	Vaalkop Dam source water to Magalies Water
D-Townlands	Purified Water from Magalies Water at Rustenburg
C-VGB	Vaal River source water at Gladdedrif bridge – Villiers
C-WF	Sample point information withheld

Table 4: List of endocrine disrupting compounds monitored

Organic contaminant	Classification	Concern to the Drinking Water Industry
17 α Ethinyl estradiol	Steroid hormone / Synthetic birth control pharmaceutical	Endocrine disruption
17 α Estradiol	Hormone	Endocrine disruption
17 β Estradiol	Hormone	Endocrine disruption
Estrone	Hormone	Endocrine disruption

4.2 Standards and sample preparation

Estrone (E1, > 99.0%), 17β-Estradiol (E2, > 99.0%), 17α-Estradiol (E3, > 99.0%) and 17α-Ethinylestradiol (EE2, > 99.0%) were purchased from Dr Ehrestorfer (Johannesburg, South Africa). Methanol (99.9%), GC and HPLC grade was purchased from Merck (Johannesburg, South Africa). HPLC grade acetonitrile (99.9%) was sourced from B & J (Johannesburg, South Africa). The method blank water was prepared in the laboratory by reverse osmosis followed by a milli-Q polishing system supplied by Merck South Africa.

The stock standard of each hormone was prepared in 50% v/v (water/methanol) by dissolving 10 mg of each pure hormone solid and made it up to a mark in a 10 mℓ volumetric flask. Further dilutions were made to reach a combined concentration of 0.01 ng/μℓ mixed solution. The target analyte concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0 ng/ℓ were prepared by transferring 20, 40, 60, 80 and 100 μℓ on 0.01 ng/μℓ mixed solution in to 1000 mℓ of deionised water and extracted under optimised conditions of SPE method. Quantities of 1000 mℓ of unknown samples were subjected to SPE under the same conditions as calibration standards.

The extracts were analysed by UFLC coupled to tandem MS for qualitative and quantitative determination of hormones.

4.3 Solid phase extraction procedure

Three aliquots of 10 mℓ methanol were used in sequence to condition the SPE (Agela PEP (C_{18}), 200 mg/6 mℓ) cartridges in an already assembled SPE manifold. A volume of 10 mℓ of blank was also added to the cartridge prior to the percolation samples and standards. An elution flow rate of 10 mℓ/min was used under a carefully controlled vacuum to extract a 1000 mℓ of a sample.

Vacuum of -20 Hg was used to dry the SPE cartridges containing the analytes over a period of 10 to 15 minutes, after the vacuum was switched off the analytes were eluted and collected slowly in to the tubes. Methanol was used to elute analytes of interest from the SPE cartridges up to a volume of 7.5 mℓ, See **Figure 6** below showing the assembled SPE manifold with conditioned SPE cartridges.

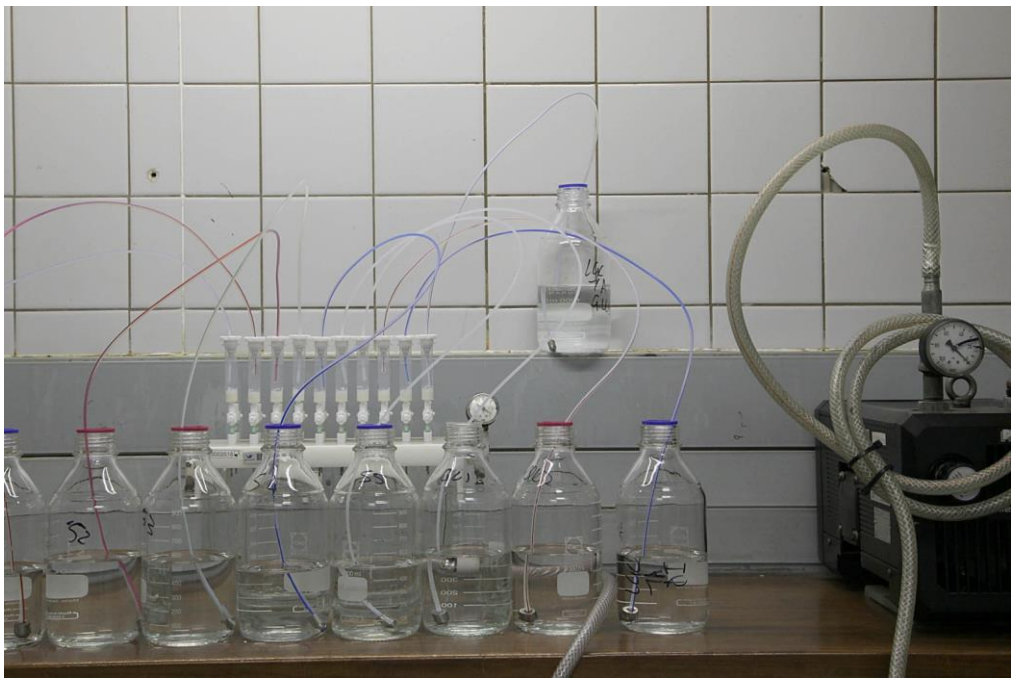


Figure 6: Assembled SPE manifold with extracting cartridges

Nitrogen gas was used to concentrate the 7.5 mℓ of the analyte extract down to the volume of less than 150 µℓ before diluting to the 250 µℓ calibration mark with 50% methanol solution using a calibrated micro-syringe. A concentrate was then subjected to a UFLC coupled to a mass spectrometer. These conditions were optimum for all analytes of interest in this study. It should also be noted that both samples and spiked standards were extracted in the same way using SPE before final analysis. Thus, accuracy of the method in this case is more important than recovery as both spiked standards for the calibration curve and samples were extracted in the same way.

4.4 Sample analysis

After analytes were subjected to UFLC-MS-MS, the qualitative analysis was performed based on retention times of the analytes as well as on the multiple reaction monitoring ratios between the precursor and the product ions. The quantification was performed using the external calibration method for standardisation; the ion of best intensity per each compound was used. The introduction of a buffer solution of 0.4% NH_3 as a modification agent was performed using an external pump system at a flow rate of 0.05 mℓ/min.

Table 1 shows the list of all hormones determined in this study with an exception of estriol due to the absence of the standard.

4.5 Detailed instrumental parameters

4.5.1 High Performance Liquid Chromatography

The separation was performed using LC-20AB Shimadzu liquid chromatograph connected to SIL-20AC auto-sampler by CBM 20A communication module. The temperature of the CTO-10AS column oven was kept constant at 40ºC.

The injection volume used was 100 µℓ for maximum sensitivity while the analytical column Kinetex C_{18}- Sorbent (100 x 4.6 mm; 2.6 µm) from Phenomenex was used for separation. The mobile phase compositions used for analyte elution were acetonitrile (with 5% water in it) and water (with 5% Acetonitrile in it) at a flow rate of 0.35 mℓ/min for 12 minutes. The UFLC gradient phase program is shown below.

Auto sampler parameter	**Magnitude**
Injection volume	100 µl
Rinsing volume	200 µl
Needle Stroke	52 mm
Rinsing Speed	35 µl/sec
Sampling Speed	15.0 µl/sec
Purge Time	5.0 min
Rinse Dip Time	0.0 sec
Rinse mode	before and after aspiration
Cooler unit at	15^0C
Control vial needle stroke	52 mm
Oven Temp	40^0C

Table 5: UFLC mobile phase program

Time	Parameter (% Water)	Parameter (% Acetonitrile)
0.10	0	100
8.00	95	5
9.00	15	85
12.00	15	85
12.10	Stop	

4.5.2 Mass Spectrometry Conditions

Table 6 below shows some of the parameters for the AB Sciex 3200 Q Trap mass spectrometry system. The instrument was optimised in negative polarity using multiple reactions monitoring mode. The variation of coin voltage for each compound determined the collision energies (CE), declustering potentials (DP) and exit potentials (EP) for these analytes, the actual figures are shown in Table 6 below.

Instrument Settings

Ion source	Turbo Spray
Scan type	MRM
Polarity	Negative
Total Scan time	0.6200 sec

Table 6: MRM transitions and retention times

R T (min)	Q1 Mass (Da)	Q3 Mass (Da)	Time (msec)	Analyte Identity	DP (Volts)	EP (Volts)	CE (Volts)
4.12	271.063	144.80	150	17-α-estradiol	-70	-70	-2.5
4.31	271.063	144.801	150	1- β-estradiol	-70	-70	-3.5
4.53	295.064	144.9	150	17-α-ethinylestradiol	-60	-60	-4
4.55	269.087	144.9	150	estrone	-40	-40	-10

4.6 Mass spectrometer optimisation

Physical and chemical properties of hormones are shown in Table 1 above, pK_a and Log K_{ow} are required as they determine the ionic prevalence of compounds as pH changes (Huerta-Fontela et al., 2011). Each 1 mg/ℓ pure hormone standard at a pH of 10.2 was infused into the mass spectrometer to optimise fragmentation parameters of ions. During the infusion of pure hormones standards, negative and positive modes were investigated, the negative mode produced optimum conditions as a result it produced intense signals that were used for quantification.

The negative ionisation was found to be better compared to the positive ionisation. The optimisation of cone voltage enhanced the sensitivity of parent ions as well as its signal for all analytes.

The optimised collision energy value differs from analyte to analyte because MRM ions were investigated independently within the same compound of interest. The different transitions investigated are shown in Table 22.

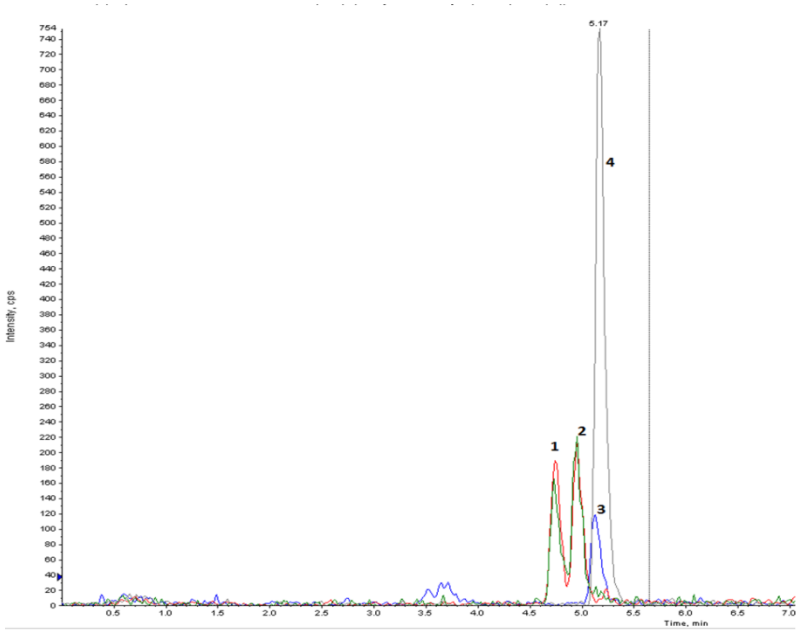


Figure 7: ***A chromatogram of compound mixture dissolved in 50%v/v of water/methanol***. Where *1*, 2, 3 and 4 represent E2 (α), E2 (β), EE2 and E1 respectively

CHAPTER FIVE– RESULTS AND DISSCUSSION

5.1 Results of estrogenic activity and mutagenicity in water samples

Table 7 shows that the estrogenic activity (using T47D-KBluc assay) was detected in all the raw water samples however; none of the samples exceeded the trigger value of 0.7 ng/ℓ for estrogenic activity in drinking water except sample C-WF in dry season. Cytotoxicity was detected in most (6 out of 12 samples) of the raw water samples during the dry season. It is also important to know that this cytotoxicity was only detected once the samples were concentrated 100 times (Table 7). It should also be kept in mind that estrogenic activity is a bioassay and the activity is measured in estradiol equivalents (Eeq's) which are only rough estimates and not concentrations. The complexity of the sample, pH, and extraction procedure of the analysis as well as the nature of the assay can have an influence on the results.

Estrogens (estradiol, estrone, estriol) are female hormones responsible for the development and maintenance of reproductive tissues and secondary sex characteristics in females (Martini, 2006). Estrone is also the main metabolite of 17 β estradiol (a natural estrogen) and reaches the environment via the sewer system or animal excretion (Ying et al., 2002; Wenzel et al., 2003).

Based on the test criteria of the Ames MPF 98/100 AQUA assay none of the raw water samples showed mutagenic activity, the absence of mutagenic effect does not mean that the sample was not mutagenic, but rather no mutagenic effect was detectable under the assay conditions of the specific test system used.

Table 7: Results of the raw water samples analysed for estrogenic activity and mutagenicity.

