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Investigation of Androgen Receptor Antagonist Compounds Present in Influent and Effluent from a Wastewater Works

By

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A Thesis Submitted for the Award of Doctor of Philosophy Degree of the University of Sussex

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Submitted October 2011

Declaration

I hereby declare that this thesis has not been and will not be, submitted in whole or in part to another University for the award of any other degree.

Signature :……(Francis O. Oladapo)

University of Sussex

Francis Olumide Oladapo

A Thesis Submitted for the Award of Doctor of Philosophy Degree

Investigation of Androgen Receptor Antagonist Compounds Present in Influent and Effluent of a Wastewater Works.

Abstract

A wide range of synthetic chemicals and their metabolites present in the environment can antagonise the receptor activity of androgen hormones present in wildlife and humans. With increasing global production of new synthetic chemicals, little is known about their environmental fate, health consequences and end-points. This study was conducted to identify and characterise chemicals with anti-androgenic activity present in wastewater influent and effluent. This study was undertaken by applying a combination of biological and analytical chemistry techniques involving Solid Phase Extraction (SPE), High Performance Liquid Chromatography (HPLC) and an in vitro steroid receptor assay for profiling and characterising extracts of grab influent and effluent wastewater samples using a toxicity identification and evaluation (TIE) procedure.

Initial work revealed variable recoveries of anti-androgenic activity from SPE of wastewaters. Therefore SPE methodology to screen wastewater samples was developed using a mixture of selected compounds which possess a range of polarities (log Kow). Their recoveries from SPE were measured by HPLC protocol and ranged from 95-100%. The mean±SD and % RSD values of the analysed wastewater replicates were 3.20±0.03 mgFeq/L and 0.78% for influent and 0.22±0.01 mgFeq/L and 3.80% for effluent samples. The recoveries of wastewater extracts after fractionation were between 78.6% and 99.6%. Fractions containing anti-androgenic activity were analysed by Gas Chromatography Mass Spectrometry (GC-MS). A number of household chemicals were detected in both influent and effluent wastewater fractions that contained anti-androgenic activity. These included the anti-bacterial agents- triclosan, chlorophene, dichlorophene, chloroxylenol, the musk fragrance galaxolide, the flame retardants-tris(1-chloro-2-propyl)phosphate (TCPP) and tris(2-butoxyethyl)phosphate (TBEP), polymer plasticizer n-butylbenzenesulfonamide (NBBSA) and bisphenol A (BPA) which is a chemical associated with the polycarbonate usage. The anti-androgenic po-

tency of pure contaminants compared with that of flutamide ranged from 0.04 (TCPP) to 13.40 (chlorophene). Anti-androgenic activity of 1.69 and 2.00% was recovered from the fractions of the effluent and influent samples respectively indicating that AA of about 98% are yet to be recovered.

This work reveals for the first time that over 12 contaminants contribute to the total anti-androgenic activity present in wastewater effluent and that a number of compounds commonly used in household products (such as chlorophene, triclosan and NBBSA) are predominant anti-androgens in wastewater effluents.

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List of Abbreviations

AA	Anti-androgenic activity
(Anti-) androgenic	Anti-androgenic and androgenic
AR	Androgen receptor
ARE	Androgen response element
AYAS	Yeast anti-androgen screen
BPA	Bisphenol A
BSTFA	N,O-bis(trimethyl-silyl)trifluoroacetamide
cAMP	Cyclic adenosine monophosphate
CPA	Cyproterone acetate
CPRG	Chlorophenol red β-D-galactopyranoside
DAG	diacylglycerol
DBP	Dibutylphthalate
DCP	Dichlorophene
DDE	1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene
DDT	1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane
DEHP	Diethylhexylphthalate
DHEA	dehydroepiandrosterone
DNA	Deoxyribonucleic acid
DHT	5α-dihydrotestosterone
E1-d_4	Deuterated estrone
EC_{50}	Effective concentration of the test compound which will generate 50% maxima of the sigma curve.

Feq	Flutamide equivalents
FSH	Follicle stimulating hormone(s)
GC	Gas chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
hAR	Human androgen receptor
HLB	Hydrophilic-lipophilic balance
HPLC	High performance liquid chromatography
HPV	High production volume
HRE	Hormone receptor elements
IP_3	Inositol triophosphate
K_{ow}	Octanol-water partition coefficient
11-KT	11-Ketotestosterone
LH	Luteinising hormone(s)
LOD	Limit of detection
LOQ	Limit of quantification
M	Moles per litre
mgFeq	Milligram in flutamide equivalents
mg/L	Milligram per litre
min	minute(s)
µgFeq	Microgram in flutamide equivalents
µg/L	Microgram per litre
MOA(s)	Modes of action
MRSA	Methicillin-resistant *Staphylococcus aureus*

NBBSA	N-butylbenzenesulfonamide
ngFeq	Nanogram in flutamide equivalent
ng/L	Nanogram per litre
ND	Not determined
NIST	National Institute of Standards and Technology
NQ	Not quantified
PAHs	Poly aromatic hydrocarbons
PCBs	Polychlorinated biphenyls
%RSD	Percentage relative standard deviation
RSD	Relative standard deviation
SD	Standard deviation
SHBG	Sex hormone-binding globulin
SIM	Selected ion monitoring
SPE	Solid phase extraction
T_2	Testosterone
TAA	Total anti-androgenic activity
TBEP	Tris(2-butoxyethyl)phosphate
TBT	Tributyltin
TCEP	Tris(2-chloroethyl)phosphate
TCPP	Tris(1-chloro-2-propyl)phosphate
TDCP	Tris-(2-chloro-, 1-chloromethyl-ethyl)phosphate
TDS	Testicular dysgenesis syndrome
T*i*BP	Tris-*iso*-butyl phosphate

TIC	Total ion chromatogram
TIE	Toxicity identification and evaluation
TMS	Trimethysilyl
T*n*BP	Tris n-butyl phosphate
TPT	Triphenyltin
WwTP	Wastewater treatment works

Glossary

Androgen	Sex hormones produced by the endocrine system and other organs in the body of male and female animals.
Anti- androgen	A substrate that blocks the action of androgens
Agonistic	Chemical activities at a receptor that produces the same or similar effect as the natural messenger.
Antagonistic	Action of a chemical at a receptor site that opposes the stimulatory effect of natural hormone or other agonist.
Cryptorchidism	The non-existence of one or both testes in the scrotal sac.
Cytotoxicity	The cellular exposure to harmful compounds which lead to serious damage to the cell and death as the endpoint.
Down-regulation	A situation where gene expression is influenced after steroid receptor binding activation by an endocrine disrupting agent.
Estrogen	Is a female hormone mainly secreted in the ovaries (gonads) and the placenta but is also produced in small quantity in the other specialised tissues and cells in the body such as the male testis and the adrenal cortex.
Feminisation	Formation of female reproductive features (e.g female egg-yolks) in male vertebrates.
Heterodimer	A protein molecule formed from paired unrelated polypeptides especially in the sequence of the amino acids.
Homodimer	A protein molecule formed from paired identical polypeptides
Hypospadias	Is a birth defect where the urethra tract opening expected to occur at the tip of the penis becomes situated anywhere on the penis.
Intersexuality	Occurrence of male and female genitalia together in a gonochoristic animal.

Lipophilicity	The ability of a compound or substance to dissolve in fatty, oily, lipid substance(s) and non-polar solvents.
Masculinisation	Formation of male phenotype or genitalia in female vertebrates.
Monomers	Are smallest representative unit of a polymer
Up-regulation	A steroid receptor binding activation by an endocrine disrupting agent which enhances positive gene expression.
Xenobiotics	The classes of foreign or exogenous compounds in an intact body.
Xenoandrogen	An exogenous androgenic compound in the body
Xenoestrogen	An exogenous estrogenic compound in the body
Xenoanti-androgen	An exogenous anti-androgenic compound in the body

Literature Review

1.0 Background

Chemical communication is the fundamental process by which all living organisms exchange vital information within their internal systemic components, with other organisms and with their immediate environment (Cheek et al., 1998; Fox, 2004). This process is basically accomplished through three major communication mechanisms: endocrine, immune and nervous systems. Although their modes of action are dissimilar, the endocrine and immune communication systems are co-ordinated through the blood (Pardridge, 1981). The presence of some man-made chemicals and their by-products in the global environment can pose a serious risk to public health when they interfere with any of these communication mechanisms (Arcand-Hoy and Benson, 2001). Given that the endocrine communication system is responsible for the regulation of metabolic processes, homeostasis, reproduction and basic developmental mechanisms (Kime, 1998), it is thus possible for some chemicals that possess hormone-like behaviours to disrupt its normal regulatory activities. Within the progressive attempts aimed at meeting the rising challenges and burden of the human needs (health, social, food and economic demands), production and use of consumer products such as pharmaceuticals, cosmetic products, laundry and washing detergents, agronomic and synthetic foodstuffs and other human consumables have increased, and their potential for environmental contamination has also heightened. As a consequence, exposure to cycles of potentially harmful chemicals can bring about endocrine modulation and genetic alterations, which may lead to dangerous health effects in humans, mammals and other living organisms. This revelation has added to the speculation that environmental chemicals may contribute to the growing cases of certain global health problems.

1.1 Endocrine System

The endocrine system is responsible for co-ordinating a wide range of complex biological processes in the body. It controls the physiological activities of the body by secreting hormones through the collection of endocrine glands and non-endocrine organs in the body. Some of these hormone-secreting bodies include the hypothalamus (which is situated above the brainstem and under the cerebrum), the pituitary glands (that are attached to the underside of the brain by stem stalks) and the various other

organs such as thyroid, kidney, pancreas, ovary and testis (Figure 1.0). Hormones are chemical messengers that communicate useful coded information to the target sites where chemical 'messages' are utilised to bring about necessary changes. For example, hormones such as gonadotropin-releasing hormones (GnRH), thyrotropin-releasing hormones (TRH) and corticotropin-releasing hormones (CRH) are secreted by the neurons in the hypothalamus and transported via the blood to encourage the pituitary glands to secrete some endocrine regulatory hormones. Hormones such as thyroid-stimulating hormone (TSH), follicle-stimulating hormone (FSH), luteinising hormone (LH), adrenocorticotropic hormone (ACTH), prolactin (PRL) and growth hormone are secreted by the anterior pituitary while antidiuretic hormone (ADH) and oxytocin (OXY) are produced by the posterior pituitary. At the particular target site where exchange of chemical messages are required, a number of functional receptors are situated either on the surface of the target cells on the plasma membrane or at the nuclear region. These facilitate the binding of hormones to the specific receptors through which chemical signalling takes place. Action of chemical communication via hormones could occur in a localised area of the body (autocrine and paracrine effects) and, sometimes, it extends to all over the body (endocrine effect) (Bigsby et al., 1999).

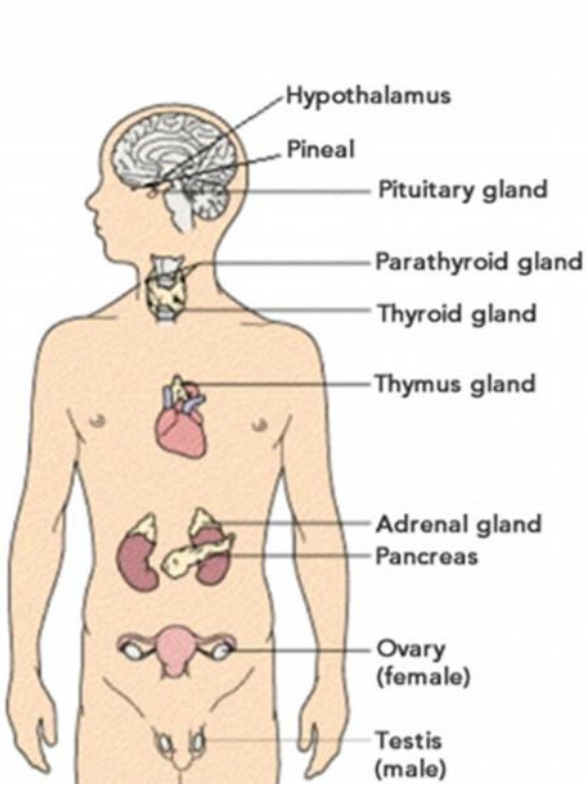


Figure 1.0: The diagram of human endocrine system showing the locations of various internal organs associated with hormone secretion in the body. Adapted from Endocrinology Medicine website, www.endoatsoim.com

1.2 Steroidal and Non-steroidal Hormones

Hormones occur as steroidal and nonsteroidal molecules with different structural forms which accounts for their diverse molecular weight. Their occurrence in different molecular weight as lipophilic or lipophobic compounds will explain why the activity of some hormones are localised to a particular region of the body. Generally, steroids are found in nature as fat-soluble, sparingly water-soluble, organic compounds (lipids) which have evolved as plant and animal hormones (Bishop and Koncz, 2002). Steroidal hormones are broadly classified into five subdivisions based on their physiological behaviours, which are: mineralocorticoids (which influence sodium retention through renal tubules), glucocorticoids (which regulate carbohydrate metabolism and inhibit the absorption of calcium in the intestine) and the three sex steroid hormones (estrogens, androgens and progestins) (Miller, 1988).

Non-steroidal hormones constitute the majority of hormone population in a category comprising proteins, peptides, amino acids and fatty acid derivatives (Hinuma

et al., 1998). Peptide hormones are water-soluble, relatively moderate molecular weight molecules (in comparison with protein) which are formed by varying chain-lengths of linear and/or ring amino acids (Neal, 2001). Some hybrid non-steroidal hormones, formed as a linkage between peptide hormones and carbohydrates, have also been identified. This class of hormones are known as glycoproteins (*ibid.*). Few common examples of glycoproteins are luteinising hormone (LH), follicle stimulating hormone (FSH), human chorionic gonadotropin (β-hcG) and thyroid-stimulating hormone (TSH) (*ibid.*). In this section, the functions of some steroid hormones especially sex steroid hormones and non-steroid hormones like GnRH, CRH and TRH are discussed.

1.2.1 Sex Steroid Hormones

The sex steroid hormones are synthesised by the endocrine system of most animal species and are responsible for the regulation of critical stages in the animals' life cycle which include gametogenesis, fertilisation, sexual development and reproduction (Gross et al., 2003). They can be divided into three major classes; namely: estrogens, androgens and progestins (or progestagens). Generally, steroid hormones have chemical structures composed of a three-dimensionally arranged four, fused carbon rings in a 6-6-6-5 ring structure with a ketonic, aldehydic or alcoholic functional group and terpenoid appendages in rare instances (Xu et al., 2003; Ying et al., 2002). This core nuclear arrangement has been described as cyclopentan-o-perhydrophenanthrene ring system, having 17 carbon atoms in total (Shen and Lin, 2006; Wynn, 1965; Ying et al., 2002). The ring nature varies from one steroid to another. Whereas the first six-carbon ring may be saturated (cyclohexane) or partly saturated in one structure, yet in another it would be fully unsaturated (benzene ring). The fourth ring is a cyclopentane, a saturated five-carbon ring (Xu et al., 2003) (see Figure 1.1).

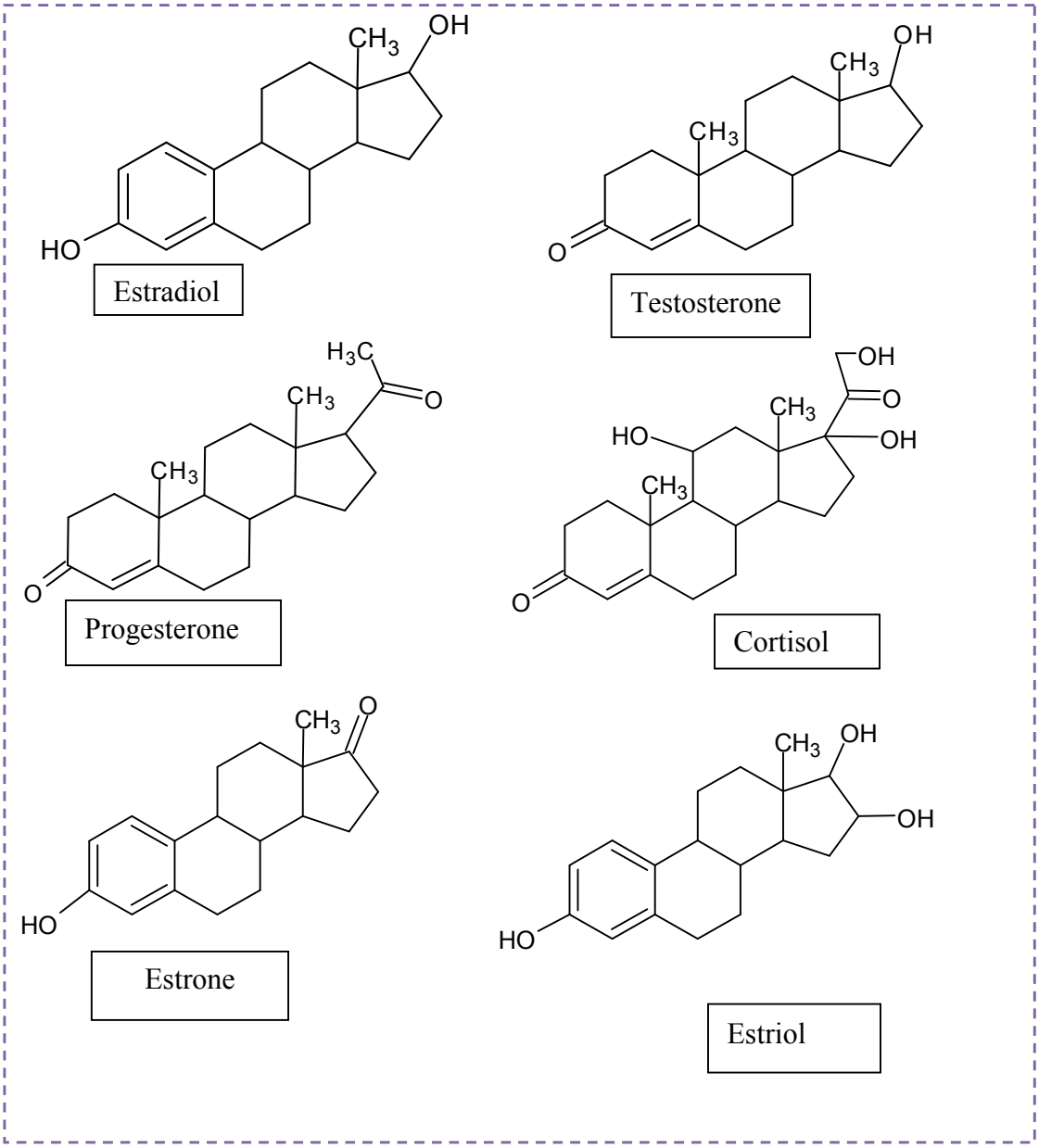


Figure 1.1: Chemical structures of common endogenous steroid hormones in the vertebrate endocrine system.

1.2.1.1 Estrogens

Estrogens are a group of steroid sex hormones which are responsible for the maintenance and regulation of growth, development, differentiation and function of reproductive organs, sexual characteristics and other reproductive processes in both mature wildlife and humans (Ciana et al., 2003). In addition, they control some important body organs such as the bones, brain and cardiovascular system (*ibid.*). Although, these hormones are found largely in females, they are present in male animals in trace amounts. In male animals, estrogen may regulate the commencement process of

spermatogenesis by facilitating spermatogonia multiplication and by enhancing the role of follicle stimulating hormone (FSH) in activating spermatogenesis (Ebling et al., 2000; Kula, 1988; Kula et al., 2001; Walczak-Jedrzejowsksa et al., 2005). They are composed of estrone, estradiol and estriol which are produced by the ovaries, adrenal glands and the fatty tissues. The first two hormones are formed in the ovaries while the last is produced in one of the reproductive tissues during luteal phase in the corpus luteum (Barlow and Logan, 1966). Estriol can also be formed through aromatisation of 16-OH-dehydroepiandrosterone sulphate (a DHEA derivative formed in the liver) during pregnancy in the placenta (Ryan, 1959).

1.2.1.2 Androgens

Androgens are a class of sex steroid hormones which are produced by both mature male and female animals. They control the development and maintenance of masculine characteristics, sperm induction and sexual differentiation as well as enhance virility and libido; in addition, they regulate water (through the synthesis of aldosterone hormone which is produced by adrenal cortex of the adrenal gland situated above the kidney) (Shigeoka et al., 1985; Milledge et al., 1983), nitrogen retention (Urban et al., 1995; Brodsky et al., 1996) and activate skeletal growth (Quigley et al., 1995). This class of hormones include androsterone, testosterone and dihydrotostesterone and they are secreted by the testis, ovaries, adrenal glands and placenta. Androgens are produced from enzymatic metabolism of cholesterol and steroid intermediates as shown in Figure 1.2.

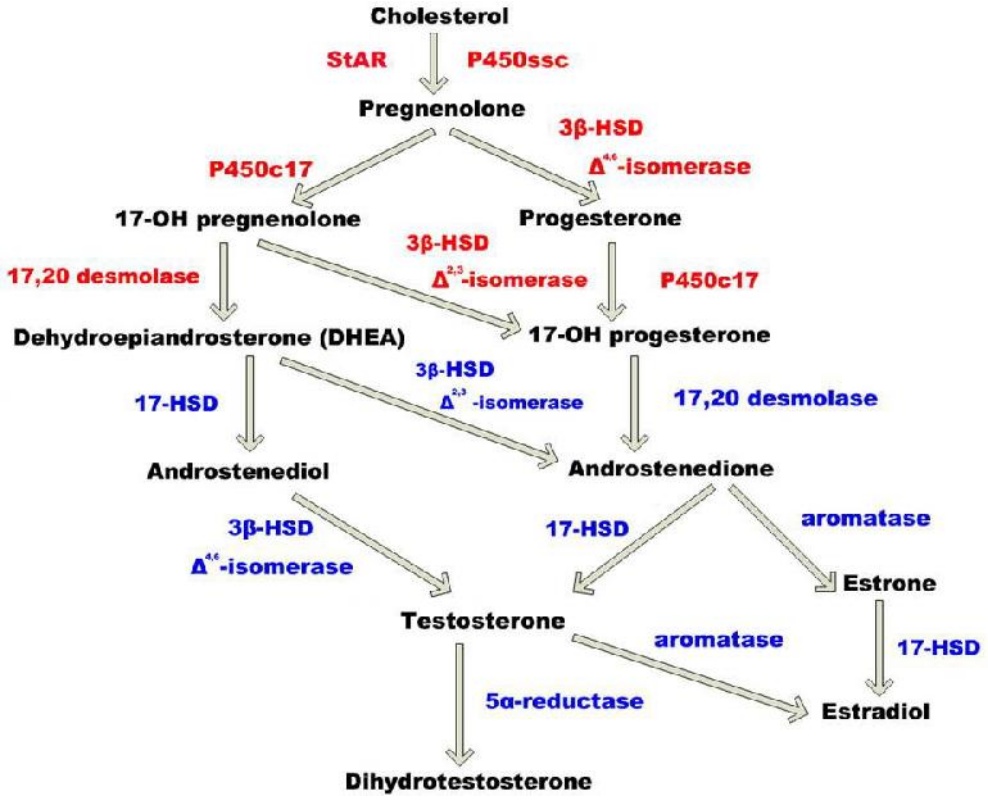


Figure 1.2: Biosynthetic pathways of some androgen hormones and their enzyme-mediated conversion products. The blue enzymes catalyse the formation and metabolism of androgen hormones including the aromatase. The red enzymes act on the pro-androgen hormone precursors and metabolites produced during steroidogenesis (adapted from, http://www.conf.ncku.edu.tw).

1.2.1.3 Progestins

Progestins are a class of steroid hormones derived from both natural and synthetic origins. They are used to sustain pregnancy to maturity (reduce preterm delivery), inhibit ovulation and menstrual cycle control, reduce the endometrial wall and stem the growth of prostate cancer (Sitruk-Ware 2004). The natural progestins (such as progesterone) are manufactured and secreted in the corpus luteum (ovary), the placenta and the adrenal cortex of female humans (Schindler et al., 2003). In the male humans, this hormone is secreted by the testes and the adrenal cortex (Simpkins et al., 2005). Comparatively, the level of progesterone in female humans is higher than that in male counterpart but this drops to the level found in the male during the follicular stage of the menstrual cycle (Chrousos et al., 2001). Apart from the natural progestins, a wide range of progestins are being manufactured on an industrial scale today for therapeutic use (e.g. manufacturing of contraceptives) (Falconer, 2006). The presence of

polychlorinated biphenyls (PCBs) in the body could facilitate the breakdown of progesterone in the liver (Colborn et al., 1997). For example, it has been documented that women who experience incessant miscarriages possess relatively higher level of PCBs in their bodies compared to those carrying normal pregnancies (Leoni et al., 1989; Saxena et al., 1981). The presence of PCBs could possibly lead to drastic reduction or non-availability of progesterone in the body system (*ibid*.). While the occurrence of PCBs may be correlated to the incessant miscarriages in pregnant women, it has not yet been proven that they are responsible for the phenomenon.

1.2.2 Non-steroidal Hormones

1.2.2.1 Gonadotropin-Releasing Hormone (GnRH)

Gonadotropin-releasing hormone (GnRH) is a decapeptide produced by the cell bodies of hypothalamic neurons present in the brain. It is released into the portal blood system and transported to the anterior pituitary to stimulate gonadotropin production. The gonadotropin FSH and LH are heteromeric glycoproteins which stimulate gonadal synthesis of sex steroid hormones (testosterone, estrogen and progesterone), which are required for spermatogenesis and oogenesis (Dalken et al., 2001). Both FSH and LH hormones have a similar α-subunit and a distinctive β-subunit (Conn, 1994). The secretion of GnRH occurs during the outset of puberty. Although the function of GnRH in the gonads (the testis, the ovary), the uterus (the placenta), the breast and the central nervous system is unknown, it has been detected in these organs (Grossman, 1998).

1.2.2.2 Corticotropin-Releasing Hormone (CRH)

Corticotropin-releasing hormone is a 41-amino acid peptide produced by the parvocellular neurons situated in the hypothalamic paraventricular nucleus (Vale et al., 1981). In addition to their presence in the hypothalamus, the CRH-secreting neurons are distributed across the central nervous system as well as the adrenal cortex, the spinal cord and the limbic regions (Brady et al., 1990; Merchenthaler et al., 1984; Swanson et al., 1983; Thompson et al., 1987). It influences the synthesis of adenocorticotropic hormone (ACTH) by activating the anterior pituitary cells. The ACTH secreted acts on the adrenal cortex and facilitates secretion of glucocorticoids (e.g. corticol in human and corticosterone in rat), the hormones which act in response to stress. The ACTH is directly responsible for regulating the effect of stress on immune system or inflammatory system (Berridge and Dunn, 1987). Although CRH is a widely known

moderator of endocrine and immune responses to stress, it is also responsible for a wide range of behaviour such as reproduction, arousal and feeding (De Souza, 1995; Dunn and Berridge, 1990). It is a key regulatory element required during human pregnancy and parturition (*ibid.*). As the period of gestation advances, the concentration of the CRH peptide in placenta increases correspondingly as that in the maternal plasma (Sasaki et al., 1987; Riley et al., 1991).