Parameter	Unit of measure	Guidelines		Analysing laboratory	Reporting Limit	Raw water samples											
						C-VGB		C-WF		B-RAW		M-CANAL		M-A18		C-VAALKOP	
			Source			Wet season	Dry season	Wet season	Dry season	Wet season	Dry season	Wet season	Dry season	Wet season	Dry season	Wet season	Dry season
ESTROGENIC ACTIVITY																	
Estradiol equivalents (YES assay)	ng/ℓ	0.7	WRC	UP		nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Estradiol equivalents (T47D-KBluc assay)	ng/ℓ	0.7	WRC	UP		0.16	0.03	0.15	nd	0.18	0.20	0.03	0.07	0.22	0.08	0.15	0.07
Cytotoxicity	ng/ℓ			UP		**d**	nd	**d**	**d**	**d**	**d**	nd	nd	**d**	nd	nd	nd
17 a Ethinylestradiol	ng/ℓ			RW	<0.30	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
17 a Estradiol	ng/ℓ			RW	<0.30	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
17 b Estradiol	ng/ℓ			RW	<0.30	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Estrone	ng/ℓ			RW	<0.30	nd	nd	0.90	nd	nd	nd	nd	nd	4.43	nd	nd	nd
MUTAGENICITY																	
AMES MPF assay				RW		nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

Table 8: Results of the drinking water samples analysed for estrogenic activity and mutagenicity

Parameter	Unit of measure	Guidelines		Analyzing laboratory	Reporting Limit	Drinking water samples															
						M-A8		D-DA8		M-B12		D-DB8		B-DOM		D-TOWNLANDS		D-MAP_S1		D-PAL_B4	
			Source			Wet season	Dry season	Wet season	Dry season	Wet season	Dry season	Wet season	Dry season	Wet season	Dry season	Wet season	Dry season	Wet season	Dry season	Wet season	Dry season
Estradiol equivalents (YES assay)	ng/ℓ	0.7	WRC	UP		nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nt	nd	nd	nd
Estradiol equivalents (T47D-KBluc assay)	ng/ℓ	0.7	WRC	UP		nd	nd	0.02	nd	nd	nd	nd	nd	nd	nd	0.04	nd	nt	nd	0.03	0.02
Cytotoxicity	ng/ℓ			UP		nd	nd	nd	nd	nd	**d**	nd	nd	nd	nd	nd	**d**	nt	**d**	nd	nd
17 a Ethinylestradiol	ng/ℓ			RW	<0.30	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nt	nd	nd	nd
17 a Estradiol	ng/ℓ			RW	<0.30	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nt	nd	nd	nd
17 b Estradiol	ng/ℓ			RW	<0.30	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nt	nd	nd	nd
Estrone	ng/ℓ			RW	<0.30	nd	nd	nd	nd	**2.88**	nd	nd	nd	nd	nd	nd	nd	nt	nd	nd	nd
MUTAGENICITY																					
AMES MPF assay				RW		nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nt	nd	nd	nd

No anti-estrogenic activity (YES assay) was detected in the drinking water samples (Table 8). Also, no estrogenic activity or cytotoxicity was detected in the water samples using the YES assay. The estrogenic activity detected by the T47D-KBluc assay were all well below the trigger value of 0.7 ng/ℓ for estrogenic activity in drinking water (Genthe and Steyn, 2008). The reason why estrogenic activity is detected by the KBluc assay and not the YES assay in a specific sample could be explained by the fact that the yeast cells contain only the ERα. The T47D-KBluc cells contain both the endogenous ERα and ERß, making the T47D-KBluc assay more sensitive than the YES assay.

It should also be kept in mind that a water sample consists of a complex mixture of chemicals with possible (anti)-androgenic and (anti)-estrogenic activity, as well as other chemicals not measured, that could affect the outcome of the assay. It should therefore be noted that estradiol equivalents are only rough estimates, as the complexity of the sample, pH, extraction procedure and the nature of the assay (i.e. a biological system) might all have an influence on the results. According to Table 8, none of the final water samples showed mutagenic activity based on the AMES MPF 98/100 AQUA assay.

5.2 Quality Control (QC) and the Analytical Method Validation

The overview of the physical and chemical properties of the compounds and some quality control parameters are shown below in Table 9. All samples and quality control verifications were analysed in the analytical range of up to 1 ng/ℓ. Any samples above this range were diluted further accordingly.

Table 9: Slope, Intercept and Physical and chemical properties of hormones (Carpinteiro et al,. 2004, Lewis et al,. 1979)

Analyte	***pK_a**	***Log K_{ow}**	**r^2**	**Slope**	**Intercept**
E1	10.7	3.7	0.998	18508.6	-318.3
E2(α)	10.7	4.1	0.997	8658.6	-57.6
E2(β)	10.7	4.1	0.996	7346.4	14.0
EE2	10.2	4.5	0.998	5239	-8.0

Table 9 shows the characteristics and method performance parameters, the coefficients of determination (r^2) of more than 0.99 were achieved indicating a good linear dynamic range from 0 to 1.0 ng/ℓ. The slope and the intercepts were recorded accordingly.

The statistical evaluation for 17-α-ethinylestradiol below is an epitome of the determination of fundamental figures of merit for the rest of the determinants in this study available in Annexure 1, involving the coefficient of determination of the calibration function, concentration range, sensitivity, uncertainty of measurements and limits of detection and quantification.

This technique involves a holistic approach to validate the analytical method, uncertainty of measurements and accuracy profiles of the analytes.

Table 10: Calibration data for 17-α-ethinylestradiol

CONCENTRATION (ng/ℓ)	RESPONSE RATIO
0.0	0
0.2	979
0.4	2060
0.6	3320
0.8	4100
1.0	5210

Table 10 shows the plot of the concentration of 17-α-ethinylestradiol versus its instrument response. These figures were subjected to the data analysis function of Microsoft (MS) excel spreadsheet and the following regression summary output was obtained.

REGRESSION ANALYSIS

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.998806003
R Square	0.997613431
Adjusted R Square	0.997016789
Standard Error	107.1946827
Observations	6

The analysis of variance (ANOVA) was determined using the MS excel to induce the slope (sensitivity/x-variable 1), intercept and standard errors that were used for the determination of limit of detection and of quantification.

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	19212984.7	19212984.7	1672.046498	2.13759E-06
Residual	4	45962.8	11490.7		
Total	5	19258947.5			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*
Intercept	-8	77.58181549	-0.103116948	0.922833133	-223.4016519
X Variable 1	5239	128.1221515	40.89066518	2.13759E-06	4883.275879

The values obtained from the ANOVA for slope (b) and y-intercept was 5239.0 and -8.0 respectively as coefficients of x and y variables, good linearity was reflected by r^2 value that was closer to 1.

In order to understand the uncertainty of regression, some of the important parameters needed to be deconstructed from the ANOVA in the form of the standard errors both from x and y variables. Therefore the regression uncertainties of random uncertainty ($S_{y/x}$), slope uncertainty (S_b) and Y-intercept uncertainty (S_a) were 107.19468270, 128.12215154 and 77.58181549 respectively.

The slope is the change in analytical response divided by change in concentration which is same as sensitivity in any given calibration curve. If the linear calibration is assumed, the sensitivity is same as slope (b) at each and every point in the analytical concentration x. In addition to that, there are two parameters that can be reciprocally derived from sensitivity, the LOD and the LOQ (Gonza´lez et al, 2007).

LOD can be expressed in response units (Y_{LOD}) and is taken typically as three times the noise level for techniques with continuous recording such as chromatography. The LOQ is estimated to be ten times signal to noise ratio hence the following calculations.

Limit of detection

$Y_{LOD}=Y_b+3S_b=bX_{LOD}+ a$

Assume $Y_b= a$

Thus, $X_{LOD}=3S_b/ b$

Therefore, $X_{LOD}= 0.073$ ng/ℓ

Limit of quantification

$Y_{LOQ}=Y_b+10S_b=bX_{LOQ}+ a$

Assume $Y_b= a$

Thus, $X_{LOQ}=10 S_b/ b$

Therefore, $X_{LOQ}= 0.245$ ng/ℓ

Accuracy

Table 11: A spiked quality sample, concentration of 0.50 ng/ℓ

DETERMINATION	CONCENTRATION	PEAK HEIGHT
#1	0.43	2240.0
#2	0.50	2590.0
#3	0.57	2970.0
#4	0.52	2690.0
#5	0.47	2460.0
#6	0.44	2280.0
#7	0.49	2520.0
#8	0.41	2110.0
Mean	0.48	2482.5
Std Deviation	0.051	275.875

The accuracy of the method was determined by analysing the reproducibility of eight replicates on the analyte over a period of time, results in Table 11 were obtained. They can be summarised as follows;

(i) RSD of concentration, (s x100/mean), % = 10.73

(ii) Mean Method Accuracy (% of true conc.), % = 95.6

(iii) Method Bias (deviation from true value), % = - 4.4

Uncertainty of measurements

The equation of the straight line for the uncertainty of regression can be expressed as follows;

$y_0=bx_0+a$

(Consider Table 11 for a spiked quality sample, concentration of 0.5 ng/ℓ)

For x_0	=	0.4779
y_0	=	2495.587125
ave.y	=	2482.50000000
y_0-ave.y	=	13.0871
(y_0-ave.y)(y_0-ave.y)	=	171.2728408

x_i	x_i-ave.x	(x_i-ave.x)*(xi-ave.x)
0.0000	-0.500	0.25
0.2000	-0.300	0.09
0.4000	-0.100	0.01
0.6000	0.100	0.01
0.8000	0.300	0.09
1.0000	0.500	0.25
	Sum =	0.7
	Sum^2=	0.49

$$Sx_0 = S_{y/x}/b \{1/m+1/n+ (y_0\text{-ave.y})^2/b^2 sum(x_i\text{-ave.x})^2\}^{1/2}$$

Substitution of the terms above where m and n are number of determination and calibration data points respectively resulted in the following answer;-

Sx_0 = 0.022100424

The **uncertainty of repeatability** was obtained by dividing standard deviation of y. by its average hence;-

Std Dev.y/ave.y = 0.111127988

The **uncertainty of purity** was obtained from the certificate of analysis as 100±0.5% and was calculated as follows assuming a rectangular distribution;-

$U(P) = 0.5/3^{1/2} = 0.29$

The **uncertainty of volume** was calculated from all volumetric apparatus used in the study taking into account the coefficient of expansion of the volume.

For the **10 mℓ volumetric flask**, the calculation was performed as follows;

Given 10±0.04mℓ, 20 ±4°C, assuming a rectangular distribution therefore;-

$U(V_1) = 0.04/3^{1/2} = 0.023094011$

Lab temperature= 20±4°C

Coefficient of volume expansion = 0.00021, assuming rectangular distribution therefore;-

$U(V_2)$ = Volume variation = (Volume*temp. var.*coefficient of volume expansion)/$3^{1/2}$

= 0.004849742

Volumetric Flask Uncertainty = $\{U(V_1)^2+U(V_2)^2\}^{1/2}$

Therefore $U(V_A)/V_A$ = 0.002359774 mℓ

For the 100 µℓ syringe, the calculation was performed as follows;

Given 100 ±1µℓ at 20 ±4°C, Assuming a rectangular distribution, the calculation was performed as follows;-

$U(V_1)$ $=1/3^{1/2}$ = 0.577350269

Lab temperature at 20±4°C

Coefficient of volume expansion = 0.00149, if Assuming a rectangular distribution, therefore;-

$U(V_2)$ = Volume variation = (Volume*temp. var.*coefficient of volume expansion)/$3^{1/2}$

= 0.34410076

Therefore for a 100 µℓ Syringe the Uncertainty $= \{U(V_1)^2+U(V_2)^2\}^{1/2}$

$U(V_B)/V_B$ = 0.006721151 µℓ

For a 500 µℓ **syringe** the calculation was performed as follows;

Given a 500 ±1 µℓ, in a room temperature at 20±4°C , Assuming a rectangular distribution

$U(V_1)=1/3^{1/2}$ = 2.886751346

Given a lab temperature of 20±4°C and coefficient of volume expansion of 0.00149 assuming rectangular distribution, therefore

$U(V_2)$ = Volume variation=(Volume*temp. var.*coefficient of volume expansion)/$3^{1/2}$

= 1.720503802

Therefore for a 500 µℓ Syringe the uncertainty = $\{U(V_1)^2+U(V_2)^2\}^{1/2}$

$U(V_C)/V_C$ = 0.003360575 µℓ

The uncertainty of the analytical balance was given as $U(V_D)/V_D$= 0.002 from the calibration certificate.

All uncertainty budgets were added and substituted accordingly as per equation below;-

$\mathbf{x_0}$ =

$U(t)/x_0$ =

$(Sx_0/x_0)^2+(SR/S)^2+(U(P)/P)^2+(U(V_A)/V_A)^2+(U(V_B)/V_B)^2+(U(V_C)/V_C)^2+(U(V_D)/V_D)^2\}^{1/2}$

U(t) = 0.049235662, this value was multiplied by the coverage factor of 2.78 for infinite degrees of freedom.

95% CL of x_0: $x_0 \pm \{t4_{;0.05} * U(t)\}$

$x_0 \pm \{2.78 * U(t)\}$

= $x_0 \pm 0.14$ ng/ℓ, therefore the result of the accuracy of 17-α-ethinylestradiol can be expressed as 0.48± 0.14 ng/ℓ.

Similar holistic approach was used when performing statistical evaluation and uncertainty of measurement for estrone, 17α-Estradiol and 17 β-Estradiol, the final calculations are shown in annexure 1.

A QC sample of 0.50 ng/ℓ was prepared by the quantitatively transferring 50 µℓ of 0.01 ng/µℓ into 1000 mℓ reagent water using a calibrated micro-syringe. This was extracted in the SPE together with standards and samples under identical instrumental conditions.

In Table 12, the percentage RSDs ranged from 9.3 to 11.4% indicating good method precision, the accuracy of the method was in the region of 93.8 to 100.9% indicating good performance achieved by the UFLC-MS-MS. The uncertainty of measurements was determined using the holistic approach of analytical method validation, it ranged between ±0.14 and ±0.15 ng/ℓ.

Table 12: Method performance parameters obtained from statistical evaluation

Analyte	**Precision** (% RSD)	**Accuracy** (%)	**Uncertainty +/-** (ng/ℓ)	**LOD** (ng/ℓ)	**LOQ** (ng/ℓ)
E1	9.3	100.9	0.15	0.07	0.30
E2(α)	9.6	93.8	0.14	0.09	0.30
E2(β)	11.4	97.6	0.15	0.10	0.32
EE2	10.7	95.6	0.14	0.07	0.24

nd – is applicable to analytes not detected.

Table 12 also gives a summary for the evaluation of LOD and LOQ in quantitative chemical analysis, based on the approach taken in the ISO "Guide to the Expression of Uncertainty in Measurement" (Ellison et al,.2011). Uncertainty of measurements was applied to at all levels of analytical results, detailed calculations were performed in Microsoft excel spread sheet as expressed in Annexure 1.