1.2.2.3 Thyrotropin-Releasing Hormone (TRH)

Thyrotropin-releasing hormone is another tropic tripeptide hormone which is produced by the paraventricular nucleus of the hypothalamic neurons. It is released at the median eminence to facilitate the secretion of thyrotrophic-stimulating hormone (TSH) at the anterior pituitary which regulates the release of triiodothyronine (T_3) and thyroxine (T_4) from the thyroid gland (Ghamari-Langroudi et al., 2010). The thyroid hormones (T_3 and T_4) secreted can also act as the negative feedback mechanism by controlling the activity of TRH-secreting neurons in the hypothalamus (*ibid.*).

1.2.3. Intracellular and Extracellular Hormone Receptors

Hormones are biological change agents that bind with specific hormone receptors to regulate some essential biological activities (such as growth, metabolism, puberty and reproduction) in the body. Extracellular messages carried by the hormones are converted to intracellular signals after forming a complex with the hormone receptors of the target cell. The nature of the receptors involved in such binding depends on the chemical structure of the hormone. Based on their cellular localisation, hormone receptors are broadly classified into two main categories, namely extracellular receptors otherwise known as cell-surface receptors (CRs) and intracellular receptors (IRs). In addition to the two major receptors, there are some receptors whose activating ligands are not known. This class of receptors is referred to as orphan receptors. CRs are glycoprotein structures located in the phospholipid bilayer of the cell membrane which explains the reason why they are referred to as cell-surface receptors. Structurally, they transverse the hydrophilic outer cell surface, the hydrophobic plasma membrane and hydrophilic inner framework of the cell cytoplasm. CRs are remarkably sensitive to different extracellular signalling molecules especially high molecular mass peptides that cannot diffuse naturally into the cell through the plasma. The commonly known

examples of CRs are G-protein coupled receptors (GPCRs), receptor tyrosine kinases (RTK), integrins and toll-like receptors.

Intracellular receptors are hormone receptors using hormone-binding to induce intracellular signals in a cellular system. Unlike the CRs, they are localised in either the inner cytoplasm or the nucleus of the cell. This class of hormone receptors has affinity for low molecular mass hormones which can easily diffuse through the plasma membrane to initiate binding in the cell. Examples of intracellular receptors include androgen receptor (AR), estrogen receptor (ER), progesterone receptor (PR), thyroid hormone receptor (THR), retinoic acid receptor (RAR), retinoid X receptor (RXR) and vitamin D receptor (VDR). Interestingly, some intracellular receptors can also bind and activate certain CRs. A member of the GPCRs family (GPR30) has recently been identified to demonstrate high binding affinity to estrogen in contrast to binding to the known cellular estrogen receptor (e.g. ERα and ERβ) (Revankar et al., 2005; Thomas et al., 2005). It was also discovered that the GPR30 binding produces multiple intracellular responses that are associated with growth, proliferation and differentiation (Filardo et al., 2000; Kanda et al., 2003; Kanda et al., 2004). This new finding is regarded as a possible significant step towards treating tumours which are not dependent on estrogen receptor agonism.

1.2.4 Mechanisms of Hormone Action

Hormones modulate gene expression via genomic and non-genomic signalling. While a genomic signalling is initiated by influencing the RNA and certain protein synthesis through receptor complex interaction with the hormone response element of the DNA, a non-genomic pathway bypasses these components by recruiting other signalling pathways (Vincent et al., 2008). Some non-steroid hormones, such as insulin, oxytocin and vasopressin, are known to elicit cellular expressions via non-genomic signalling but steroid hormones (e.g. estrogen, progesterone and androgen) can regulate cellular expressions via both genomic and non-genomic mechanisms (Losel et al., 2003; Simoncini and Genazzani, 2003; Vincent et al., 2008). In this Section, both transcriptional and non-transcriptional mechanisms of steroid and non-steroid hormone actions are briefly described.

When steroid hormones are released into the blood stream after secretion, they are transported to the target cells where the process of biological changes is set to occur. At these cells, they locate and bind to the functional intracellular receptors, known as

steroid receptors, which are present either in the cytoplasm (cytosol), when in free state or in the nucleus, when they occur as complex (see Figure 1.3; Ing and O'Malley, 1995; Jacobson et al., 1995; Kemppainen et al., 1992; Waller et al., 2000). Steroid hormone receptors are biological co-change agents with hormones which can facilitate internal communication and transformation. Steroid hormone receptors are made up of amino- and carboxyl-terminus, the DNA and ligand binding domains (LBD) (Beato, 1989). Generally, they exist in the cytoplasm as a monomer with perinuclear distribution as well as homodimers or heterodimers (Jacobson et al., 1995; Kemppainen et al., 1992; Waller et al., 2000). After the formation of steroid receptor complexes, the heat shock proteins (HSP) detached from the receptors while the receptor complexes formed diffuse through the cytoplasm and the nuclear pores as they translocate into the nucleus (Keller et al., 1996). In the nucleus, the receptor complexes bind to the human response elements (HREs) on the DNA. Prior to the binding, the receptor complexes undergo a series of conformational changes which modify them into a form that will interact with the hormone response elements (HREs) (see Figure 1.3; Beato et al., 1987; Beato, 1989; Mckenna et al., 1999; Mckenna and O'Malley, 2002). To initiate transactivation, the HREs attract coactivators or corepressors which induce the transcription of downstream DNA to messenger RNA. The necessary gene expressions are stimulated in the target cells by the protein formed through the RNA transcription (Ing and O'Malley, 1995). The choice of either coactivators or corepressors is determined by the nature of the ligand that bounds to the receptor. An agonist signalling could sometimes recruit coactivators which will lead to increased expression of one or more genes and the proteins which they encode. The process is referred to as gene up-regulation. For instance, increase in concentration of testosterone in the bloodstream induces production of more androgen receptor and hence activates gene up-regulation that would bring about skeletal muscle development in animals (e.g. cattle) (Squires, 2003). Conversely, decrease in certain gene expression and their encoding protein are induced when an agonist signalling occurs by recruiting corepressors. The phenomenon is known as gene down-regulation. The build-up of progesterone concentration in the uterine and endometrial regions leads to the reduction of progesterone receptors and hence causes gene down-regulation (Bazer, 1998). While some knowledge of the genetic behaviour of agonist signalling exists, little is known about genetic behaviour of antagonist signalling.

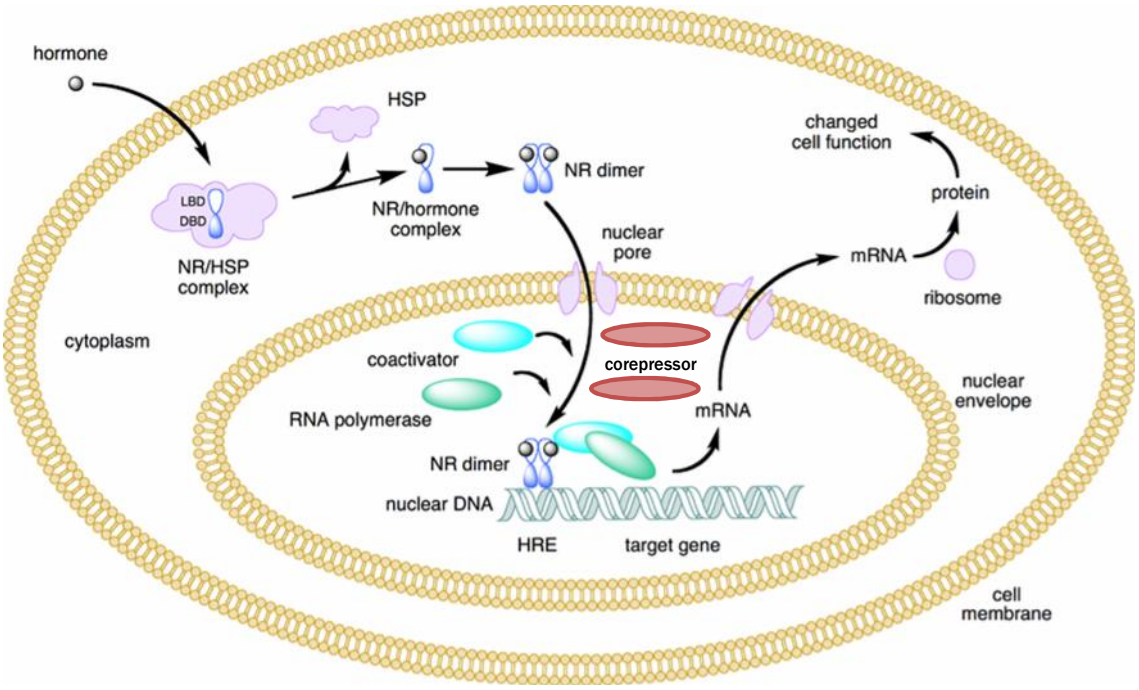


Figure 1.3: Structural configuration and nature of a typical nuclear (e.g. androgen) receptor: showing the location of nuclear receptor (NR) in the cytosol in absence of ligand, the dissociation of heat shock proteins (HSP), dimerization and nuclear translocation of activated receptor as well as the coactivator (or corepressor) recruitment, the DNA binding of the translocated receptor and the translation of downstream DNA to RNA which would effect change in the cell function (adapted from Wikimedia Commons, http://www.commons.wikimedia.org and reconstructed).

The mechanisms by which non-steroid hormones carry out their actions are somewhat complex compared to the modalities involved in steroid hormones. Given the relatively large size of their molecules, it is difficult for non-steroid hormones to diffuse through the cell wall in order to bind intracellular receptors. Rather, they interact with membrane receptors on the surface of the cell to form a complex which generates signal that alters the conformation of the receptor. G-protein coupled receptors (GPCRs) are just one example of a number of protein hormone receptors present in animal tissues. The receptor in such modified configuration facilitates coupling to the G-protein to form hormone-receptor-G protein complex (Birnbaumer and Birnbaumer, 1995). Non-activated G proteins are heterotrimeric guanine nucleotide-binding protein which has three subunits (Gα, Gβ and Gγ subunits) and the guanosine diphosphate (GDP) moiety (Chedrese, 2009). The direct binding of the receptor complex to the Gα-subunit of the

G-protein leads to the activation of G-protein and causes the dissociation of G protein into Gα and Gβγ subunits (Neer, 1995; Surya et al., 1998; Strader et al., 1994). Hitherto activation, the Gα of the G-protein is bound to the GDP as Gα-GDP but upon activation, it becomes Gα-GTP. The activation of G-protein is presumed to facilitate the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) (Neer, 1995; Surya et al., 1998). The activated G protein dissociates from the receptor to modulate the activity of the effector protein, which could be an ion channel or an enzyme (e.g. adenylyl cyclase, phospholipase C and guanylyl cyclase), to produce certain levels of second messengers (e.g. cyclic adenosine monophosphate (cAMP), diacylglycerol (DAG) and Inositol triphosphate (IP_3)) and transduction of signals (Chedrese, 2009).

However, given that Gα subunits carry some G-protein signalling modulating proteins, referred to as GTPase activating protein, GTP can be hydrolysed to GDP (Melmed and Conn, 2005). This same GTPase activating property is possessed by some effector proteins such as adenylyl cyclase (Melmed and Conn, 2005). Upon activation, these effector proteins will interact with Gα–GTP and convert it to Gα-GDP thus making the Gα inactive (Chedrese, 2009; Melmed and Conn, 2005). The Gα reassociates with Gβγ subunits of the G protein, fully ready to repeat the cycle (Chedrese, 2009). Hormones associated with these cell surface receptors (and second messengers) include TSH and catecholamine. As hitherto indicated (Section 1.2.3), CR signalling can also be modulated via other molecular mechanisms such as ligand-gated ion exchange (e.g. nicotinic acetylcholine receptors), receptor serine/threonine kinases (e.g. receptors of activins and inhibins), receptor tyrosine kinases (RTK), receptor guanylate cyclase (e.g. atrial natriuretic factor receptor), integrins and toll-like receptors beside using G-protein coupled receptors (Kronenberg et al., 2008).

1.3 Endocrine Communication Pathways, Feedback Mechanism and Reproduction End-points.

Endocrine communication involves concerted contributions and collaboration of the central nervous system, the hypothalamus, the pituitary and the target tissues. The central nervous system is a major division of the nervous system that controls and co-ordinates dissemination of information useful for regulating biological processes of the whole body. Given that the brain and the spinal cord constitute the central nervous system, it directly supervises the activities of the hypothalamus and the pituitary gland which co-ordinate and regulate the production of endocrine hormones. The

hypothalamus, the pituitary glands and the effector organ or tissue are frequently, conveniently addressed as a single entity because they work in co-operation to produce the desired effects. Thus, many triangular communication partnerships between the hypothalamus, the pituitary gland and any of the different effector organs and tissues in the body have been recognised but this study will be limited to three major ones associated with reproduction, immune system and growth. These are the hypothalamic-pituitary-gonadal (HPG), the hypothalamus-pituitary-adrenal (HPA) and the hypothalamus-pituitary-thyroidal (HPT) axes. It is widely held that for the reproductive system to function normally the activities of the HPG, the HPA and the HPT axes must be balanced out (Simon and Polan, 1994). The HPG axis constitutes one of the major endocrine signalling pathways in which the endocrine hormones are deployed for specific localised actions on the body's reproductive processes (see Figure 1.4). The activities of the hypothalamus, the pituitary and the endocrine effector organs/tissues are complementary. The preoptic region of the hypothalamus is composed of gonadotropin-releasing hormone (GnRH) neurons, which induces the secretion of both luteinizing hormone (LH) and follicle-stimulating hormone (FSH). They work in concert to provide both positive and negative feedbacks to the hypothalamus and the pituitary gland (Conn and Crowley, 1994; Shupnik, 1996). In females, both the FSH and LH function essentially to stimulate the ovaries to produce estrogen and inhibin (Finkelstein et al., 1991; Billiar et al., 2003). They are also responsible for regulating the menstrual and ovarian cycles. While estrogen is known to contribute effectively to the negative feedback mechanism leading to inhibition of GnRH production in the hypothalamus, the inhibin is responsible for constraining activin which induces the production of GnRH producing cells (Holdcraft and Braun, 2004; Drummond and Findlay, 1999). In males, the LH controls the production of testosterone by activating the interstitial cells situated in the testis and the FSH contributes to spermatogenesis (Luconi et al., 2002). As in female, gonadal androgens can regulate cell growth, sensitivity to GnRH and level of expression of the gonadotropins by feeding back directly on the pituitary gonadotropes (Melmed and Conn, 2005). Generally, feedbacks on the hypothalamus and pituitary sites are activated by the gonadal hormones at high concentrations in order to regulate the secretion and release of gonadotropin (Melmed and Conn, 2005) (See Figure 1.4).

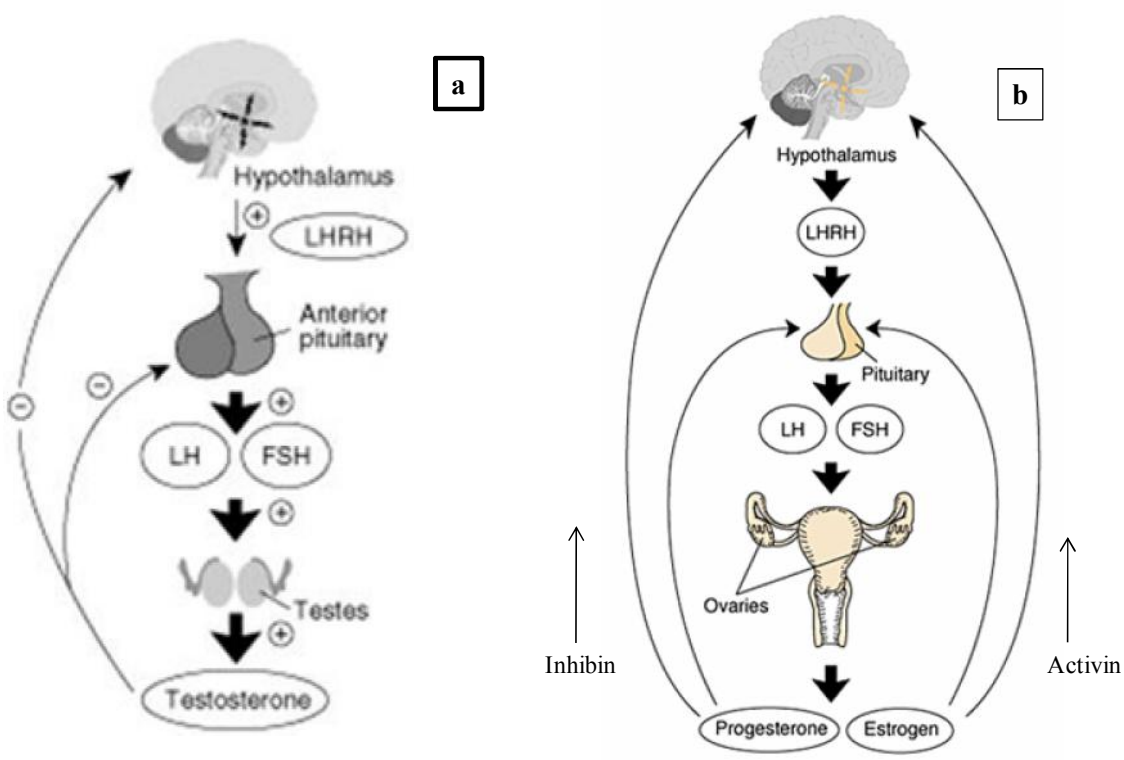


Figure 1.4: A diagram showing the transition of activity and feedback network (cross-talks) between the brain and hypothalamus-pituitary-gonad (HPG) axis during endocrine hormone regulatory activity in the body. In Figure 1.4(a), the + and – symbols in the diagram stand for and trace the positive and negative feedback pathways respectively in the male body. Figure 1.4(b) shows similar progression of activity and feedback mechanism in the female body (both diagrams were adapted from National Institute of Alcohol Abuse and Alcoholism publications, http://pubs.niaaa.nih.gov/publications). The feedback pathways are indicated by the upward arrows. In the two diagrams, the activin and inhibin are secreted by the gonads to regulate the feedback mechanism. The transition occurs via the action of luteinising hormone releasing hormone (LHRH) on the anterior pituitary through to the gonads.

The regulatory ability of the HPG axis could be influenced by the other two axes identified in this study. The HPA axis is a control pathway which links the hormone system and the central nervous system. It has also demonstrated the ability to influence the activity of HPG axis. Stress serves as a major mechanism by which the HPA axis is activated. Some HPA components via which such activation is induced include CRH, corticotropin, β-endorphin and glucocorticoid (Weinstock, 1997). CRH and GnRH are

two peptide hormones that are secreted in the hypothalamus which are indirectly responsible for adrenal and gonadal activities respectively (Rivest and Riviest, 1995). Moreover, a direct neural linkage between the two hormones has been reported (*ibid.*). In addition to HPA regulation, CRH has also been shown to regulate proopiomelanocortin-derived peptides, such as β-endorphin, which inhibit the secretion of GnRH in the hypothalamus (Chrousos et al., 1998). This leads to non-secretion of LH and FSH which are responsible for gonadal reproductive activity (Chen et al., 1992). Likewise, the adrenal glucocorticoid can influence the HPG axis via positive and negative feedback mechanisms at the level of the hypothalamus, pituitary, gonad and other tissues (Chatterjee and Chatterjee, 2009; Dubey and Plant, 1985). It has been reported that long-term elevation of glucocorticoid concentration can lead to the suppression of HPG functions and the immune system (Melmed and Conn, 2005). Glucocorticoids can feedback on the gonads to suppress gonadotropin synthesis (*ibid*.)

The HPT axis constitutes another vital feedback mechanism which can influence the HPG axis. The process (described schematically in Figure 1.5 and Section 1.2.2.3) leads to the formation of thyroid hormones (thyroxine, T_4 and triiodothyronine, T_3) and regulation of hormone equilibrium in the body. The thyroid hormones are secreted to regulate development, reproduction, metabolism, behaviour and growth in vertebrate animals (Gorbman et al., 1983). The thyroid hormones influence the ovaries indirectly by reducing the gonadotropin-releasing hormones (GnRH) in anestrous ewe (Clark, 1988). It is also likely responsible for the seasonal reproductive patterns in *Bos indicus* cattle and Welsh Mountain ewe including development of refractoriness at the end of the reproductive season (De Moraes et al., 1998; Follet and Potts, 1990). When thyroid hormones are in excess of requirement during regulatory activities, they form a negative feedback network which will influence the collective processes that facilitate their secretion in the thyroid glands (Figure 1.5). Such a negative feedback mechanism will down-regulate the activities of the hypothalamus and the pituitary, and in turn, affect the activities along the HPG axis. For example, the relationship of the HPT with reproductive activities in some vertebrate fish species has been established. Cyr and Eales (1996) showed, over some specific phases in the reproductive cycle of some teleosts, that there exists temporal relationship between the thyroidal and the gonadal states. It was widely reported from studies on a wide variety of teleost species that the increased thyroidal activity observed during early gonadal development is usually

sustained during reproduction (*ibid.*). Conversely, the activity in both the thyroid and gonad reduces during the spawning and post-spawning stages (*ibid.*).

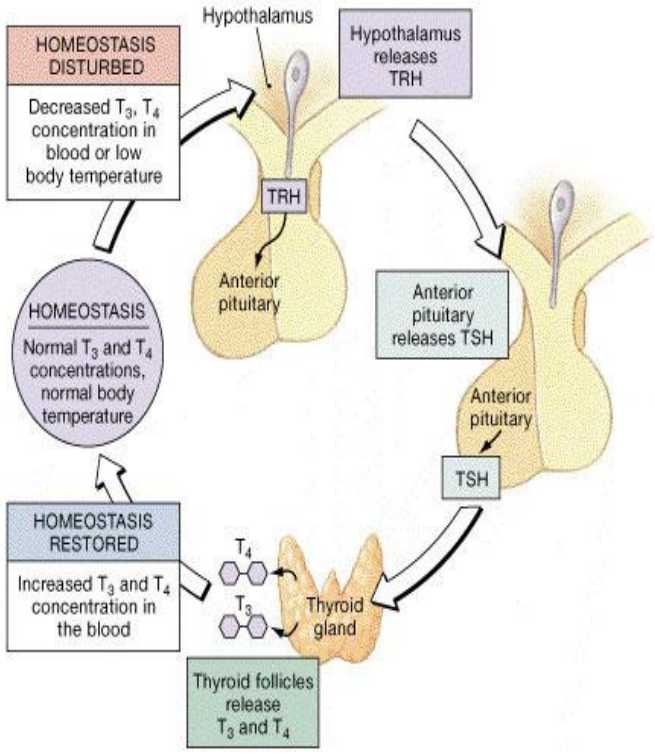


Figure 1.5: A diagram illustrating the positive and negative feedback mechanisms of the hypothalamus-pituitary-thyroid (HPT) axis between the brain, pituitary and thyroid organs of the body (by courtesy Zoe).

1.4 Endocrine Disrupting Chemicals

A wide variety of anthropogenic chemicals, and some exogenous natural chemicals, can influence the course of endocrine communication processes in humans and wildlife. This class of bioactive chemicals are referred to as endocrine disrupting chemicals, or simply as endocrine disruptors. An endocrine disruptor is broadly defined as any exogenous agent that interferes with the normal processes of production, secretion, transportation and receptor activation of natural hormones which are responsible for the maintenance of homeostasis, growth, reproduction, and other internal activities (Kavlock et al., 1996; USEPA, 1998). Endocrine disrupting chemicals are mostly lipophilic in nature, bioaccumulative and environmentally persistent while some are characterised with low vapour pressure (Colborn et al., 1995). At low

concentrations, endocrine chemicals have potentials of inducing biological effects. The likely reason for this is associated with the ability of these endocrine disrupting compounds to act via multiple mechanisms. The effects of endocrine disrupting chemicals and their biological end-points in living organisms may be irreversible. Therefore, there are possibilities that these biological end-points may be expressed during developmental and reproductive stages (Rhind, 2002). Controlled laboratory exposure of fetal rodents to p,p'-DDT has resulted in reproductive defects such as reduced and impaired fertility, decreased viability of the offspring, variation in hormonal level and sexual behaviour of adult males and occurrence of some developmental abnormalities such as cryptorchidism, hypospadias, disruption in the network of intercellular bridges conjoining the germ cells in the testes and anomalies in the acrosome, the nucleus and the shape of the sperms in these adults (Veeramachaneni, 2008). Effects such as increased anogenital distance (AGD), vagina agenesis, masculinisation of female offsprings and induced male-like accessory tissues have also been reported in female rats exposed *in utero* to β-trenbolone (Hotchkiss et al., 2007a; Wilson et al., 2002). It has also been reported that most environmental chemicals exhibiting endocrine disrupting characteristics have dissimilar chemical structures (*ibid.)*.

1.4.1. Behavioural and Structural Diversity of Endocrine Disrupting Chemicals

Although most disrupting activities of environmental chemicals are presumed to occur via steroid receptor activation, it is also possible for these chemicals to alter the synthesis, transport and metabolism pathways of hormones. The steroid hormone production process is largely controlled by receptor-based feedback mechanisms and consists of networked pathways of precursors, enzymes and products. In addition to steroidogenesis (steroid hormone synthesis), endocrine chemicals can also alter the level of hormone excretion and steroid biotransformation mechanisms. In gastropod mollusks (e.g. dogwhelk, *Nucella lapillus*), tributyltin (TBT) can obstruct the formation of sulphate conjugates of testosterone and its related metabolites regarded as less potent and easily excreted forms of testosterone (Nelson et al., 1996; Ohno et al., 2005; Ohhira et al., 2006). TBT can also inhibit aromatase cytochrome P450 enzymes involved in biotransformation of androgens to estrogens. In most cases, environmental chemicals having endocrine disrupting ability are different in structure when compared to the structures of endogenous hormones modulating similar effects (McLachlan, 2001;

Mantovani, 2002). While ethynylestradiol (EE2) and estrogen possess structural similarities that could be receptor active, compounds such as nonylphenol (NP) and dibutylphthalate (DBP) possess structures that are relatively different from that of estrogen and yet are estrogen receptor(ER)-active (Sonnenschein and Soto, 1998). In addition, chiral compounds may exhibit different biological activities based on their enantiomeric forms (Garrison et al., 2000; Puttmann et al., 1989; Rodman et al., 1991; Ulrich et al., 2001; Shen et al., 2006). Studies of induction of ethoxyresorufin-O-deethylase (EROD) and benzphetamine Ndemethylase (BPDM), the cytochrome P450-dependent enzymes, by 2,2',3,4,6-pentachlorobiphenyl (PeCB), 2,2',3,4,4',6-hexachlorobiphenyl (HeCB), and 2,2',3,3',4,4',6,6'-octachlorobiphenyl (OCB) showed that their (-)-enantiomers are more potent than the (+)-enantiomers except for HeCB where the reverse is the case (Rodman et al., 1991). Therefore, it may be practically difficult to design models for predicting the steroid receptor activity of compounds based on their structures. Some of the diverse chemical structures are highlighted in Figure 1.6.