The recovery of spiked samples was not determined since calibration standards and analytical samples were extracted in the similar manner. Analysis was performed under similar SPE and optimised instrumental conditions. Instead of using recovery to measure accuracy, the quality control sample, internally prepared in the laboratory, analysed independently over a period of three non-consecutive days was used to determine the accuracy of the method.

5.3 Optimisation of the analytical method

Following the understanding of the effects of pKa and Log K_{ow} in relation to their ionic prevalence (Huerta-Fontela et al., 2011), each 1 mg/ℓ pure hormone standard at a pH of 10.2 was infused into the mass spectrometer to optimise fragmentation parameters of ions. Various parameters of the mass spectrometer were explored, negative mode produced optimum conditions as a result it produced intense signals that were used for quantification, See Annexure 3.

Two transition ions were selected; the more intense fragmentation ion was used for quantification of analytes and less intense one for confirmation. It was therefore equally important to have transition ions that are stable and intense. The mass spectrometer was optimised by investigating the change in collision energy versus the production of the product ion (MRM 1 and 2) formed after each parent ion was fragmented. There was a direct correlation between the collision energy variation's influence on the precursor ion and the production of product ions.

The co-elution of peaks existed when the mixture of hormones was analysed. The co-elution of isomers 17β-Estradiol (β-E2) and 17α -Estradiol (α-E2) was due to similar chemical properties. Analytical gradient conditions were further optimised to achieve the separation of two peaks at 4.12 and 4.31 minutes (Figure 7). The co elution of E1 and EE2 at 4.53 minutes was insignificant since their product ions are different. Details of the separation for the four compounds are shown along with their fragmentation ions (Refer to Figure 26 for the detailed transition profiles of parent and product ions).

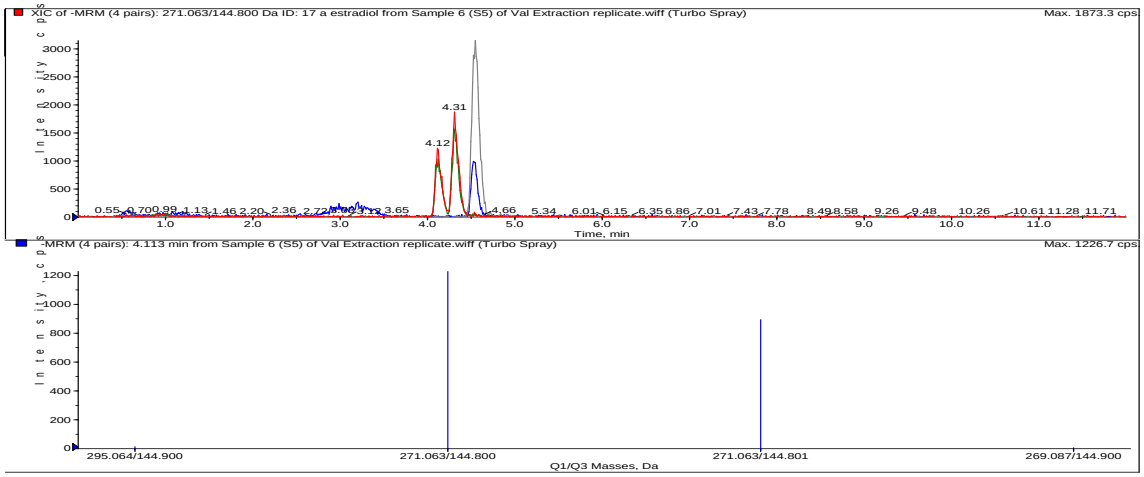


Figure 8: Retention Time 4.12, 17-α- estradiol

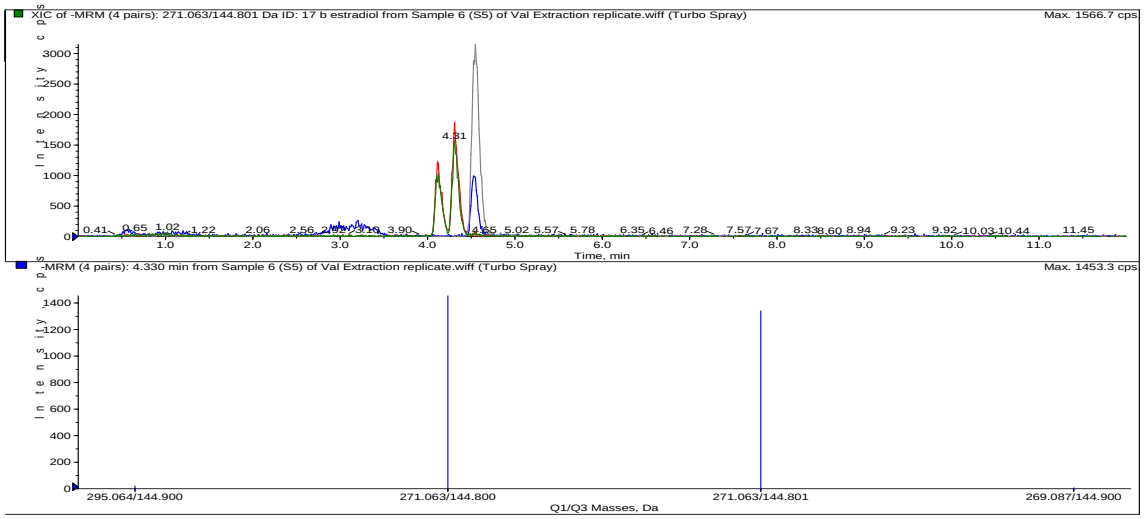


Figure 9: Retention Time 4.31, 17-β- estradiol

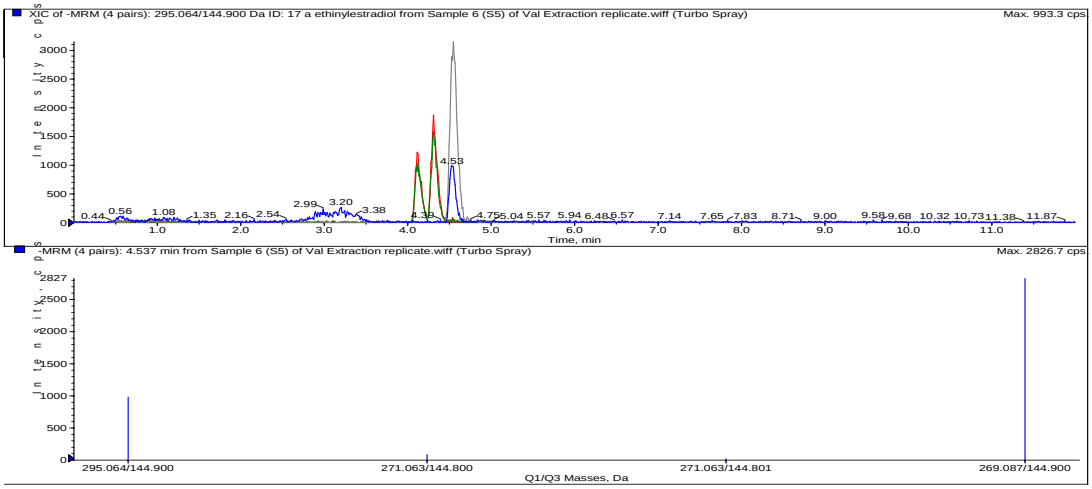


Figure 10: Retention Time 4.53, 17-α-ethinylestradiol

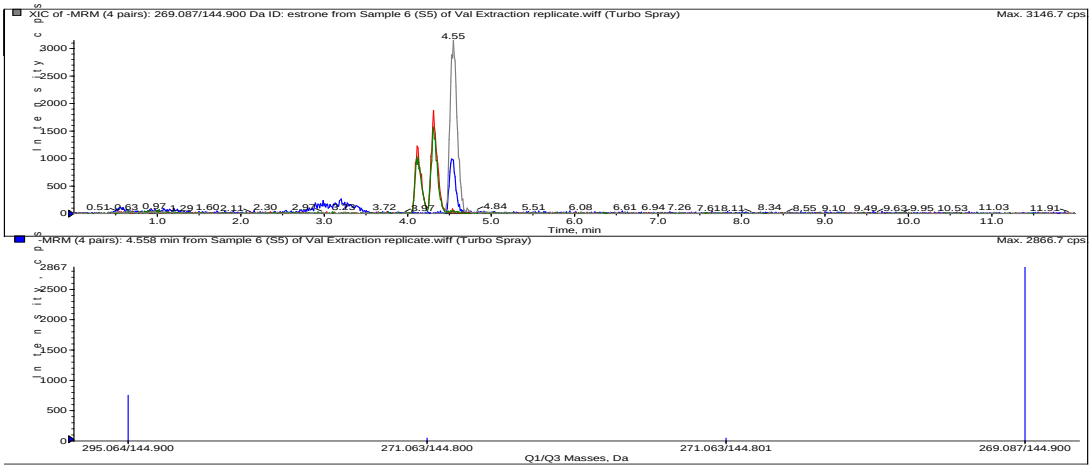


Figure 11: Retention Time 4.55, Estrone

5.4 Analysis of hormones in environmental water samples

To the best of our knowledge, this is the first time that hormones are investigated in Vaal Rivers' catchments, raw and treated waters in South Africa. This is also one of few studies of these compounds in South African aquatic ecosystem.

Regarding water analysis, earlier studies have shown that these compounds are only partially eliminated during conventional (coagulation, sand filtration) water treatment (Huerta-Fontela et al., 2011).

There is little information about the occurrence of hormones in ground waters and drinking waters for public health issues. Hohenblum et al. (2004) detected 17β-Estradiol in the range 0.1 to 0.8 ng/ℓ in Austrian groundwater. Various estrogenic compounds were detected in ground and surface waters water of France within the range of 0.1 to 1 ng/ℓ even though the fate and impact of these compounds are not clearly known on public health (Vulliet and Cren-Olivé, 2011).

After the method development, here we further conducted a study between 2013 and 2014 periods where samples were taken in summer (wet season) as well as in winter (dry season); a total combined number of 28 samples were taken for endocrine disrupting compound determination in both seasons. The bi-annual assessment results for the wet and dry seasons of raw water samples are presented in Table 7.

No estrogen hormone was detected in any of the eight drinking water samples in the south of Johannesburg region during both seasons with an exception of one sample at a concentration of 2.88 ng/ℓ estrone. According to a study conducted by Dévier et al. (2013) these four estrogenic hormones were also not detected in Evian and Volvic (bottled natural mineral waters), the presence of this hormone in treated water however came as a surprise and has not yet been established.

However, estriol has been detected at the drinking water treatment plant of Abrera in Spain at a concentration of 11.60 ng/ℓ (Kuster et al., 2008).

Table 13: Summary of results for the raw water samples where the hormones of interest were detected

Parameter	Unit of measure	Raw water samples			
		Sample B		Sample E	
		Wet season	Dry season	Wet season	Dry season
E1	ng/ℓ	0.90	nd	4.43	nd
E2(α)	ng/ℓ	nd	nd	nd	nd
E2(β)	ng/ℓ	nd	nd	nd	nd
EE2	ng/ℓ	nd	nd	nd	nd

nd – is applicable to analytes not detected.

The endocrine disruptor estrone was detected during the wet season in Sample B and Sample E which are raw water samples at a concentration of 0.90 and 4.43 ng/ℓ respectively (Table 13). According to a study performed by Pool et al. (2012) estrone levels above 5 ng/ℓ can adversely affect reproduction of aquatic animals.

The availability of estrone in water bodies did not come as a surprise because it is also the main metabolite of 17β-Estradiol (a natural estrogen) and reaches the environment via the sewer system or animal excretion (Wenzel et al., 2003).

Table 14: Summary of availability of hormones in South Africa compares to other countries surface waters

Country	α -E2 (ng/ℓ)	β-E2 (ng/ℓ)	EE2 (ng/ℓ)	E1 (ng/ℓ)	Reference
Netherlands	0.3 – 3.0	0.3 – 5.5	0.1 – 4.3	0.1 – 3.4	Belfroid et al.,1999
China	0 – 1.8	-	0 – 2.7	0.5 – 3.1	Jiang et al.,2011
German	-	-	-	0 – 1.6	Ternes et al.,1999
South Korea	-	-	-	1.8 – 5.0	Kim et al.,2007
South Africa	-	-	-	0.9 - 4.4	This Study

A study conducted in the Netherlands also shows that generally, concentrations of hormones in surface water range between 1 and 5 ng/ℓ with estrone being the most frequent observed estrogen hormone.

During the Vaal River catchment study the concentration level of estrone was detected in the range of 0.90 to 4.43 ng/ℓ which is not significantly different from the result of 1 to 5 ng/ℓ obtained in the Netherlands and in other countries (Table 14). Guang-Guo Ying et al. (2002) reported that estrogens in humans and animals undergo various degradation mechanisms taking place mainly in the liver. They are frequently oxidized, hydroxylated, deoxylated and methylated prior to the final conjugation with glucuronic acid or sulphate. E2 is rapidly oxidized to estrone, which can be further converted into estriol, the major excretion product which has not been determined in this study. A study performed on Canadian and German waste water samples detected estrone at a maximum concentration level of 70 ng/ℓ. However, a concentration of 1.6 ng/ℓ estrone was detected in the surface and stream water samples in Germany (Ternes et al., 1999). This can support the rapid degradation of estrone into estriol (Guang-Guo Ying et al. 2002).

CHAPTER SIX – CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

A sensitive analytical method has been developed and validated to identify and quantify four hormones in raw and treated drinking water samples with the accuracy ranging between 93.8 – 100.9% and the limit of quantification in the range of 0.24 - 0.32 ng/ℓ for all analytes.

The study reveals that in South Africa the occurrence of hormones in raw water is variable depending on the particular season, the absence of hormones in drinking water reveals that current levels of hormones are removed during conventional water treatment processes.

Based on method statistical analysis the high accuracy, good precision, limits of detection, limits of quantification and overall good performance in terms of consistency in results obtained by SPE and UFLC interfaced to a MS-MS, Consequently the evidence demonstrates that the developed method is suitable for determination of estrogen hormones in source and drinking water samples.

6.2 Recommendations

It is recommended that the method for wastewater effluents be developed and validated to determine the availability of these compounds in water treatment plants of South Africa. The application of the developed methods to other waste, raw water and drinking water sources in the country is required in order to establish a high priority list of estrogens to be monitored and regulated. This will be followed by necessary studies for the removal of these contaminants if they are available in large quantities in South African water streams.

The second recommendation is to perform qualitative analysis of other classes of emerging contaminants such as PPCPs. In determining these contaminants, the inside out approach should be used, whereby screening can be done in all source water points and the list compiled with the identified contaminants. This priority list should then be compared with the international data available in the literature. The criteria for monitoring should be triggered by relevance, toxicological effects, national interest (SA) and legislation in order to develop a nationwide monitoring framework for endocrine disrupting compounds.

CHAPTER SEVEN– PRESENTATIONS AND PUBLICATIONS

7.1 Conference Presentations

SB Mnguni, C Schoeman, SS Marais, E Cukrowska, L Chimuka Determination of Estrogen Hormones in Raw and Treated Water Samples by Reverse Phase Ultra-Fast Liquid Chromatography Mass Spectrometry. SACI Convention 2015. 29th – 04th December 2015. Elangeni Hotel, Durban (South Africa)

S. Mnguni, L. Chimuka. Determination of estrogen hormones in raw and treated water samples by reverse phase ultra-fast liquid chromatography mass spectrometry. Chromsa Postgraduate Seminar 2015. 11th August 2015. University of the Witwatersrand, Johannesburg (South Africa)

S. Mnguni, L. Chimuka, C. Schoeman. Determination of Endocrine Disrupting Compounds such as hormones in environmental samples by HPLC-MS. NLA Test and Measurement Conference. 20th October 2013. Misty Hills Conference Hotel, Muldersdrift (South Africa)
http://www.nla.org.za/conferences/proceedings_archive/2013/Presentations/Tuesday,%208%20October/T107%20Determination%20of%20endocrine%20disruptive%20compounds%20such%20as%20hormones%20in%20environmental%20water%20samples%20by%20HPLC-MS.pdf

http://www.nla.org.za/conferences/proceedings_archive/2013/Manuscripts/Tuesday,%208%20October/T107%20Determination%20of%20Endocrine%20Disrupting%20Compounds%20such%20as%20Hormones%20in%20Environmental%20Water%20Samples%20by%20HPLC-M.pdf

7.2 Publications emanating from this project

SB Mnguni[1*], C Schoeman[1], SS Marais[1], E Cukrowska[2], L Chimuka[2] Determination of Estrogen Hormones in Raw and Treated Water Samples by Reverse Phase Ultra-Fast Liquid Chromatography Mass Spectrometry – a Case Study in Johannesburg South, South Africa. (Submitted to Water SA), Review number - Water SA 3251

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ANNEXTURES

Annexure 1: Statistical analysis and uncertainty of measurements calculations

Table 15 : Analysis of Variance of 17-a-ethinylestradiol

17 a ethinylestradiol

1. RANGE OF STANDARDS

0-1.0 ng/ℓ

2. CALIBRATION DATA

CONCENTRATION (ng/ℓ)	RESPONSE RATIO
0.0	0
0.2	979
0.4	2060
0.6	3320
0.8	4100
1.0	5210

3. REGRESSION ANALYSIS

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.998806003
R Square	0.997613431
Adjusted R Square	0.997016789
Standard Error	107.1946827
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	19212984.7	19212984.7	1672.046498	2.13759E-06
Residual	4	45962.8	11490.7		
Total	5	19258947.5			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	-8	77.58181549	-0.103116948	0.922833133	-223.4016519	207.40165
X Variable 1	5239	128.1221515	40.89066518	2.13759E-06	4883.275879	5594.7241

4.1 Slope (b) **5239.00000000**

4.2 Y-intercept (a) **-8.00000000**

4.3 Linearity **F_{calc} = 1672**

F_{calc} large , therefore signifigant linearity

5. REGRESSION UNCERTAINTIES

5.1 Random Uncertainty ($S_{y/x}$) **107.19468270**

5.2 Slope Uncertainty (S_b) **128.12215154**

5.3 Y-intercept Uncertainty (S_a) **77.58181549**

6. LIMIT OF DETECTION

$Y_{LOD}=Y_B+3S_B=bX_{LOD}+a$

Assume $Y_B=a$

Thus, $X_{LOD}=3S_b/b$,ng/ℓ

0.073 ng/ℓ

6. LIMIT OF QUANTITATION

$Y_{LOQ}=Y_B+10S_B=bX_{LOQ}+a$

Assume $Y_B=a$

Thus, $X_{LOQ}=10S_b/b$,ng/ℓ

0.245 ng/ℓ

7. ACCURACY

A spiked quality sample, concentration of **0.5 mg/L**

DETERMINATION	CONCENTRATION	PEAK HEIGHT
#1	0.43	2240.0
#2	0.50	2590.0
#3	0.57	2970.0
#4	0.52	2690.0
#5	0.47	2460.0
#6	0.44	2280.0
#7	0.49	2520.0
#8	0.41	2110.0
Mean	0.48	2482.5
Std Deviation	0.051	275.875

7.1 RSD of concentration, (sx100/mean), %

10.73

7.2 Mean Method Accuracy (% of true conc.), %

95.6

7.3 Method Bias (deviation from true value), %

-4.4

8. UNCERTAINTY

8.1 Uncertainty of Regression

$y_0=bx_0+a$

For x_0= 0.4779

y_0= 2495.587125

ave.y = 2482.50000000

y_0-ave.y = 13.0871

$(y_0\text{-ave.y})(y_0\text{-ave.y})$ 171.2728408

x_i	x_i-ave.x	$(x_i\text{-ave.x})*(xi\text{-ave.x})$
0.0000	-0.500	0.25
0.2000	-0.300	0.09
0.4000	-0.100	0.01
0.6000	0.100	0.01
0.8000	0.300	0.09
1.0000	0.500	0.25

Sum = 0.7

Sum^2= 0.49

$Sx_0= S_{y/x}/b\{1/m+1/n+(y_0\text{-ave.y})^2/b^2 sum(x_i\text{-ave.x})^2\}^{1/2}$

m = determinations = 1

n = calibration points =6

Sx_o= 0.022100424

8.2 Uncertainty of Repeatability

Std Dev.y/ave.y= 0.111127988

8.3 Uncertainty of Purity

Purity=100%+-0.5%

Assume rectangular distribution

U(P)=0.5/$3^{1/2}$= 0.29

8.4 Uncertainty of Volume

8.4.1 10mℓ volumetric flask, S.N 0134

10mℓ +-0.04mℓ , 20DC

Assume rectangular distribution

$U(V_1)=0.04/3^{1/2}$= 0.023094011

Lab temperature= 20DC+- 4

Coefficient of volume expansion= 0.00021

Assume rectangular distribution

$U(V_2)$= Volume variation= (Volume*temp. var.*coefficient of volume expansion)/$3^{1/2}$ 0.004849742

Volumetric Flask Uncertainty= $\{U(V_1)^2+U(V_2)^2\}^{1/2}$

$U(V_A)/V_A$= 0.002359774 mℓ

8.4.2 100ul syringe

100ul+-1ul, 20DC

Assume rectangular distribution

$U(V_1)=1/3^{1/2}$= 0.577350269

Lab temperature= 20DC+- 4

Coefficient of volume expansion= 0.00149

Assume rectangular distribution

$U(V_2)$= Volume variation= (Volume*temp. var.*coefficient of volume expansion)/$3^{1/2}$ 0.34410076

100ul Syringe Uncertainty= $\{U(V_1)^2+U(V_2)^2\}^{1/2}$

$U(V_B)/V_B$= 0.006721151 ul

8.4.3 500ul syringe

500ul+5ul, 20DC

Assume rectangular distribution

$U(V_1)=5/3^{1/2}$= 2.886751346

Lab temperature= 20DC+- 4

Coefficient of volume expansion= 0.00149

Assume rectangular distribution

$U(V_2)$= Volume variation= (Volume*temp. var.*coefficient of volume expansion)/$3^{1/2}$ 1.720503802

500ul Syringe Uncertainty= $\{U(V_1)^2+U(V_2)^2\}^{1/2}$

$U(V_C)/V_C$= 0.003360575 ul

8.5 Uncertainty of Balance

$U(V_D)/V_D$= **0.002** g

8.6 Total Uncertainty, at x_0= **0.5**

$U(t)/x_0 = \{(Sx_0/x_0)^2+(SR/S)^2+(U(P)/P)^2+(U(V_A)/V_A)^2+(U(V_B)/V_B)^2+(U(V_C)/V_C)^2+(U(VD)/VD)^2\}^{1/2}$

U(t)= **0.049235662** **ng/ℓ**

95% CL of x_0: **x_0+/-{$t4_{,0.05}$*U(t)}**

x_0+/-{2.78*U(t)}

0.5 **ng/ℓ** **+/-** **0.1** **ng/ℓ**

Table 16: Analysis of Variance of 17-a-ethinylestradiol

17 a estradiol

1. RANGE OF STANDARDS

0-1.0 ng/ℓ

2. CALIBRATION DATA

CONCENTRATION (ng/ℓ)	RESPONSE RATIO
0.0	0
0.2	1640
0.4	3220
0.6	5180
0.8	7190
1.0	8400

3. REGRESSION ANALYSIS

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.9982507 58
R Square	0.9965045 76
Adjusted R Square	0.9956307 2
Standard Error	214.52383 59
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	52479601.43	52479601.43	1140.353 29	4.58709E-06
Residual	4	184081.90 48	46020.476 19		
Total	5	52663683.33			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	-57.619047 62	155.26095 36	0.3711109 99	0.729374 349	488.69256 23	373.454 47	488.6925 62	373.4544 671
X Variable 1	8658.5714 29	256.40502 61	33.769117 4	4.58709E -06	7946.6769 49	9370.46 59	7946.676 95	9370.465 908

K	6
sum(x)	3.0000
sum(x)2	2.2
SS(X)	0.7
sumY	25630
	16214650
sum(y)2	0
	52663683
SS(Y)	.33
sumXY	18876
SP(XY)	6061
	8658.571
B (SLOPE)	429

4.1 Slope (b) **8658.57142857**

4.2 Y-intercept (a) **-57.61904762**

4.3 Linearity **F_{calc} = 1140**

F_{calc} large , therefore signifigant linearity

5. REGRESSION UNCERTAINTIES

5.1 Random Uncertainty ($S_{y/x}$) **214.52383595**

5.2 Slope Uncertainty (S_b) **256.40502611**

5.3 Y-intercept Uncertainty (S_a) **155.26095362**

6. LIMIT OF DETECTION

$Y_{LOD}=Y_B+3S_B=bX_{LOD}+a$

Assume $Y_B=a$

Thus, $X_{LOD}=3S_b/b$,ng/ℓ

0.089 ng/ℓ

6. LIMIT OF QUANTITATION

$Y_{LOQ}=Y_B+10S_B=bX_{LOQ}+a$

Assume $Y_B=a$

Thus, $X_{LOQ}=10S_b/b$,ng/ℓ

0.296 ng/ℓ

7. ACCURACY

A spiked quality sample, concentration of **0.5 mg/L**

DETERMINATION	CONCENTRATION	PEAK HEIGHT
#1	0.5	3880.0
#2	0.4	3800.0
#3	0.5	4640.0
#4	0.5	3920.0
#5	0.5	4140.0
#6	0.4	3330.0
#7	0.5	4330.0
#8	0.4	3770.0
Mean	0.5	3976.3
Std Deviation	0.045	395.725

7.1 RSD of concentration, (sx100/mean), %

9.55

7.2 Mean Method Accuracy (% of true conc.), %

93.8

7.3 Method Bias (deviation from true value), %

-6.2

8. UNCERTAINTY

8.1 Uncertainty of Regression

$y_0=bx_0+a$

For x_0= 0.4690

y_0= 4003.250952

ave.y = 3976.25000000

y_0-ave.y = 27.0010

$(y_0\text{-ave.y})(y_0\text{-ave.y})$ 729.0514295

x_i	x_i-ave.x	$(x_i\text{-ave.x})*(xi\text{-ave.x})$
0.0000	-0.500	0.25
0.2000	-0.300	0.09
0.4000	-0.100	0.01
0.6000	0.100	0.01
0.8000	0.300	0.09
1.0000	0.500	0.25

Sum = 0.7

Sum^2= 0.49

Sx_0= $S_{y/x}/b\{1/m+1/n+(y_0\text{-ave.y})^2/b^2 sum(x_i\text{-ave.x})^2\}^{1/2}$

m = determinations = 1

n = calibration points =6

Sx_0= 0.02676125

8.2 Uncertainty of Repeatability

Std Dev.y/ave.y= 0.099522143

8.3 Uncertainty of Purity

Purity=100%+-0.5%

Assume rectangular distribution

$U(P)=0.5/3^{1/2}$= 0.29

8.4 Uncertainty of Volume

8.4.1 10mℓ volumetric flask, S.N

0134

10mℓ +-0.04mℓ , 20DC

Assume rectangular distribution

$U(V_1)=0.04/3^{1/2}$

= 0.023094011

Lab temperature= 20DC+- 4

Coefficient of volume expansion= 0.00021

Assume rectangular distribution

$U(V_2)$= Volume variation= 0.004849742 (Volume*temp. var.*coefficient of volume expansion)/$3^{1/2}$