1.4.2: Mechanisms of Endocrine Disruption Action

There is a wide range of means by which bioactive xenobiotics could disrupt the normal endocrine activities. However, this review will be restricted to cellular-based endocrine disrupting mechanisms which may or may not involve steroid hormone-receptor sites and these include ligand receptor agonism, ligand receptor antagonism, biosynthetic interruption, hormone transport interference and hormone metabolism.

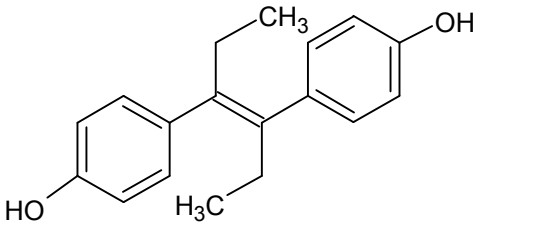
1.4.2.1 Steroid Hormone-Receptor Agonism and Antagonism

Hormones produce effect-directed responses after successfully binding to the targeted hormone receptor site. Hormone receptor sites are generally made of flexible, broad domains with unique size and structure where interactions with a wide range of hormone-like chemicals can take place. This tolerance behaviour of steroid hormone receptors (Cooper and Kavlok, 1997) explains why xenobiotics would be able to disrupt the activities of the endocrine system. Steroid hormone-receptor interaction can be classified broadly into four categories based on the nature and type of ligand, and the eventual hormone-receptor complexes formed: direct-acting agonists, direct-acting antagonists, indirect-acting agonists and indirect-acting antagonists (Cooper and Kavlock, 1997). Akin to endocrine hormones, bioactive xenobiotics can activate the

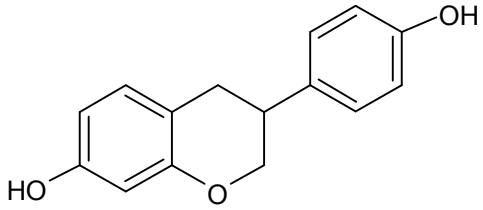
hormone receptor sites to produce similar effects caused by endogenous hormones. The principle associated with the receptor binding of endogenous hormone is the same as that involving a direct-acting agonism except for the biochemical response. The level of response induced corresponds to the potency of such compounds and the effectiveness (success) of such binding. It must be stated that binding effectiveness accomplished by a xenobiotic is determined by how closely fit in size and shape the ligand is to the receptor in the ligand-receptor complex. Xenobiotics with higher potency are expected to produce stronger physiological response than endogenous hormones of the same concentration. Examples of xenobiotics known to possess hormone receptor agonism include estrogen agonists diethylstilbestrol (DES), nonylphenol (NP), octylphenol (OP), nonylphenol polyethoxylates (NPE), genistein, bisphenol-A (BPA), paraben and tamoxifen (Fisher et al., 1999; Gray et al., 2001a; Snyder et al., 1999) and androgen agonists 17α-and 17β-trenbolone, PCB-169, androstenedione and androstadienedione (Ankley et al., 2003; Denton et al., 1985; Gray et al., 1999; Hewitt et al., 2000; Hotchkiss et al., 2007a,b). The structures of some estrogen agonists, estrogen antagonists and mixed agonist-antagonist are shown in Figure 1.6.

However, some receptor binding will not elicit response. This receptor-binding effect is referred to as direct-acting antagonism and can be via competitive or non-competitive antagonism. In the former, the hormone antagonists compete with agonists for hormone binding sites. If the antagonist-receptor binding was successful, it will be practically impossible for the binding to transform to signal. Chemical compounds that can exhibit this form of behaviour are tamoxifen (a breast cancer drug), linuron, cyproterone acetate, vinclozolin, dibutylphthalates (DBP), diethylhexylphthalate (DEHP) and nafoxidine (Baker, 2001; Clark et al., 1973; Mantovani, 2002). In non-competitive antagonist binding, the hormone antagonist can work in two possible ways: inhibition of structural alteration at the receptor site which will cause ligand binding to occur or inhibition of post-binding interactions with the HRE of the DNA that would normally produce a response to the ligand. Vinclozolin derivatives (M1 and M2) have been reported to demonstrate the potentials of impeding androgen-dependent gene expression activation by effectively blocking androgen-induced steroid-steroid receptor complexes (AR) binding to the HRE on the DNA (Kelce et al., 1994; Wong et al., 1995). Some synthetic chemicals can express dual behavioural responses in different biochemical environments. Such chemicals can be classified into partial agonist-partial antagonist and mixed agonist-antagonist. The action of the latter produces different

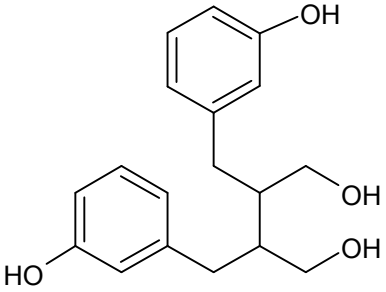
binding competence in different environmental conditions but sometimes the former can act under such different environmental conditions too. The former acts as a partial agonist by binding to the target receptors as a surrogate ligand in absence of the endogenous hormone. Most often, such binding occurs at concentrations comparatively lower than the effective concentrations of the endogenous ligand and the biological responses produced are usually very weak (De Castro et al., 1991). However, as partial antagonist, the exogenous ligand competitively binds the receptors at elevated concentrations compared to that required for binding when it was acting as partial agonist as well as that required by endogenous ligand (Ayd, 2000). When the endogenous hormone is involved, the binding interaction mostly favours the exogenous ligand due to its comparative advantage (i.e. high concentration). The biological response induced in the process falls below that produced if endogenous ligands were to act alone. In succinct description, partial agonist-partial antagonist compounds usually produce agonistic response that increases as the concentration of the compound increases at the receptor until it reaches a plateau where further increase in concentration will no longer raise the level of the response (De Castro et al., 1999). Any further increase in concentration will only elicit antagonistic response at that same receptor (*ibid.*). Sometimes, the responses displayed by compounds having partial agonist-partial antagonist characteristics are induced by the ionic strength of the environment they operate in. Common examples of compounds acting relative to this environmental condition are olanzapine and clozapine (Bymaster and Falcone, 2000). Mixed agonist-antagonist phenomenon is expressed by tamoxifen which acts as anti-estrogen in the breast tissues but also exhibits agonist behaviour in the bone and uterine environments (Lewis-Wambi and Jordan, 2005; Fawell et al., 1990; Figure 1.6). Raloxifene is another mixed agonist-antagonist compound which is highly effective as an antagonist in the reproductive tissues as well as an agonist in the bone (Draper et al., 1996; Gustafsson, 1998; Jordan, 1998). An example of a partial agonist-partial antagonist is 2-arylpyrazolo[4,3-c] quinolin-3-ones, a derivative of benzenediazepines (Yokoyama et al., 1982).



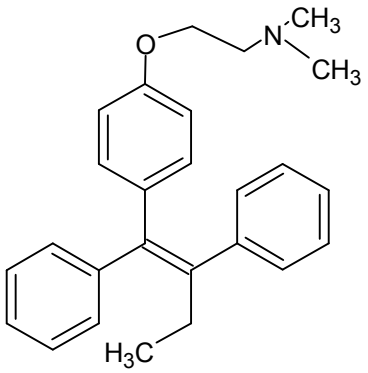
Diethylstilbestrol



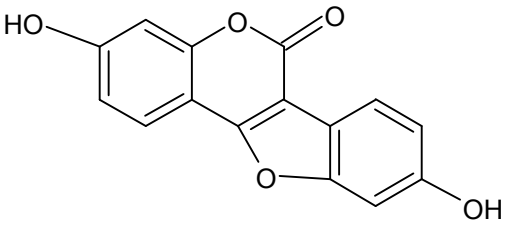
Equol



Enterodiol



Tamoxifen



Coumestrol

Figure 1.6: Structures of some endocrine disrupting chemicals behaving as estrogen receptor-binding agonist (diethylstilboestrol, enterodiol and coumestrol) and estrogen antagonist (equol). Tamoxifen acts as estrogen agonist and antagonist (or estrogen mixed agonist-antagonist).

1.4.2.2 Disruption of Steroidogenesis

Hormone biosynthesis and metabolism are significant molecular processes relevant to regulation and maintenance of cellular activities. With cholesterol as the starting materials, a wide array of hormones can be produced by the action of enzymes either directly on cholesterol or indirectly via other intermediates of steroid hormone biosynthesis (see Figure 1.2). It is possible for xenobiotics to interfere with any of these vital enzymatic pathways to induce endocrine-related health problems (Sanderson et al., 2006). The broad range of enzymes involved in steroidogenesis is classified into four

basic categories: aromatase, demolases, hydroxylases and hydroxysteroid dehydrogenases. The biosynthetic pathways in Figure 1.2 reveal the many potential disruption points where enzymatic functions may be altered during steroidogenesis. A known enzymatic disruption pathway that has been reported in wildlife occurred in dogwhelk (Bettin et al., 1996; Oehlmann et al., 1996). Bettin and colleagues (1996) showed that TBT can inhibit P450 aromatase enzyme (known to be responsible for the conversion of testosterone to estradiol) in dogwhelks when exposed to it. The study also indicated that the aromatase inhibition by TBT must have led to the accumulation of testosterone which is responsible for the formation of penis (imposex) in female dogwhelks (*ibid*.). Ketoconazole, an antifungal antibiotic, is another compound that can inhibit certain mammalian cytochrome P450-dependent enzyme such as $C_{17,20}$-lyase and 17-hydroxylase (which are responsible for biosynthesis of androgens in the animal) at elevated concentration (Vanden Bossche et al., 1987). It can also inhibit the production of cortisol in the human adrenal glands (Engelhardt et al., 1985; Mantovani, 2002).

1.4.2.3 Interference with Hormone Transport

Steroid hormones are lipophilic biological molecules which account for why they are sparingly soluble in water. They are transported in free or bound state to their binding destination via the blood plasma. Special hormone carrier proteins such as albumins and globulins are manufactured in the liver and the steroids are transported in conjugated form as glucoronides and sulphates or as the lipid-soluble hormones themselves. These globulin proteins occur as steroid hormone-binding globulin (SHBG) or testosterone-estrogen-binding globulin (TEBG) and they may bind to either testosterone or estrogen (Pertschuk et al., 1980; Roy et al., 2008). Since hormones are transported in small concentrations, any slight increase in globulin concentration is expected to reduce the chances of hormone availability for binding. Steroid hormones are temporarily stored in the blood carrier proteins where they are shielded from enzymatic activities which may lead to metabolism (*ibid.*). The carrier protein may release these hormones, from time-to-time whenever there is a short-fall in free-state concentration present in the blood. The role of the blood carrier proteins is to shield the hormones from enzymatic action (e.g. deactivating activity of the liver) and this contributes to why they are able to persist longer in the body. When hormones occur below the receptor binding concentration, there is a possibility that they may be less effective and hence have a shorter half-life *in vivo*. With the presence of circulating

xenobiotics in the body, the transport protein binding sites are subjected to competition with other chemicals and so may reduce binding of endogenous hormones to the carrier protein and disrupt the transport of the hormone. The implication of disrupting the hormone transportation mechanism is that the biological process of hormone complex formation which induces gene expression could be significantly impaired or affected. This may lead to serious health problem.

1.5 Endocrine Disruption Phenomena in the Environment

Every living organism requires the functionality of an effective chemical communication mechanism to regulate and facilitate its complex internal arrays of activities (MacLachlan 2001). These complex networks of biological activities include cellular growth, sexual differentiation, reproduction and biological development. In addition to normal intracellular regulation, the activities of organs and some specialised systems are also co-ordinated. In recent times, environmental agents of both natural and synthetic origins have come under intense investigation, especially concerning their role in disrupting chemical signalling and inducing adverse health effects in wildlife and humans. Interdisciplinary studies conducted to date have increasingly linked the growing endocrine-related health cases in wildlife to this chemical disruption phenomenon.

1.5.1 Evidence of Endocrine Disruption in Invertebrates.

A wide range of biological effects associated with the exposure of some species of invertebrate phyla to endocrine disrupting chemicals has been identified. The most comprehensive account of these environmental disturbances is summarised in the exposure of oyster (*Crassostrea gigas*), female marine gastropods (molluscs) and dog-whelk snail (*Nucella lapillus*) to organotin compounds (TBT). These compounds were used as biocides in marine antifouling paints for ship hulls, boats and aquaculture pen nets but also used in wood preservatives, agricultural pesticides, textiles and as UV stabilizers in some plastics (Oehlmann et al., 1998). A wide spread occurrence of imposex phenomenon, also known as pseudohermaphroditism, has been reported globally in marine gastropod species. Molluscs account for the majority of marine invertebrates found to be sensitive to TBT exposure. The term imposex defines the reproductive defect where the male reproductive organs (penis and vas deferens) are superimposed on the female marine species (Bryan et al., 1986; Horiguchi, 2006).

Additional effects linked to imposex include increased concentration of testosterone in the tissue of marine snail (Spooner et al., 1991). About 150 species of gonochoristic prosobranch gastropods worldwide are found to exhibit this reproductive abnormality (*ibid.*; Matthiessen and Gibbs, 1998). At low concentration (1-2ng/l), female dogwhelk exposed to TBT developed a blockage of oviduct, otherwise known as sterility (Bryan et al., 1986). Further elevation in concentration (3-8ng/l) led to diminished juvenile recruitment, induced masculinisation, reproductive difficulties, population reduction and extinction (Matthiessen and 1998; Fox, 1992). These morphological abnormalities are thought to be the consequence of inhibiting the aromatase (enzyme), which is responsible for the conversion of testosterone to 17β-estradiol (Oehlmann et al., 1996). The increased concentrations of testosterone induced biological effects which resulted in this masculinisation phenomenon (Matthiessen and Gibbs, 1998). With competing arguments suggesting a wide range of other possible modes of action of TBT such as its action as a retinoid X receptor agonist (Nishikawa et al., 2004), interruption of neuropeptide signaling pathways and modification of testosterone metabolism, the most likely mechanism of action based on causal-effect evidence comes from aromatase (CYP19) inhibition of testosterone metabolism. In oyster (*Crassostrea gigas*), the consequences of TBT include shell deformation and decimation of adult population as reproductive failure increased. Following worldwide confirmation of the long-term toxicological implication associated with continued use of TBT, the International Maritime Organisation (IMO) treaty in 2001 ratified the imposition of a partial and total worldwide ban on its use on boats and ships which indeed took effect from 2003 and 2008 respectively (Antizar-Ladislao, 2008).