Volumetric Flask Uncertainty= $\{U(V_1)^2+U(V_2)^2\}^{1/2}$

$U(V_A)/V_A$= 0.002359774 mℓ

8.4.2 100ul syringe

100ul+-1ul,

20DC

Assume rectangular distribution

$U(V_1)=1/3^{1/2}$= 0.577350269

Lab temperature= 20DC+- 4

Coefficient of volume expansion= 0.00149

Assume rectangular distribution

$U(V_2)$= Volume variation= 0.34410076 (Volume*temp. var.*coefficient of volume expansion)/$3^{1/2}$

100ul Syringe Uncertainty= $\{U(V_1)^2+U(V_2)^2\}^{1/2}$

$U(V_B)/V_B$= 0.006721151 ul

8.4.3 500ul syringe

500ul+-5ul,

20DC

Assume rectangular distribution

$U(V_1)=5/3^{1/2}$= 2.886751346

Lab temperature= 20DC+- 4

Coefficient of volume expansion= 0.00149

Assume rectangular distribution

$U(V_2)$= Volume variation= 1.720503802 (Volume*temp. var.*coefficient of volume expansion)/$3^{1/2}$

500ul Syringe Uncertainty= $\{U(V_1)^2+U(V_2)^2\}^{1/2}$

$U(V_C)/V_C$= 0.003360575 ul

8.5 Uncertainty of Balance

$U(V_D)/V_D$= **0.002** g

8.6 Total Uncertainty, at x_0= **0.5**

$U(t)/x_0$= $\{(Sx_0/x_0)^2+(SR/S)^2+(U(P)/P)^2+(U(V_A)/V_A)^2+(U(V_B)/V_B)^2+(U(V_C)/V_C)^2+(U(VD)/VD)^2\}^{1/2}$

U(t)= **0.051194144** **ng/ℓ**

95% CL of x_0: **x_0+/-{$t4_{;0.05}$*U(t)}**

x_0+/-{2.78*U(t)}

0.5 **ng/ℓ** **+/-** **0.1** **ng/ℓ**

Table 17: Analysis of Variance of 17-b-estradiol

17 b estradiol

1. RANGE OF STANDARDS

0-1.0 ng/ℓ

2. CALIBRATION DATA

CONCENTRATION (ng/ℓ)	RESPONSE RATIO
0.0	33
0.2	1290
0.4	3260
0.6	4360
0.8	5770
1.0	7410

3. REGRESSION ANALYSIS

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.997978582
R Square	0.995961225
Adjusted R Square	0.994951563
Standard Error	195.7011236
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	37778274.29	37778274.29	986.4054825	6.12507E-06
Residual	4	153195.71	38298.92		
Total	5	37931470.01			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	14.00476191	141.6380745	0.098877099	0.925992834	-379.2455769	407.2551007	-379.2455769	407.2551007
X Variable 1	7346.357143	233.9075818	31.40709287	6.12507E-06	6696.925582	7995.788703	6696.925582	7995.788703

K	6
sum(x)	3.0000
sum(x)2	2.2
SS(X)	0.7
sumY	22123
	11950339
sum(y)2	5.6
	37931470
SS(Y)	.01
sumXY	16204
SP(XY)	5142.45
	7346.357
B (SLOPE)	143

4.1 Slope (b) **7346.35714286**

4.2 Y-intercept (a) **14.00476190**

4.3 Linearity F_{calc} = **986**

F_{calc} **large , therefore signifigant linearity**

5. REGRESSION UNCERTAINTIES

5.1 Random Uncertainty ($S_{y/x}$) **195.70112356**

5.2 Slope Uncertainty (S_b) **233.90758176**

5.3 Y-intercept Uncertainty (S_a) **141.63807455**

6. LIMIT OF DETECTION

$Y_{LOD}=Y_B+3S_B=bX_{LOD}+a$

Assume $Y_B=a$

Thus, $X_{LOD}=3S_b/b$,ng/ℓ

0.096 ng/ℓ

6. LIMIT OF QUANTITATION

$Y_{LOQ}=Y_B+10S_B=bX_{LOQ}+a$

Assume $Y_B=a$

Thus, $X_{LOQ}=10S_b/b$,ng/ℓ

0.318 ng/ℓ

7. ACCURACY

A spiked quality sample, concentration of **0.5 mg/L**

DETERMINATION	CONCENTRATION	PEAK HEIGHT
#1	0.5	3500.0
#2	0.5	3750.0
#3	0.6	4320.0
#4	0.5	3360.0
#5	0.5	3870.0
#6	0.4	3170.0
#7	0.5	3620.0
#8	0.4	2990.0
Mean	0.5	3572.5
Std Deviation	0.056	419.651

7.1 RSD of concentration, (sx100/mean), %

11.39

7.2 Mean Method Accuracy (% of true conc.), %

97.6

7.3 Method Bias (deviation from true value), %

-2.4

8. UNCERTAINTY

8.1 Uncertainty of Regression

$y_0=bx_0+a$

For x_0= 0.4879

y_0= 3598.108753

ave.y = 3572.50000000

y_0-ave.y = 25.6088

(y_0-ave.y)(y_0-ave.y) 655.808229

x_i	x_i-ave.x	(x_i-ave.x)*(xi-ave.x)
0.0000	-0.500	0.25
0.2000	-0.300	0.09
0.4000	-0.100	0.01
0.6000	0.100	0.01
0.8000	0.300	0.09
1.0000	0.500	0.25

Sum = 0.7

Sum^2= 0.49

Sx_0= $S_{y/x}/b\{1/m+1/n+(y_0\text{-ave.y})^2/b^2 sum(x_i\text{-ave.x})^2\}^{1/2}$

m = determinations = 1

n = calibration points =6

Sx_0= 0.028773937

8.2 Uncertainty of Repeatability

Std Dev.y/ave.y= 0.1174671

8.3 Uncertainty of Purity

Purity=100%+-0.5%

Assume rectangular distrobution

U(P)=0.5/$3^{1/2}$= 0.29

8.4 Uncertainty of Volume

8.4.1 10mℓ volumetric flask, S.N 0134

10mℓ +-0.04mℓ , 20DC

Assume rectangular distrobution

$U(V_1)=0.04/3^{1/2}$

= 0.023094011

Lab

temperature= 20DC+- 4

Coefficient of volume expansion= 0.00021

Assume rectangular distrobution

Volume (Volume*temp. var.*coefficient of volume

$U(V_2)=$ variation= expansion)/$3^{1/2}$

0.004849742

Volumetric Flask Uncertainty= $\{U(V_1)^2+U(V_2)^2\}^{1/2}$

$U(V_A)/V_A=$ 0.002359774 mℓ

8.4.2 100ul syringe

100ul+1ul,

20DC

Assume rectangular distrobution

$U(V_1)=1/3^{1/2}=$ 0.577350269

Lab

temperature= 20DC+- 4

Coefficient of volume expansion= 0.00149

Assume rectangular distrobution

Volume (Volume*temp. var.*coefficient of volume

$U(V_2)=$ variation= expansion)/$3^{1/2}$

0.34410076

100ul Syringe Uncertainty= $\{U(V_1)^2+U(V_2)^2\}^{1/2}$

$U(V_B)/V_B=$ 0.006721151 ul

8.4.3 500ul syringe

500ul+-5ul,

20DC

Assume rectangular distrobution

$U(V_1)=5/3^{1/2}=$ 2.886751346

Lab

temperature= 20DC+- 4

Coefficient of volume expansion= 0.00149

Assume rectangular distrobution

Volume (Volume*temp. var.*coefficient of volume

$U(V_2)=$ variation= expansion)/$3^{1/2}$

1.720503802

500ul Syringe Uncertainty= $\{U(V_1)^2+U(V_2)^2\}^{1/2}$

$U(V_C)/V_C=$ 0.003360575 ul

8.5 Uncertainty of Balance

$U(V_D)/V_D=$ 0.002 g

8.6 Total Uncertainty, at x_0= **0.5**

$U(t)/x_0 = \{(Sx_0/x_0)^2+(SR/S)^2+(U(P)/P)^2+(U(V_A)/V_A)^2+(U(V_B)/V_B)^2+(U(V_C)/V_C)^2+(U(VD)/VD)^2\}^{1/2}$

$U(t)$= **0.054138708** **ng/ℓ**

x_0+/-

95% CL of x_0: **$\{t4_{;0.05}*U(t)\}$**

x_0+/-{2.78*U(t)}

0.5 **ng/ℓ** **+/-** **0.2** **ng/ℓ**

Table 18: Analysis of Variance of estrone

estrone

1. RANGE OF STANDARDS

0-1.0 ng/ℓ

2. CALIBRATION DATA

CONCENTRATION (ng/ℓ)	RESPONSE RATIO
0.0	36
0.2	3240
0.4	6640
0.6	11000
0.8	14200
1.0	18500

3. REGRESSION ANALYSIS

SUMMARY OUTPUT

Regression Statistics	
	0.9988159
Multiple R	6
	0.9976333
R Square	23
Adjusted R	0.9970416
Square	53
Standard	377.11688
Error	22
Observations	6

ANOVA

K	6
sum(x)	3.0000
sum(x)2	2.2
SS(X)	0.7
sumY	53616
	71947849
sum(y)2	6

	df	SS	MS	F	Significance F
Regression	1	239797051.4	239797051.4	1686.133237	2.10209E-06
Residual	4	568868.5714	142217.1429		
Total	5	240365920			

SS(Y)	240365920
sumXY	39764
SP(XY)	12956
B (SLOPE)	18508.57143

	Coefficients	Standard Error	t Stat	P-value	Lower 95%	Upper 95%	Lower 95.0%	Upper 95.0%
Intercept	-318.2857143	272.937161	-1.166150161	0.308342689	-1076.080759	439.50933	-1076.08076	439.5093305
X Variable 1	18508.57143	450.7408867	41.06255273	2.10209E-06	17257.1141	19760.029	17257.1141	19760.02876

4.1 Slope (b) **18508.57142857**

4.2 Y-intercept (a) **-318.28571429**

4.3 Linearity **F_{calc} = 1686**

F_{calc} large , therefore signifigant linearity

5. REGRESSION UNCERTAINTIES

5.1 Random Uncertainty ($S_{y/x}$) **377.11688222**

5.2 Slope Uncertainty (S_b) **450.74088670**

5.3 Y-intercept Uncertainty (S_a) **272.93716104**

6. LIMIT OF DETECTION

$Y_{LOD}=Y_B+3S_B=bX_{LOD}+a$

Assume $Y_B=a$

Thus, $X_{LOD}=3S_b/b$,ng/ℓ

0.073 ng/ℓ

6. LIMIT OF QUANTITATION

$Y_{LOQ}=Y_B+10S_B=bX_{LOQ}+a$

Assume $Y_B=a$

Thus, $X_{LOQ}=10S_b/b$,ng/ℓ

0.244 ng/ℓ

7. ACCURACY

A spiked quality sample, concentration of **0.5 mg/L**

DETERMINATION	CONCENTRATION	PEAK HEIGHT
#1	0.5	8640.0
#2	0.5	9270.0
#3	0.6	10300.0
#4	0.5	8710.0
#5	0.5	9300.0
#6	0.4	7160.0
#7	0.5	9270.0
#8	0.5	8630.0
Mean	0.5	8910.0
Std Deviation	0.047	894.204

7.1 RSD of concentration, (sx100/mean), %

9.33

7.2 Mean Method Accuracy (% of true conc.), %

100.9

7.3 Method Bias (deviation from true value), %

0.9

8. UNCERTAINTY

8.1 Uncertainty of Regression

$y_0=bx_0+a$

For x_0= 0.5044

y_0= 9016.975

ave.y = 8910.00000000

y_0-ave.y = 106.9750

(y_0-ave.y)(y_0-ave.y) 11443.65062

x_i	x_i-ave.x	(x_i-ave.x)*(xi-ave.x)
0.0000	-0.500	0.25
0.2000	-0.300	0.09
0.4000	-0.100	0.01
0.6000	0.100	0.01
0.8000	0.300	0.09
1.0000	0.500	0.25

Sum = 0.7

Sum^2= 0.49

Sx_0= $S_{y/x}/b\{1/m+1/n+(y_0\text{-ave.y})^2/b^2 sum(x_i\text{-ave.x})^2\}^{1/2}$

m = determinations = 1

n = calibration points =6

Sx_0= 0.022008435

8.2 Uncertainty of Repeatability

Std Dev.y/ave.y= 0.100359546

8.3 Uncertainty of Purity

Purity=100%+-0.5%

Assume rectangular distrobution

U(P)=0.5/$3^{1/2}$= 0.29

8.4 Uncertainty of Volume

8.4.1 10mℓ volumetric flask, S.N 0134

10mℓ +-0.04mℓ , 20DC

Assume rectangular distrobution

$U(V_1)=0.04/3^{1/2}$

= 0.023094011

Lab temperature= 20DC+- 4

Coefficient of volume expansion= 0.00021

Assume rectangular distrobution

$U(V_2)$= Volume (Volume*temp. var.*coefficient of volume

variation= expansion)/$3^{1/2}$

0.004849742

Volumetric Flask Uncertainty= $\{U(V_1)^2+U(V_2)^2\}^{1/2}$

$U(V_A)/V_A$= 0.002359774 mℓ

8.4.2 100ul syringe

100ul+1ul,

20DC

Assume rectangular distrobution

$U(V_1)=1/3^{1/2}$= 0.577350269

Lab temperature= 20DC+- 4

Coefficient of volume expansion= 0.00149

Assume rectangular distrobution

$U(V_2)$= Volume variation= 0.34410076 (Volume*temp. var.*coefficient of volume expansion)/$3^{1/2}$

100ul Syringe Uncertainty= $\{U(V_1)^2+U(V_2)^2\}^{1/2}$

$U(V_B)/V_B$= 0.006721151 ul

8.4.3 500ul syringe

500ul+5ul,

20DC

Assume rectangular distrobution

$U(V_1)=5/3^{1/2}$= 2.886751346

Lab temperature= 20DC+- 4

Coefficient of volume expansion= 0.00149

Assume rectangular distrobution

$U(V_2)$= Volume variation= 1.720503802 (Volume*temp. var.*coefficient of volume expansion)/$3^{1/2}$

500ul Syringe Uncertainty= $\{U(V_1)^2+U(V_2)^2\}^{1/2}$

$U(V_C)/V_C$= 0.003360575 ul

8.5 Uncertainty of Balance

$U(V_D)/V_D$= 0.002 g

8.6 Total Uncertainty, at x_0= 0.5

$U(t)/x_0$= $\{(Sx_0/x_0)^2+(SR/S)^2+(U(P)/P)^2+(U(V_A)/V_A)^2+(U(V_B)/V_B)^2+(U(V_C)/V_C)^2+(U(VD)/VD)^2\}^{1/2}$

U(t)= **0.053789473** **ng/ℓ**

95% CL of x_0: x_0+/-

$\{t4_{;0.05}*U(t)\}$

x_0+/-{2.78*U(t)}

0.5 ng/ℓ +/- 0.1 ng/ℓ

Table 19: The table of calibration standards vs Area counts

		17 a ethinylestradiol	17 a estradiol	17 b estradiol	estrone
Area	**S0**	0.00	0.00	33.10	36.00
	S1	979.00	1640.00	1290.00	3240.00
	S2	2060.00	3220.00	3260.00	6640.00
	S3	3320.00	5180.00	4360.00	11000.00
	S4	4100.00	7190.00	5770.00	14200.00
	S5	5210.00	8400.00	7410.00	18500.00
Concentration (ng/ℓ)	**S0**	0.00	0.00	0.00	0.00
	S1	0.20	0.20	0.20	0.20
	S2	0.40	0.40	0.40	0.40
	S3	0.60	0.60	0.60	0.60
	S4	0.80	0.80	0.80	0.80
	S5	1.00	1.00	1.00	1.00

Table 20: The table of precision and accuracy of quality control standards

		17 a ethinylestradiol			17 a estradiol			17 b estradiol			estrone		
Expected Conc. (ng/ℓ)		0.20	0.50	1.00	0.20	0.50	1.00	0.20	0.50	1.00	0.20	0.50	1.00
Observed Std Conc. (ng/ℓ)	#1	0.199	0.522	0.990	0.30	0.47	0.96	0.20	0.50	0.89	0.26	0.46	0.88
	#2	0.25	0.53	0.91	0.25	0.47	0.88	0.29	0.43	0.86	0.22	0.46	0.89
	#3	0.18	0.41	0.87	0.31	0.47	0.89	0.25	0.52	0.91	0.09	0.46	0.90
	#4	0.21	0.40	0.93	0.25	0.50	0.90	0.19	0.37	0.94	0.21	0.46	0.90
	#5	0.25	0.46	0.93	0.22	0.47	0.87	0.20	0.47	0.88	0.23	0.46	0.95
	#6	0.21	0.46	0.83	0.25	0.44	0.91	0.18	0.50	0.91	0.13	0.48	0.94
	#7	0.21	0.49	0.94	0.35	0.47	0.90	0.15	0.43	0.83	0.17	0.50	0.96
	#8	0.12	0.42	0.81	0.24	0.46	0.84	0.17		0.83	0.21	0.46	0.93
	Average	0.20	0.46	0.90	0.27	0.47	0.89	0.20	0.46	0.88	0.19	0.47	0.92
	Std. Dev.	0.04	0.05	0.06	0.04	0.02	0.03	0.04	0.05	0.04	0.06	0.01	0.03
	%RSD	20.3	10.7	6.7	15.4	3.5	3.8	21.8	11.1	4.5	30.2	3.0	3.1
	% Accuracy	102.4	92.3	90.1	135.3	93.8	89.4	101.8	92.1	88.0	94.5	93.6	91.8

Table 21: Determination of robustness of quality control sample over a period of three days

	17 a ethinylestradiol			17 a estradiol			17 b estradiol			estrone		
Day	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
#1	0.43	0.52	0.47	0.46	0.47	0.38	0.48	0.50	0.63	0.49	0.46	0.41
#2	0.50	0.53	0.50	0.45	0.47	0.61	0.51	0.43	0.69	0.52	0.46	0.43
#3	0.57	0.41	0.39	0.54	0.47	0.23	0.59	0.52	0.55	0.58	0.46	0.50
#4	0.52	0.40	0.38	0.46	0.50	0.42	0.46	0.37	0.59	0.49	0.46	0.44
#5	0.47	0.46	0.41	0.49	0.47	0.53	0.53	0.47	0.58	0.53	0.46	0.47
#6	0.44	0.46	0.49	0.40	0.44	0.34	0.44	0.50	0.57	0.41	0.48	0.49
#7	0.49	0.49	0.29	0.51	0.47	0.35	0.49	0.43	0.50	0.52	0.50	0.47
#8	0.41	0.42	0.41	0.45	0.46	0.22	0.41		0.34	0.49	0.46	0.46
Average	0.48	0.46	0.42	0.47	0.47	0.38	0.49	0.46	0.56	0.50	0.47	0.46
Nominal	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
n	8	8	8	8	8	8	8	7	8	8	8	8
Std. Dev.	0.0513	0.049	0.067	0.045	0.016	0.135	0.056	0.051	0.104	0.047	0.014	0.030
s/√n	0.0181	0.017	0.024	0.016	0.006	0.048	0.020	0.019	0.037	0.017	0.005	0.011
t_{calc}	-1.22	-2.22	-3.51	-1.96	-5.34	-2.42	-0.62	-2.05	1.55	0.26	-6.48	-3.92
t_{crit}	2.36	2.36	2.36	2.36	2.36	2.36	2.36	2.45	2.36	2.36	2.36	2.36
Significant difference	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO

Table 22: Summary of basic figures of merit

COMPOUND	Range, (ng/ℓ)	F Factor	Limit Of Detection, (ng/ℓ)	Limit Of Quantitation, (ng/ℓ)
17 a ethinylestradiol	0-1.00	1672	0.07	0.24
17 a estradiol	0-1.00	1140	0.09	0.30
17 b estradiol	0-1.00	986	0.10	0.32
estrone	0-1.00	1686	0.07	0.24

Table 23: Summary of basic method performance parameters

COMPOUND	Precision, (%)	Accuracy, (%)	Bias, (%)
17 a ethinylestradiol	10.7	95.6	-4.4
17 a Estradiol	9.6	93.8	-6.2
17 b Estradiol	11.4	97.6	-2.4
Estrone	9.3	100.9	0.9

Table 24: Summary of measurement uncertainty for each analyte

COMPOUND	Uncertainty	
	Concentration (ng/ℓ)	Uncertainty (ng/ℓ), +/-
17 a ethinylestradiol	0.48	0.14
17 a estradiol	0.47	0.14
17 b estradiol	0.49	0.15
estrone	0.50	0.15

Annexure 2: Optimum conditions of measured parameters

Table 25: Multiple Reaction Monitoring ions for hormones (Optimised conditions)

Analyte	Precursor ion	Product ion	Units
Estrone	269.087	144.900	Da
		183.100	Da
Ethinylestradiol	295.064	144.100	Da
		183.100	Da
Estradiol	271.063	144.800	Da
		182.800	Da

Annexure 3: Infusion and tuning conditions of the spectrometer

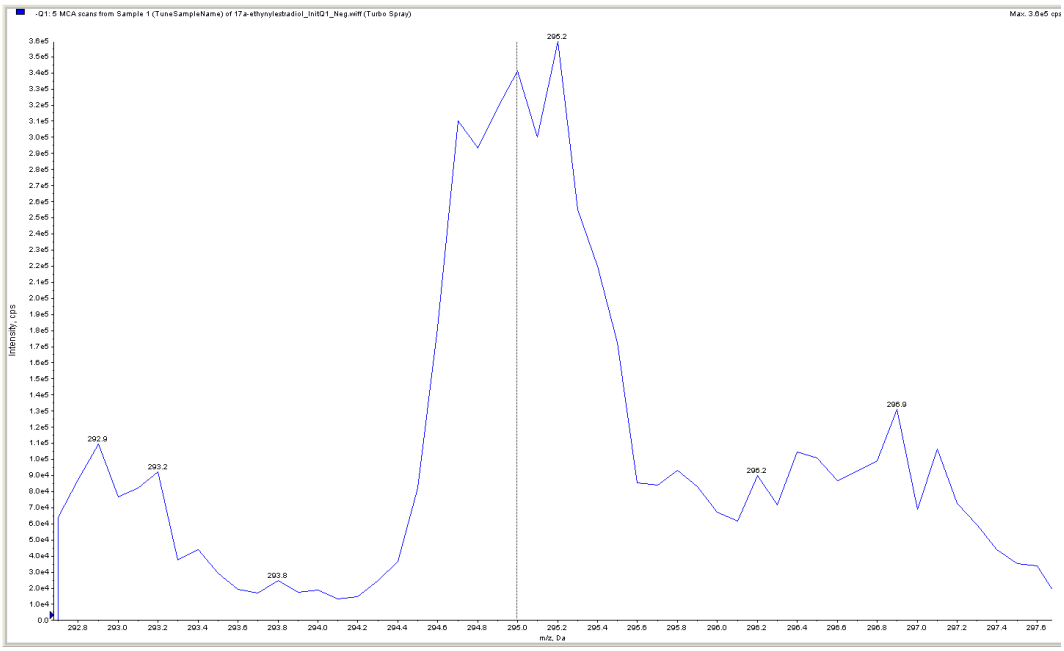

Figure 12: EE2, initial Q1

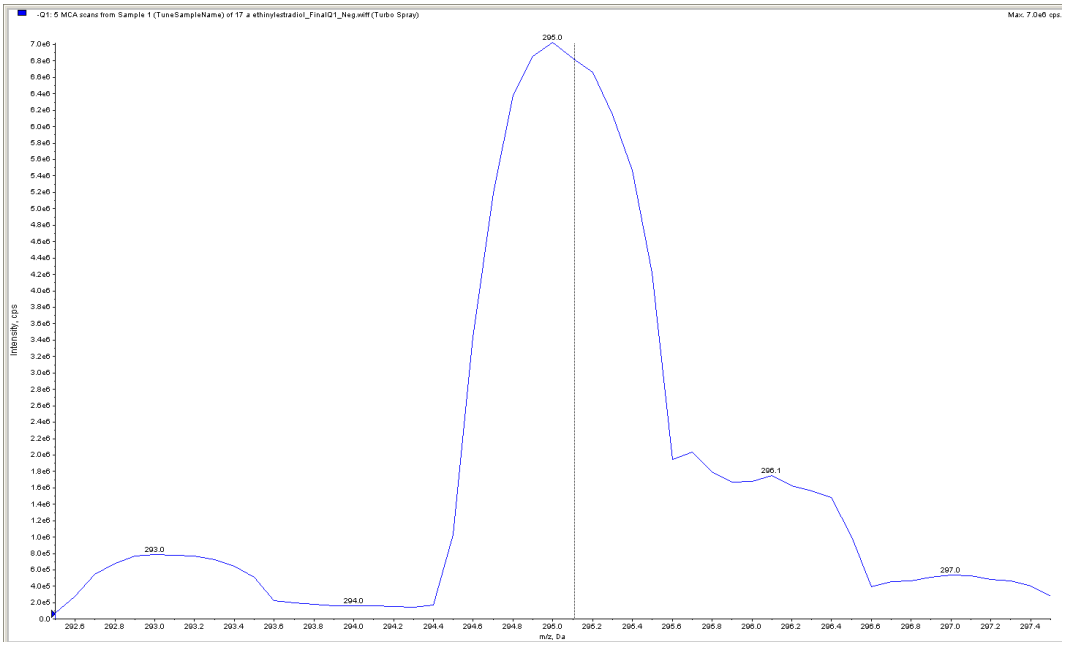

Figure 13: EE2, Final Q1

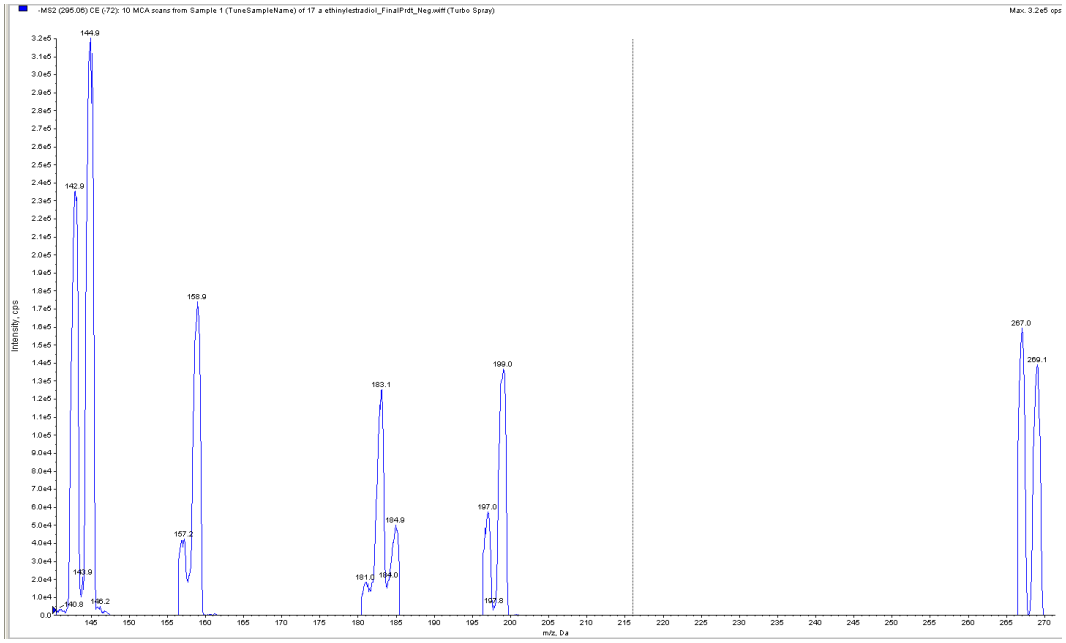

Figure 14: EE2, Final Product ion.