Similar to TBT, triphenyltin, a compound used in similar capacity as TBT in the production of certain antifouling paint, has shown the potential of inducing imposex phenomenon in some species of marine snail. It is significant to state that triphenyltin (TPT) usage is not restricted to the production of antifouling paint alone (Crompton, 1998). In the 1960s, TPT was the main constituent of triphenyltinhydroxide and triphenyltinacetate used as fungicides to treat potato blight, leaf spot and powdery mildew (Keijzer and Loch, 1995). Schulte et al. (2000) reported imposex in female freshwater ramshorn snail (*Marisa cornuarietis*), an ampillariidae-mesogatropod, exposed to TPT. Some male sexual abnormalities associated with TPT have been reported to include azoospermia (spermatogenesis impairment) and partial infertility. These experimental discoveries underscore the endocrine disrupting impact of TPT

across both sexes of freshwater ramshorn snail (*Marisa cornuarietis*) (*ibid.*). While no significant negative effects were recorded in female netted whelk (*Hinia reticulate*), dogwhelk (*Nucella lapillus*) showed abnormal sexual features highlighting impairment of oogenesis and spermatogenesis when exposed to TPT.

Another biological effect of endocrine disruption in invertebrates is intersexuality. Intersex reproductive abnormality was reported as the phenotypic interference of sex determining organs occurring between the gonad and genital tracts (Oehlmann et al., 1994; Bauer et al., 1995). Intersex males and females retained phenotypic identity but are differentiated by the presence of additional reproductive features specific to their opposite sex (Ford et al., 2004). While the female intersexed species possess one or two penis or vas deferens (genitalia), the male species carry rudimentary oostegites, the female brood plates (*ibid.*). It is reported that periwinkles (*Littorina littorea*) developed intersexuality effects when exposed to TBT, a phenomenon that contrast with the outcome of the previous exposures (Matthiessen et al, 1995). Intersex phenomenon has been reported in crustaceans (*harpacticoid copepods*) exposed to sewage effluents along the east coast of Edinburgh, Scotland (Moore and Stevenson, 1994). Although the mechanisms surrounding the phenomenon is unclear, factors responsible for intersex vary and may include age and the species (De Bock and Greco, 2010), parasitism (Ford et al., 2004), temperature (Vazquez et al., 2004), bacterial infection (Rigaud and Juchault, 1998), genetic control (Lebederf, 1939), environmental sex determination (Dunn et al., 1996; Dunn et al., 2005), protandrous hermaphroditism (Yaldwyn, 1966), exogenous chemicals (Olmstead and Leblanc, 2007) and pollution (Moore and Stevenson, 1991; Ford et al., 2004a). The following copepods were discovered to exhibit intersexuality: *Paramphiascella hyperborea* and *Stenhelia gibba* and it was also evident in two species of *Halectinosoma* (*Similidistinctum* and *finmarchicum*). Intersex contrasts with imposex in their reproductive morphology. While imposex phenomenon can be described as complete superimposition of the female reproductive organs with the penis or the vas deferens (the male reproductive organs), intersex phenomenon is characterised with deepening development of abnormal pallial oviduct by the female reproductive organs.

1.5.2 Evidence of Endocrine Disruption in Vertebrates.

1.5.2.1 Reptiles:

Accidental spillage of a mixture of chemicals containing diclofol, DDT, DDE and sulphuric acid, into Lake Apopka in Florida induced a wide range of reproductive and developmental abnormalities in juvenile alligators (*Alligator mississippiensis*) living in the lake (USEPA, 1979). In addition to the spill, the lake is known to receive agricultural wastewaters which have further caused the level of its contamination over time to be complicated (Guillette et al., 1994). Three years post-spillage review of the alligator densities in the lake showed 90% reduction in the population of juvenile alligators in the lake (Guillette et al., 1995; Jenning et al., 1988). This noticeable decline in the alligator population has been attributed to reproductive failure modulated by DDT and its metabolites, DDE and DDD. Woodward et al. (1993) reported reduced hatching success from 80% to about 20% of the eggs taken from alligators harvested from Lake Apopka. The juvenile male alligators that matured from these hatchlings were discovered to have developed small penis size. High mortality rate of hatchlings (about 50%) occurred after 14 days when the concentration of bioaccumulated pesticides and their metabolites recorded in the eggs doubled (*ibid.*). Abnormal morphological effects attributed to these endocrine disrupting chemicals in both adult male and female alligators include demasculinisation of male alligator (where increased length of testicular phalluses was developed), malformation of cell structures in seminiferous tubules, ovarian defects in the female alligators (*ibid*.). Plasma hormone concentration studies of the gonad region have reportedly confirmed elevation of estradiol-testosterone ratio (E2/T2) by a factor of 2 in female alligators and quadrupled factor from 0.5 in the male reptiles under the influence of DDT and their metabolites stressing the biological implication of xenoestrogens *in vivo* (Milnes et al., 2002; Guillette et al., 1994). Laboratory studies revealed that o, p'-DDE which is suspected to be responsible for most hormonal effects (estradiol) in alligator could effect sex differentiation when eggs coated with chemical was incubated in the hatchery (Hileman, 1994). At normal male hatching temperature, DDE coated eggs produced a mixture of male, female and intersex in ratio 2:1:2 against 100% male recorded by DDE-free eggs (*ibid*.).

Another known example of environmental occurrence of endocrine induced chemical effects detected in Lake Apopka occurred in red-eared slider turtle (*Trachemys scripta*) population. Investigation conducted on the eggs, juvenile and adults of this turtle species in Lake Apopka revealed some developmental and reproductive

abnormalities which have been attributed to exposure to chemical contaminants in the lake. Seven years review of the turtle densities after the spillage revealed an alteration in the hormonal balance and the trend of androgen synthesis. Most male sexed turtles are progressively demasculinised while hatchlings either result in abnormal male or intersex according to histopathological studies (Guillette et al., 1994). Studies have shown that two hydroxylated PCB(2',4',6'-trichloro-4-biphenylol and 2',3',4',5'-tetra-4-biphenylol), which may be detected as metabolites of PCBs in the marine environment, could activate estrogenic characteristics in eggs of red-eared male turtles that are pre-dosed (coated) with these compounds when incubated at male-producing temperature (Bergeron et al., 1994). Their endocrine disrupting characteristic could induce sex reversal in male but the end-results in female turtles are unknown (*ibid.*).

1.5.2.2 Amphibians:

The declining profile of amphibians in the wild has raised questions about the role of endocrine disrupting chemicals in the reduction of their population. Currently, the available evidence implicating xenobiotic chemicals for the extinction of some wildlife species is so far limited. Exposure of some amphibian species {e.g. northern leopard frogs (*Rana pipiens*), green frogs (*Rana clamitans*) and mink frogs (*Rana septentironalis*)} to agricultural chemicals has resulted in some developmental deformities in addition to some physiological and immunological abnormalities and propensities such as alteration in developmental hormones, increased susceptibility to diseases, missing or supernumerary limbs, bony limb-like projections, digit and muscle defects as well as those associated with central nervous system (Ankley and Giesy, 1998). Chemical-induced developmental and reproductive malformations have further suggested that chemical exposure may be responsible for the decline of some amphibian populations. Metamorphosis is a crucial developmental process by which eggs of a wildlife species are transformed to the adult wildlife. Each of these stages is mediated by the endocrine system through the endogenous hormones. Although the role of endogenous hormones on the formation and transformation of larvae is not known, it is suspected that sex steroid hormones can hinder larval development in some amphibian species (Gray and Janssens, 1990; Hayes, 1997; Richards and Nace, 1978). Long-term exposure of amphibians to estrogen and estrogen-like chemicals can modulate sex differentiation during metamorphosis (Bevan et al., 2003; Christensen et al., 2005; Goto et al., 2006; Mackenzie et al., 2003). Studies of cricket frog (*Acris crepitans*) taken from

a PCBs- and polychlorinated dibenzofuran (PCDF)-contaminated site in Illinois, USA revealed that male frog species showed sex ratio alteration. The additional reproductive abnormalities reported include formation of testicular oocytes, incomplete metamorphosis, and feminisation. Common morphological abnormalities documented in female frogs comprise female-biased sex ratio and gonadal malformation (Jofre and Karasov, 2007). Similar malformations have been reported in Northern leopard frogs (*Rana pipiens*) captured in Ontario, USA.

1.5.2.3 Aves (Birds):

The best known examples of endocrine disrupting effects of environmental chemicals in wild birds pertain to egg shell thinning, supernormal clutches and sex differentiation. Widespread DDT and DDE pollution across the Pacific coast of the United States of America between the 1950s and 1970s has been linked with some developmental and reproductive abnormalities. Roughly 2 million kilograms of industrial DDT and its metabolites were released into the coastline via the sewers leading to bioaccumulation and bioconcentration of their particulates in the tissues of fish, sea birds and sea lions. The effect-based ecological response to these contaminants led to breeding failure in double-crested cormorants (*Phalacrocorax auritus*), brown Pelicans (*Pelecanus occidentalis*) of Anacapa Island, common egrets (*Casmerodius albus*) in California, sparrowhawks (*Accipiter nisus*) of South Scotland, fish-eating Caspian and Foster's terns (*Stern caspia and Stern forsteri*), white-tailed eagles (*Haliaeetus albicilla*) of Schleswig Hostein, herring gulls (*Larus argentatus*) of Lake Ontario and shoreline-nesting bald eagles (*Haliaeetus leucocephalus*) in the Great Lake Basin. These and some other chlorinated pesticides were linked to the general decline in the population of wild birds (Fry and Toone, 1981; Palmiter and Mulvhill, 1978). The step-wise formation process of medullary bone (which is recognised as the primary source of calcium during formation of eggs and eggshells) and calcium metabolism are regulated by the sex steroid hormone, estrogen. It is possible for bioaccumulated DDT and its metabolites in the tissues to act against the transportation of calcium across eggshell gland mucosa given that some chlorinated pesticides have been reported to have estrogenic characteristic. This calcium transportation inhibition leads to eggshell thinning in predatory and non-predatory birds (Taylor and Harrison, 1999; Risebrough et al., 1968). In addition to eggshell thinning, seen to be the prevalent symptom in most exposed bird species, sex differentiation (skewed sex ratios) such as feminisation and

intersexuality, have been reported to occur in some breeding populations of some wild birds. Such bird species include Japanese quails (Bryan et al., 1989), Western gulls and Herring gulls (Fry et al., 1981). Further long-term effects of morphological abnormalities reported in wild birds include development of malformed ovarian tissues and oviducts in male embryos (Fry et al., 1981), hematology (Bryan et al., 1989), developmental abnormalities, such as feather malformation and growth retardation, and mortality (Safe et al., 2000).

Another illustrative evidence of endocrine disruption reported in wild birds is the incidence of supernormal clutches, where the average number per clutch (nest) rose from three to between four and six (Dawson, 2000; Kovacs and Ryder, 1985; Ryder and Somppi, 1979). Due to biased sex ratio in the population density (which resulted in the shortage of male population density), it is observed that two female members could come together (in what has been termed female-female pairing effect) to lay eggs in common nest and share the responsibility of rotational incubation which was usually undertaken with the male (Hunt and Hunt, 1977; Kovacs and Ryder, 1985). This phenomenon was triggered by the high level of o, p'-DDT contaminants which induced feminisation of male Western gull embryos found in the heavily contaminated Santa Barbara Island (Fry and Toone, 1981). Supernormal clutches in member birds was caused by skewed sex ratio in the population. Another explanation tenable for this behaviour, which has not been investigated, is the masculinisation of female Western gulls' behaviour. The effect was no longer noticed after a worldwide ban was imposed on the production and use of DDT as pesticide.

1.5.2.4 Pisces (Fish):

Among the five classes of vertebrate animals, fish ranks as one of the most widely studied to illustrate endocrine disrupting effects of environmental chemicals. This is because the aquatic environment, which is their natural habitat, is regarded as "the ultimate sink" for natural and anthropogenic chemicals (Sumpter, 1998). Studies have also shown that wastewater effluents are one of the major sources of chemical contaminants of the aquatic environment (Desbrow et al., 1998; Snyder et al., 1999; Baronti et al., 2000). There is a general view that severe endocrine disrupting consequences, which could threaten the reproductive health and population sustainability of fish, would not only spell their extinction but could also have adverse consequences on the population and health of humans, predatory animals and other

living organisms. Studies of the physiology and morphology of fish species caught downstream of some wastewater-receiving rivers and lagoons and some laboratory exposures showed diverse degrees of abnormalities (Kime, 1998; Tyler et al., 1998). Gonad analysis of caged and wild male roach (*Rutilus rutilus*) exposed to wastewater effluents during sexual differentiation revealed the induction of protein egg-yolk, known as vitellogenin, a phospholipoprotein manufactured in the liver of egg-laying female vertebrates (Folmar et al., 1996; Larsson et al., 1999; Purdom et al., 1994; Rodgers-Gray et al., 2000, 2001). This egg-yolk formation has been defined as feminisation of the male fish. Similarly, formation of vitellogenin has been reported in juvenile male rainbow trout (*Oncorhynchus mykiss*), Japanese medaka (*Oryzias latipes*), fathead minnows (*Pimaphales promelas*), zebrafish (*Danio rerio*) and cyprinid fish species exposed to laboratory dosage of estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) (Brion et al., 2001; Harries et al., 2000; Orn et al., 2006; Purdon et al., 1994; Routledge et al., 1998; Thorpe et al., 2000, 2003). The presence of nonylphenol, a metabolite of nonylphenol ethoxylate surfactants, in wastewater effluents induces the egg yolk formation and inhibits testicular growth (Harries et al., 1995). However, other variables such as water temperature, the migratory habits of fish, the nature and dosage of EDC exposed to (including the diverse past EDCs exposure which can leverage the fresh exposure threshold) can also contribute to the level of vitellogenic response (Purdom et al., 1994; Kirby et al., 2004; Pait and Nelson, 2003; Panter et al., 2002).