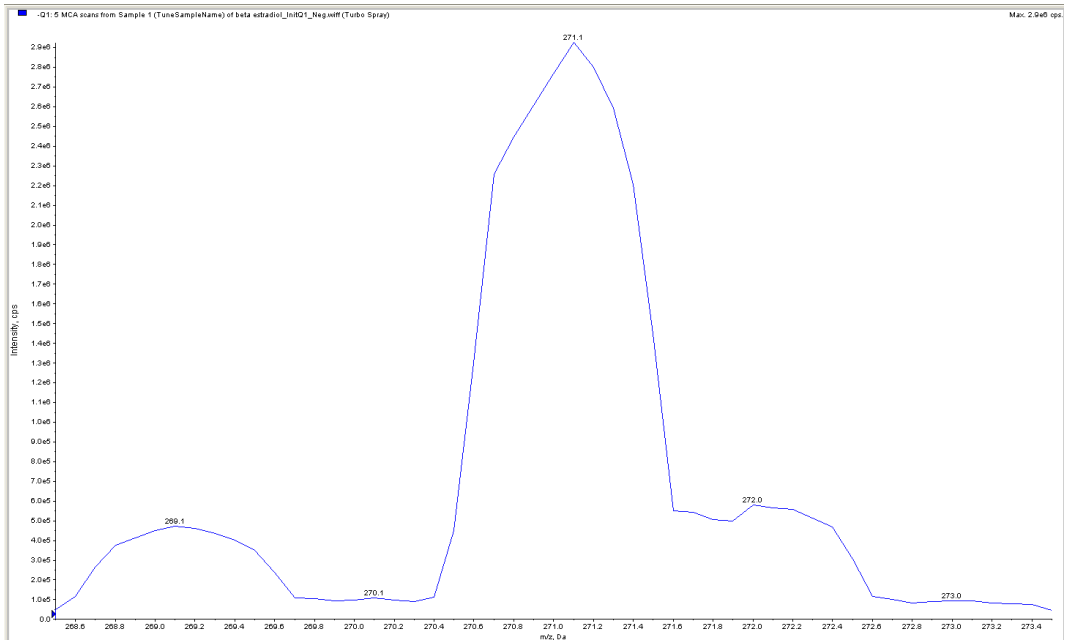

Figure 15: E2, Initial Q1

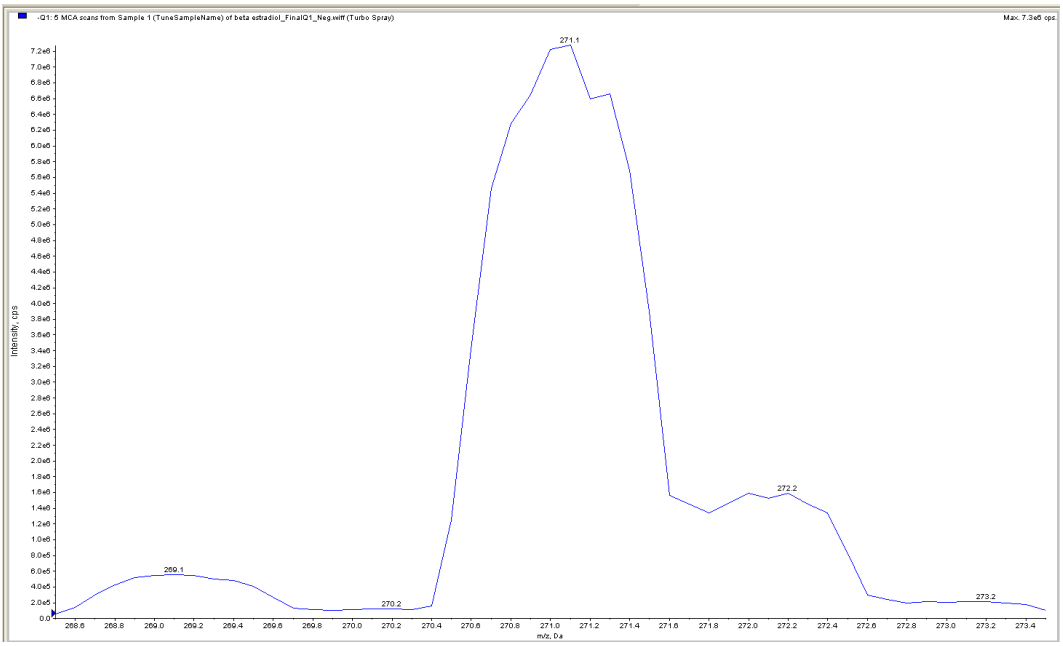

Figure 16: E2, Final Q1

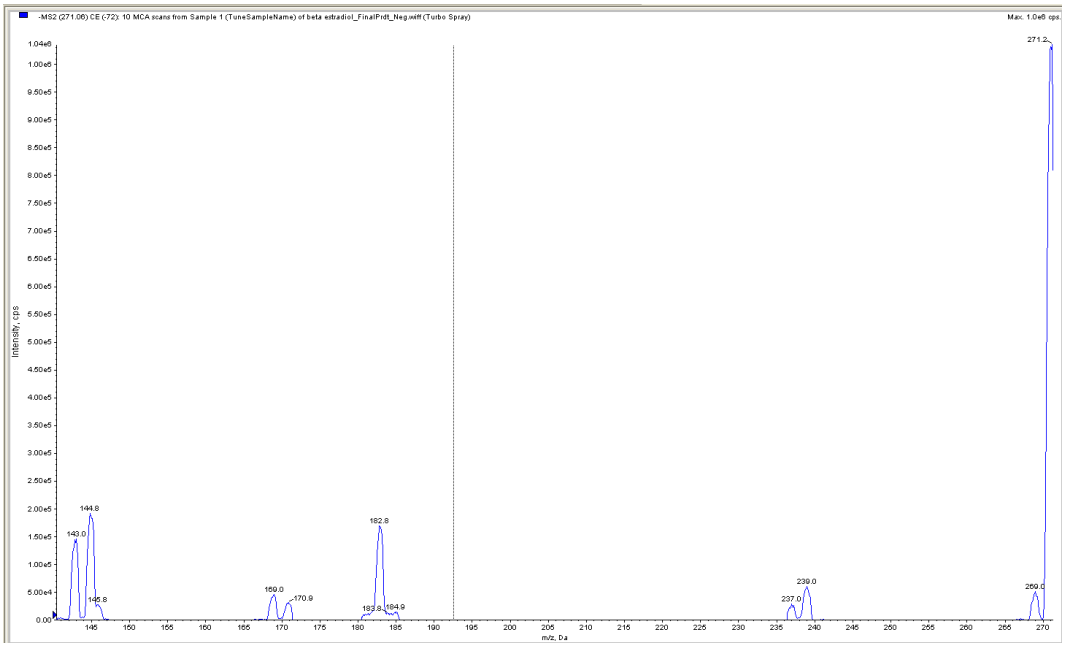

Figure 16: E2, Final product ion

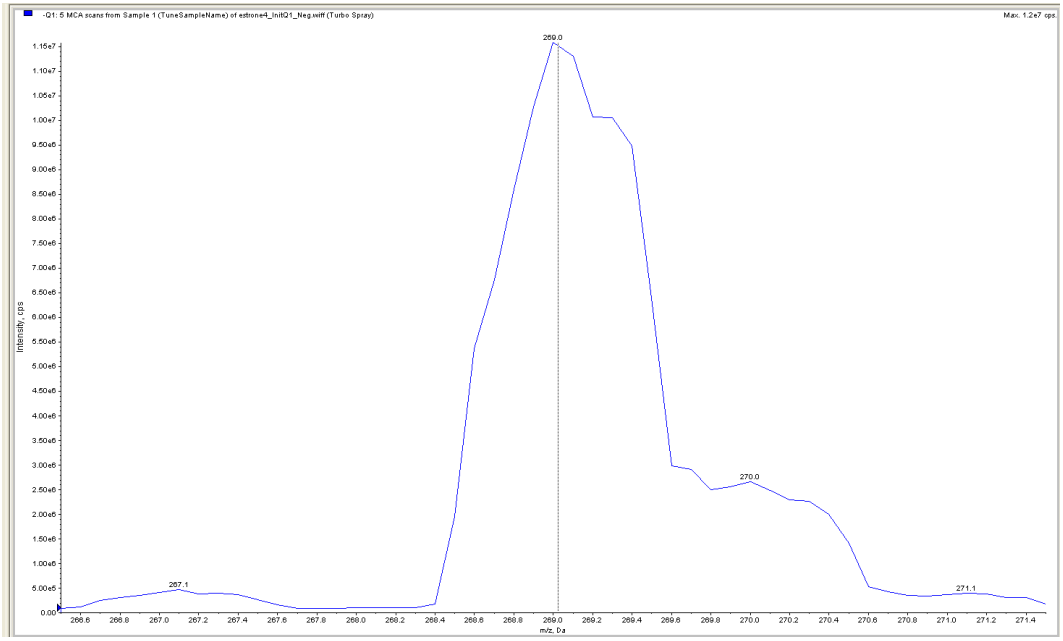

Figure 17: E1, Initial Q1

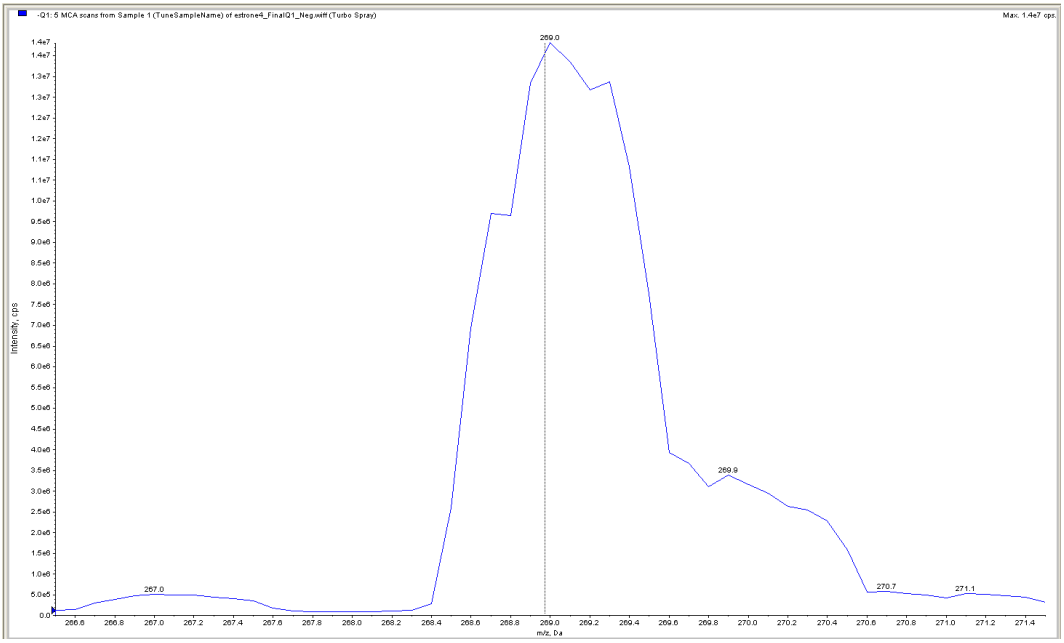

Figure 18: E1, Final Q1

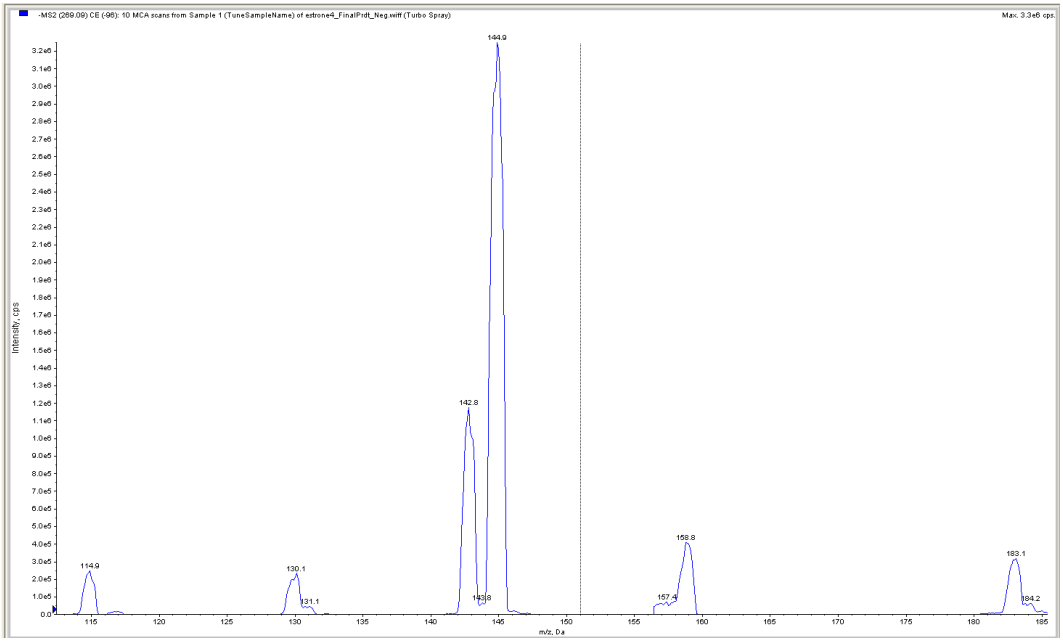

Figure 19: E1, Final Product ion

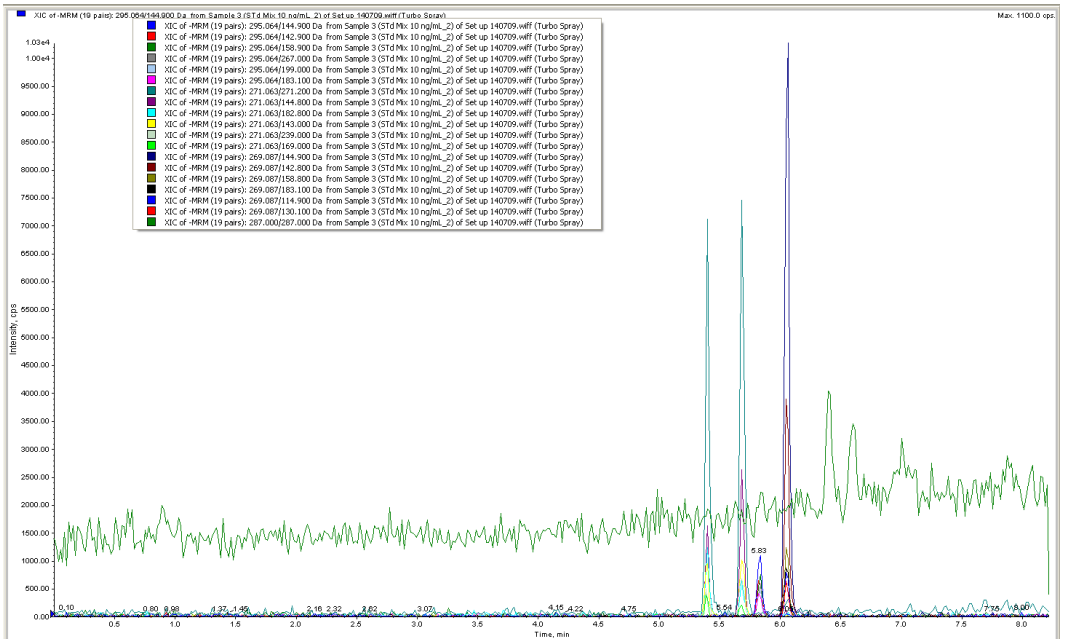

Figure 20: Infused mixture of hormones (10ng/mℓ)

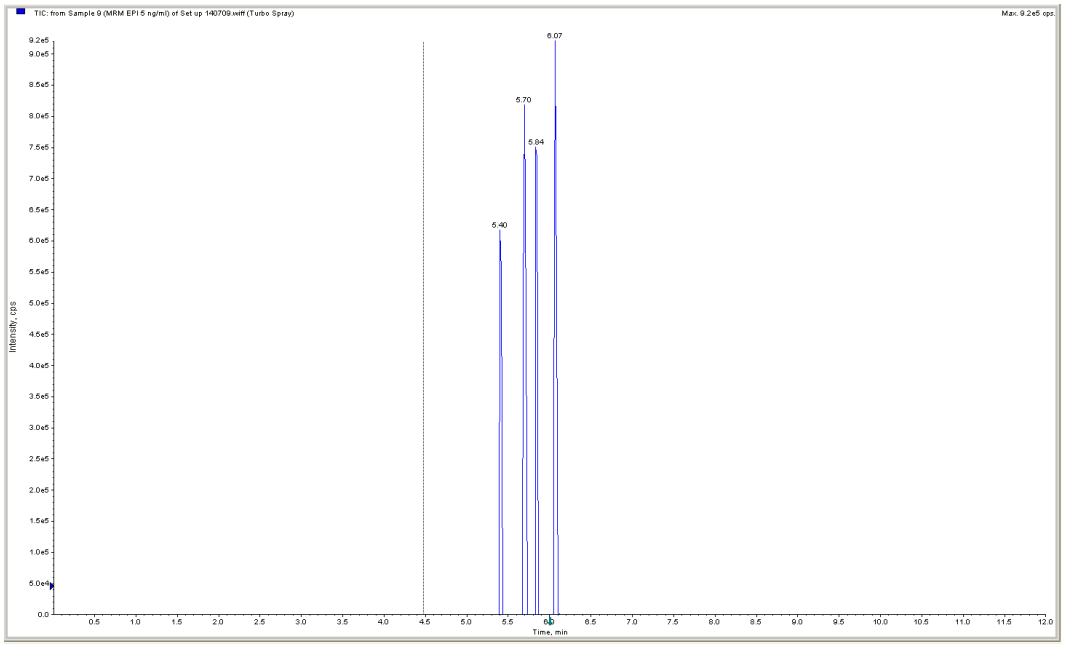

Figure 21: MRM EPI for 15 ng/mℓ

Annexure 4: Chromatograms of hormones

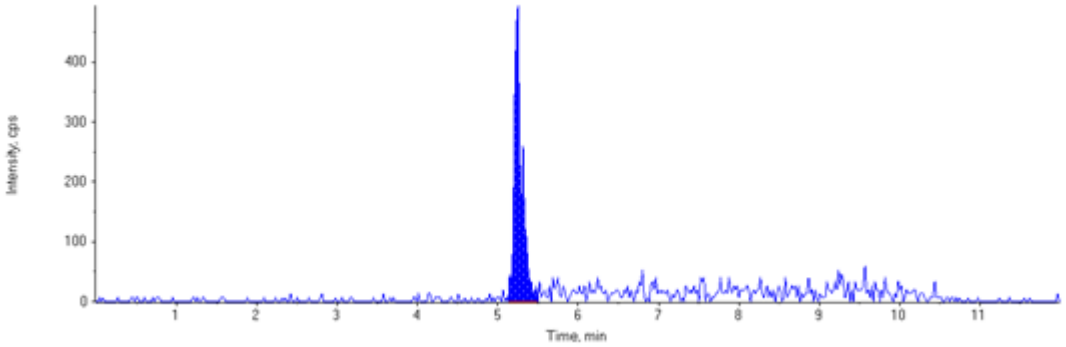

Figure 22: Quantitation ion of estrone (269.087/144.900 Da)