In addition to feminisation effect in male fish, formation of cysteine-rich glycoprotein called spiggin in female fish species, as yet another reproductive abnormality, has been reported. At the biochemical level during the breeding period, the male stickleback fish develops kidney hypertrophy under the influence of androgen thereby causing the epithelial cells of the kidney to produce hydrophobic protein named spiggin (Jakobsson et al., 1999; Sanchez et al., 2008). This 230kDa protein is released into the urinary bladder where it is constituted as a structural thread (or glue) that is used for the purpose of nest-building preparatory to the egg-laying process by female fish (Jakobsson et al., 1999). Conventionally, kidneys of female sticklebacks are not known to produce spiggin until recently when the spiggin-forming genes in female stickleback were found in dormant (latent) form (Sanchez et al., 2008). As in male stickleback, these dormant genes in the female can be induced by androgen receptor agonists to produce spiggins. It is realised that spiggin formation via androgen induction

in young and immature female stickleback fish could serve as a reliable end-point which can be used to screen (xeno)-androgens and anti-androgens in the laboratory and the field (Allen et al., 2002; Bjorkblom et al., 2007; Katsiadaki et al., 2002, 2006; Jones et al., 2001) .

In Florida, USA, female mosquito fish (*Gambusia affinis*) harvested in rivers downstream of pulp and paper mills were observed to exhibit masculinisation phenomenon which affected its behavioural pattern too (Howell et al., 1980). In addition, the female mosquito fish was reported to develop elongated anal fins which are a replica of male gonopodium (*ibid*.). The males caught in the same rivers were also reported to show hypermasculinisation which makes them develop a more hyperaggressive mating behaviour than the normal males (*ibid.*). Other abnormalities identified include reduced sex hormone level, delayed sexual maturity, reduced and abnormal sexual organs, reproductive difficulties, reduced fecundity and intersexuality (Gray et al., 1999; Hirai et al., 2006; Lange et al., 2001; Robinson et al., 2007). Although, the environmental chemicals responsible for these reproductive effects were not immediately known as studies indicated that such chemicals have hormonal characteristics similar to endogenous androgens.

A wide range of scientific studies undertaken have identified some of the xenobiotic chemicals involved in some of these reproductive endpoint abnormalities to include PCBs (mostly components or metabolites of known pesticides e.g. DDT, DDE and chlordane), natural estrogens (e.g. 17β-estradiol, phytoestrogen) and other unknown chemicals (Allen et al., 1999; Sumpter and Jobling, 1995). Significantly, laboratory studies have further advanced the knowledge on endocrine disrupting effects of environmental chemicals as most of the observed effects have been replicated. Incidences of vitellogenin production and gonadosomatic index development in juvenile Atlantic cod (*Gadus morhua*) and flounder (*Platichthys flesus*) have been demonstrated in the laboratory (Allen et al., 1997; Ilylland et al., 1997).

1.5.2.5 Mammals:

A spectrum of well-established evidences of endocrine disrupting chemicals in mammals has been reported. Most examples documented were based on the reproductive endpoints and population reduction in terrestrial and aquatic mammals. For instance, Florida panthers (*Felis concolor coryi*), grey seal (*Halichoerus grypus*), common seal (*Phoca vitulina*), Baltic ringed seal (*Phoca hispicla botnica*), bottlenose

dolphin (*Tursiops truncatus*), striped dolphins (*Stenella coeruleoalba*) and beluga whales (*Delphinapterus leucas*) exhibit a wide range of developmental abnormalities and reproductive dysfunction. Following progressive declines in their populations, investigation revealed that a high level of organochlorines was found in the tissues of the Florida panthers (Facemire et al., 1995). The organochlorines detected are suspected to originate from raccoon (*Procyon lotor*) food items contaminated by mercury and pesticides during production (*ibid.*). Florida panthers (*Felis concolor coryi*) have also shown reproductive and morphological abnormalities such as low ejaculation volume, diminished sperm concentration, sperm characterised with high morphological anomaly, cryptorchidism and male infertility (Facemire et al., 1995; Roelke, 1990). Declining trends of grey and ringed seals' population in the Baltic Sea has likewise been linked to endocrine disruptive effects of environmental chemicals (Taylor and Harrison, 1999). Increased concentration of PCBs, DDT and its metabolites in the tissues of seals has also been detected, some of which have developed abnormalities such as testicular steroidogenetic defects, immune dysfunction and infertility linked with these endocrine disrupting chemicals. Similar abnormalities occurred when seals harvested in the heavily polluted Wadden Sea were analysed (*ibid.*). Additional evidences documented from this experimental analysis include high incidence of fatality and reduced concentrations of estradiol resulting in imbalances in this endogenous hormone. For example, polar bears (*Ursus maritimus*) in Svalbard, Norway recorded a drastic decline in female population following chemically-induced hormonal imbalance that nearly wiped out the female bear population from age sixteen upward (Derocher et al., 2003). Occurrence of pseudo-hermaphrodites, a phenomenon synonymous to females developing small penis in front of their vagina, has been documented in female polar bears (Wiig et al., 1998). The elevated PCB concentration in polar bears may be the potential causal agent responsible for dwindling younger polar bear population in Svalbard although there is no enough evidence yet to suggest that these are the key agents (*ibid.*).

1.5.2.6 Evidence of Endocrine Disrupting Phenomena in Human.

The best known evidence of endocrine disruption in human beings involved use of diethylstilbestrol (DES). DES is a popular anabolic agent widely used in the USA to improve the economic values of livestock. However, DES was administered to pregnant women between late 1940s and early 1970s to prevent spontaneous abortion and this led

to a myriad of irreversible reproductive abnormalities, many of which affected the unborn baby directly (Marselos and Tomatis, 1992). The outcome of long-term *in utero* studies of DES exposure, which has widened through meat and dairy-related products, has been characterised. Similar to laboratory animals, risks of human exposure to DES include suppression of lactation, testicular malformation, underdevelopment or absence of vas deferens, gynecomastia (development of abnormally large breast tissue in men), retention of Mullerian ducts, deteriorated sperm quality and formation of epididymal cysts in matured male progeny (McLachlan et al., 2001; Steinberger and Lloyd, 1985). Structural malformations such as cryptorchidism, hypoplastic testis and penis and meatal stenosis (narrowing urethra tube leading to the penis opening) have been similarly reported in children male born after their parents' exposure to DES (Gill et al., 1977; Bibbo et al., 1977; Gill et al., 1979; Stillman et al., 1982). Female children that emerged after *in utero* exposure in their parents' womb have been shown to develop reproductive and developmental malformations. Some of such effects include subfertility and virilisation (Kunz et al., 2004), vaginal adenocarcinoma, premature birth, pseudo precocious puberty (Felner and White, 2000), vaginal adenosis, elevated serum testosterone, structural abnormalities in the uterus, ectopic pregnancy, hood and polyps of cervical and vaginal organs, anatomical masculinisation and malformation of cervical canal, reduced libido, sexual impotence and tumours of reproductive organs in adult women (Steinberger and Lloyd, 1985; Newbold, 1995; Grajewski et al., 1996; Whelan et al., 1996).

1.5.3 Environmental Sources of Exposure to Endocrine Disruptors

A wide range of synthetic and natural chemicals which are capable of modulating endocrine-related reproductive and developmental health problems have been detected in the environment. Synthetic chemicals find their way into the environment via accidental and deliberate human and non-human activities from several point and non-point sources. Some of these sources identified include agricultural, industrial, commercial and residential outlets. For instance, pesticide applications (to improve crop yields in plantations), industrial by-products discharges, landfill leachates, combustion process, sewage sludge and domestic wastes discharges are commonly known means of chemical sources in the environment. The fact that endocrine disruptors possess a wide range of physicochemical properties will suggest the possibility of exhibiting different modes of behaviour in the environment. For instance,

most EDCs are known to be lipophilic but have variable chemical and biological transformation pathways in the environment which are dependent on their structures and other chemical properties. Moreover, catabolic processes such as chemical degradation often are altered by environmental factors (e.g. aerobic and anaerobic conditions, elevated temperature and photocatalysis, and hydrolysis) to change the form and, sometimes, the state of these chemical compounds. However, some of these compounds are relatively stable as they do not undergo any degradation. For this reason, they tend to bioaccumulate in the environment, maintain a high shelf-life and long enduring transportation away from their sources. These are expected to impact on the general mechanism of distribution. Human and wildlife exposure to endocrine disrupting chemicals can take place through contact with food, water, air, personal care products (consumer products), soil and aquatic sediments. These exposure sources are broadly classified into two: indoor and outdoor sources. Generally, endocrine disruptors enter into the blood streams of human and wildlife through ingestion, inhalation and direct access across the cell membrane via skin and gill contacts.

1.5.4 Anti-androgens in the Environment.

The word ‘anti-androgen’ can be defined, in a short phrase, as “action taken against androgen”. Androgens are useful hormones secreted by the gonads in conjunction with the pituitary glands to induce chains of biochemical processes in the body of vertebrate animals for the purpose of reproduction and reproductive activities. The word ‘androgen’ originated from the combination of two Greek words, “andros” (man) and “genein” (to produce). Androgens mediate biological responses through interaction with androgen receptor (AR) as described in Section 1.4.2. Anti-androgenic chemicals simply act by blocking the biological process of complex formation and/or transcription, which are strategic for activating biological responses. Exogenous anti-androgens are also referred to as xenoanti-androgens and they describe the endocrine disrupting chemicals that are responsible for interrupting these internal biologically strategic processes.

Therefore, broadly expressed, anti-androgens are any group of androgen (receptor) antagonists, hormone synthesis inhibitors, hormone metabolism interrupters or hormone transport modulators capable of obstructing partially or totally the biological responsibilities of any natural or synthetic chemical compounds (androgens), which act on appropriate target cells in the body system to promote and activate the

production, development and maintenance of masculine sex organs and sundry secondary sexual characteristics in vertebrates.

Anti-androgens existing in the environment originate generally from natural and synthetic sources as steroid and non-steroid compounds. Most known naturally occurring anti-androgens are derived from plant sources and are substantially non-steroid in form (Section 1.5.4.1). The knowledge of natural steroid and non-steroid anti-androgens from animal sources is currently not available. A wide range of exogenous anti-androgens, occurring in steroidal and non-steroidal forms, have been reported among the myriads of anthropogenic chemicals found in the environment. Presently, the only known synthetic anti-androgenic compound having steroidal property is cyproterone acetate (CPA)(Figure 1.9). From this analysis, it may be appropriate to say that most synthetic anti-androgens known are non-steroidal.

Nonsteroidal synthetic anti-androgens (NSA) are synthesised for myriads of human and non-human use, many of which cut across agriculture, medicine, environment, industries and domestic life.

1.5.4.1. Plant and Fungal Anti-androgens

Anti-androgens that occur in nature are limited and most of those reported so far originate from plant sources. Some of the exogenous anti-androgenic compounds reported in nature include permixon (an extract derived from an American dwarf palm tree, *Serenoa repens B*) (Wilt et al., 1998), a non-steroidal CpdA [2-(4-acetoxyphenyl)-2-chloro-*N* methylethylammonium chloride -a stable derivative of the African shrub, *Salsola tuberculatiformis* Botsch, prepared from less stable hydroxyphenyl aziridine] (Tanner et al., 2003) and isolates of three leaves -beech, oak and reed extracts (Hermelink et al., 2010) (see Figure 1.7). Also, estrogenic effects sometime reported in poultry and livestock have been linked with feeds contaminated with Zearalenone, a fungal mycotoxin produced by the action of *Fusarium sp* on carbohydrate feeds (Manfred, 2001; Meronuck et al., 1970; Roine et al., 1971). Studies have shown that zearalenone and its metabolites (zearalanone, zearalanol and zearalenol) have anti-androgenic potency almost equivalent to flutamide (Paris et al., 2005). This discovery has shown that some estrogens can act as also anti-androgens at receptor level. Although, the AR binding mechanism is currently poorly understood, zearalenone has been reported to demonstrate hepatic ER binding (Powell-Jones et al., 1981). There are *in vivo* data indicating that *in utero* exposure could lead to sex differentiation and

serious disruption of reproductive competence (*ibid.*). Equol (7-hydroxy-3[4'-hydroxyphenyl]-chroman) [see Figure 1.6] has also been investigated as a biologically active compound with anti-androgenic property (Lund et al., 2004). Equol is a chief component of daidzein, a phytoestrogenic derivative of soyabeans. It is found that equol has poor AR affinity but bind selectively, with high affinity, to 5α-dihydrotestosterone (DHT) to deny DHT from AR binding opportunity (*ibid.*). Binding affinity of equol to DHT is partly accountable for lean epididymal and prostate weight after exposure (*ibid*.).