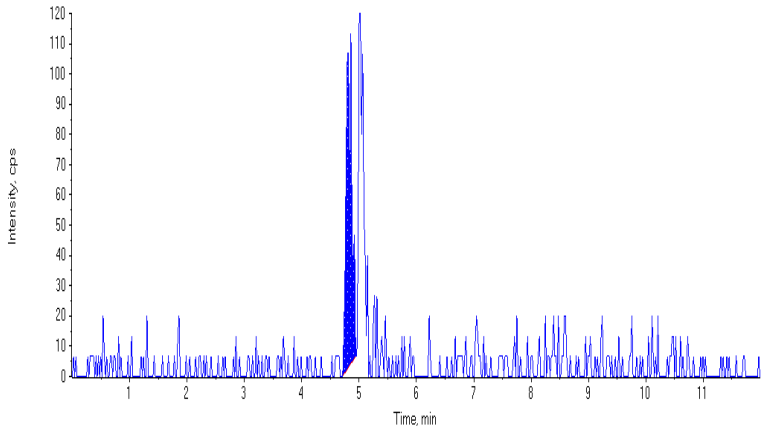

Figure 23: Quantitation ion 17 α estradiol (271.063/144.800 Da)

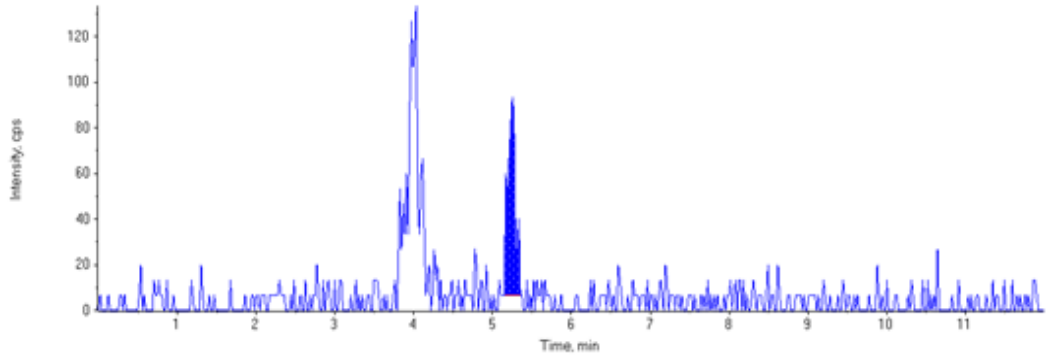

Figure 24: Quantitation ion 17 α ethinylestradiol (295.064/144.900 Da)

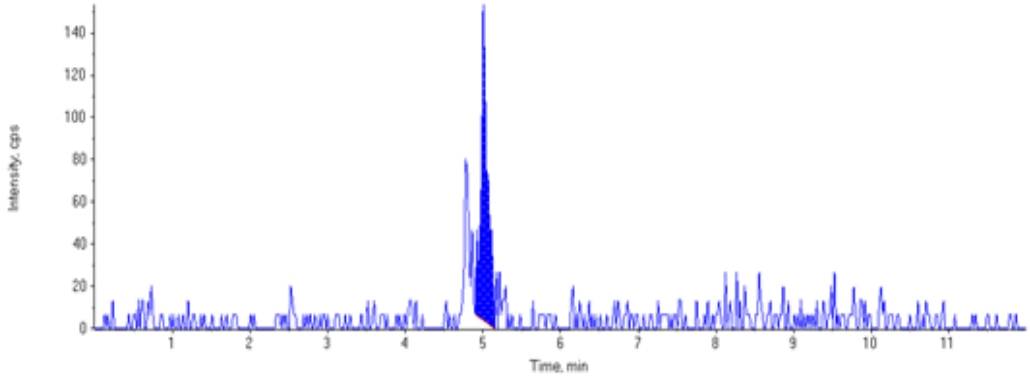

Figure 25: Quantitation ion 17 β estradiol (271.063/144.801 Da)

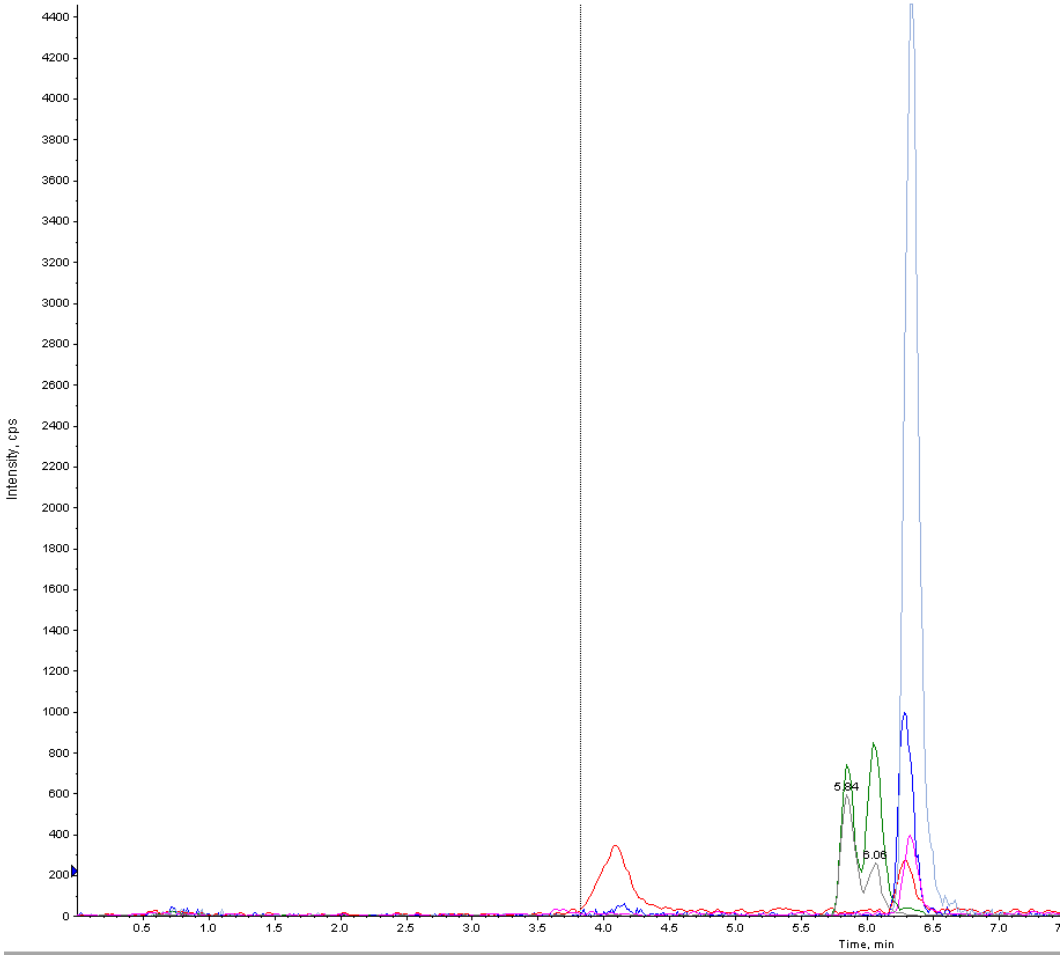

Figure 26: Parent and daughter ions of estrogens

Top: Green – parent ion, E2 (271.063/144.8), Bottom: Gray – product ion, E2 (271.063/182.8), Top: Blue – parent ion, EE2 (295.064/144.9), Bottom: Red – product ion, EE2 (295.064/183.1), Top: Powder blue – parent ion, E1 (269.087/144.9), and Bottom: Pink – product ion, E1 (2693087/183.1).