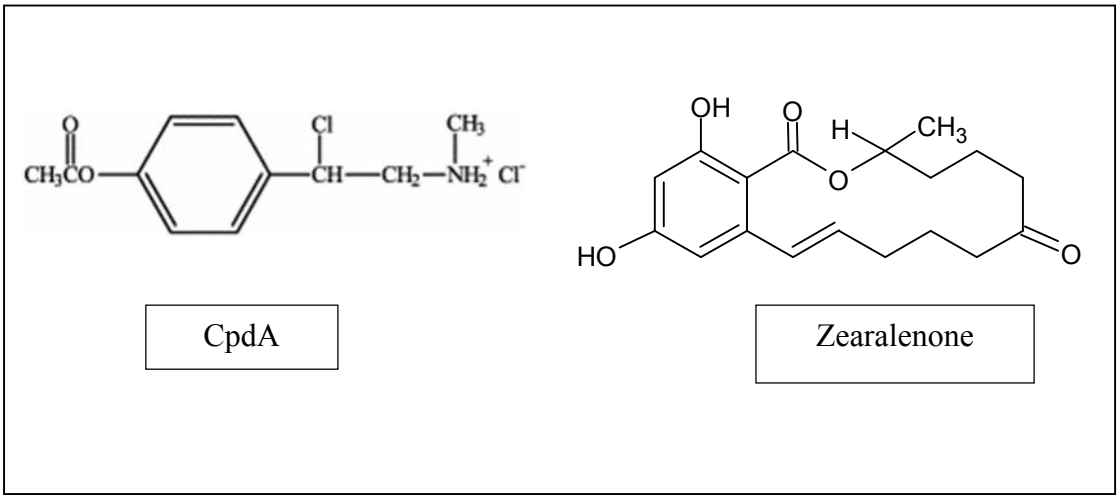


Figure 1.7: The chemical structures of 2-(4-acetoxyphenyl)-2-chloro-*N*-methyl-ethylammonium chloride (CpdA) and Zearalenone.

1.5.4.2 Agricultural Anti-androgens

A wide range of chemicals that are associated with agricultural practices have been reported to possess anti-androgenic properties. Effect-based studies of the anti-androgenic activities of some of these agro-allied chemicals under *in vivo* and *in vitro* experimental conditions have been conducted. Gray and co-workers (1994) discovered that, at cell receptor level, some pesticides possess anti-androgenic characteristics *in vitro* or *ex vitro* and, in some, with a high degree of potency. The majority of these pesticides have been discovered in the environment in their original or metabolised form and their mechanism of anti-androgenic action is expressed via aromatase inhibition. For most environmental compounds that modulate anti-androgenic effects, aromatase induction clears away the competitive binding hurdles posed by the endogenous androgen hormones at the target receptors and makes passage to the receptors free for the chemical anti-androgens to bind to. Classical examples of anti-

androgens in the environment include insecticides DDT and its metabolite-DDE (Kelce et al., 1995a,b), fenitrothoin, chlorpyrifos-methyl (CPM) (Kang et al., 2004), toxaphene and metachlor (its metabolite, HPTE), fungicide vinclozolin and metabolites M1 and M2, procymidone (Kelce et al, 1994; Ostby et al., 1999; Gray et al., 2001), prochloraz (Gray et al., 2001) and herbicide linuron (Lambright et al., 2000; McIntyre et al., 2000; Vinggaard et al., 2005) (Figure 1.8). *In utero* exposure of some male terrestrial mammals (e.g. rat, dog, sheep and pig) to vinclozolin and p, p'-DDE induces reduced anitogenital distance (AGD), reduced weight of sex accessory glands, hypospadias, ectopic and undescended testes, vaginal pouch and retained nipples (Gray et al., 2001; Wolf et al., 1999; Kelce et al., 1995a, b). No knowledge of phenotypic effect has so far been reported in the females of all the laboratory animals exposed to procymidone, p,p'-DDE and vinclozolin. Identified effects of developmental exposure to each of the three compounds include testicular cancer (Shakkebaek, 1972), erectile dysfunction and infertility in rats (Brien et al., 2000). Given that anti-androgenic response of vinclozolin is very weak relative to its metabolites (M1 and M2), the potency is comparatively low. Although, linuron and methoxychlor also exhibit AR antagonism *in vivo* and *in vitro*, and induce reproductive-based effects similar to vinclozolin, procymidone and p,p'-DDE, the fact that their bioactive concentration are higher implies low potency.

Most of these agricultural anti-androgens have been detected in the environment either in their original or metabolised form. Due to their non-persistence in nature, residues of a wide range of pesticides have been discovered in water, air, soil and food samples. Organochlorine chemicals such as DDT, linuron, procymidone, metachlor, vinclozolin and methoxychlor are widely used on agricultural farmlands to control broad range of pests. Although DDT is either completely banned or restricted in some countries across the globe, its residues are still being recovered in previously exposed soil layers years afterward. For instance, DDT detected in Tianjin soil samples analysed in China occurred in the range of 628.1-2840.5ng/g decades after its application was banned (Gong et al., 2002). In Argentina where DDT usage has been restricted, traces of it at levels of 0.0204-2.123ppm have been reported in dairy by-products such as butter (Lenardon et al., 1994). Similar investigation in Spain has also confirmed the occurrence of DDT in trace quantity in pasteurised milk (Martinez et al., 1997). Investigation carried out in Ayeduasi, KNUST and K-Poly in Kumasi, Ghana has also reported the occurrence of DDT in yoghurt at concentrations of 8.96µg/kg, 4.09µg/kg and 7.52µg/kg respectively (Darko and Acquaah, 2008). In addition, studies further

indicate that traces of DDT were detected in fresh milk at a concentration of 12.53µg/kg in KNUST samples and in cheese sampled from Aboabo, Tafo and Asawasi at concentrations of 14.02µg/kg, 298.57µg/kg and 42.17µg/kg respectively (Darko and Acquaah, 2008). In an experimental study undertaken thirty-days after procymidone application to four supervised leek fields in China, an average range of 0.033-0.17mg/kg and 0.020-1.75mg/kg of its residues, corresponding to 74.9-100% and 82.5-92.5%, were detected in leeks and soil samples respectively (Chen et al., 2010). Procymidone residues have been discovered in wine grapes and vegetables harvested from exposed farmlands (Urruty et al., 1997; Rodriguez et al., 2002). Linuron is a herbicide that is used extensively for cultivating tuber and vegetative crops such as onions, carrots and potatoes (Sanchez-Camazano et al., 2000). About 70% of linuron was reported to leach into the soil when four natural soil samples taken from the Province of Salamanca, Spain were investigated (*ibid.*). There was a significant variation in the result outcome when the organic content of the soil samples was modified indicating that the level of linuron retention in the soil changes with different soil profiles (*ibid.*). Prochloraz is a fungicide for controlling eyespot disease and powdery mildew in cereals and fungal diseases in vegetables and fruits (Birchmore et al., 1977; Lafuente and Tadeo, 1985; Kapteyn et al., 1992). A multiresidue analysis carried out on pesticide-contaminated soil samples taken from Sao Paolo state, Brazil reported a recovery range of 54-73% of prochloraz between 55-73^{0}C temperatures. During the analysis, recoveries of linuron and vinclozolin were also measured at the respective range of 55-70% and 69-73% under the same temperature conditions (Risatto et al., 2005). Prochloraz-formylurea and prochloraz-urea are two metabolites of prochloraz that have been detected at different concentrations of various subsoil layers (Hollrigl-Rosta et al., 1999). The level of prochloraz degradation is a function of the texture, pH and the temperature of the soil (*ibid*.).

Analytical studies of horticultural soil samples taken in five different locations in northern Portugal showed the occurrence of chlorpyrifos (a derivative of chlorpyrifos-methyl) in 33.8µg/kg respectively (Goncalves and Alpendurada, 2005). While chlorpyrifos-methyl has been shown to possess anti-androgenic activity, the biological activity of chlorpyrifos is yet to be known. In Saudi Arabia, residues of toxaphene were detected in subsoil samples of contaminated farmlands (30-60cm) at concentration between 0.006-0.162mg/kg (Al-Wabel et al., 2011). Another commonly known anti-androgenic organochlorine pesticide is methoxychlor. It is used in isolation

or in combination with other chemicals to control cereal and fruit pests especially during storage and planting seasons (ATSDR, 1994). It is also used on agricultural fields planted with vegetable, soyabeans and nuts to control insect pests (*ibid.*). Its mode of action is known to be similar to that of DDT. Traces of methoxychlor residue have been detected in food stuff, water and soil. Due to its less persistence, methoxychlor is rarely found in the environment but could be detected near the disposal, dispensal or the manufacturing premises. In soil, methoxychlor has an anaerobic half-life of 30 days and an aerobic half-life over 100 days (Muir and Yarechewski, 1984). Fenitrothion is a non-persistent organophosphorus insecticide that has been identified to exhibit anti-androgenic property. It is commonly used to control insects associated with cereals, vegetables, stored grains and cotton on agricultural fields (Tamura et al., 2001). It undergoes biodegradation under aerobic condition to produce toxic nitrophenolic metabolites in soil and water (Mikami et al., 1985). One major metabolite generated during its aerobic biodegradation, which only occur via artificial means, is 3-methyl-4-nitrophenol (MNP) (Keith and Telliard, 1979). Due to the possibility of producing myriads of toxic metabolites aerobically, extensive use of fenitrothion has become the likely channel that could constitute environmental health concerns in humans and wildlife (Tago et al., 2006). The structures of these agricultural anti-androgenic compounds are provided in Figure 1.8.

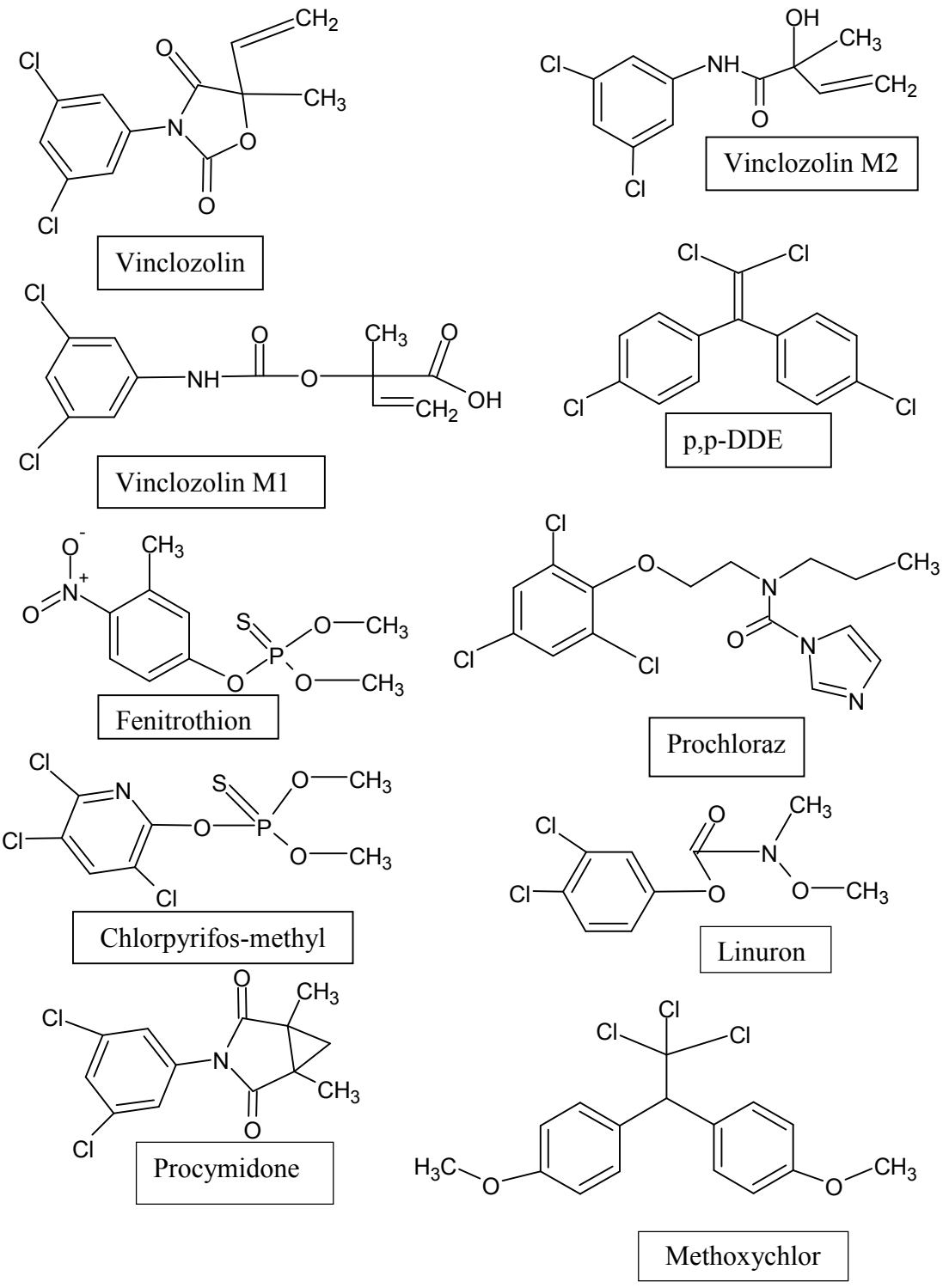


Figure 1.8: A diagram showing examples of agricultural anti-androgens found in the environment.

1.5.4.3 Clinical Anti-androgens

In utero administration of DES to pregnant women, as estrogenic supplement, for three decades in the USA led to a wide range of phenotypic end-points that indicated compromise in health conditions. Cases of cryptorchidism, hypospadias, and diminished volume and quality of sperm in adult laboratory animals have been reported (Imperato-McGinley et al., 1992; van der Schoot, 1992; Silversides et al., 1995; Sharpe et al., 2000). For the fact that an estrogen could induce all the effects highlighted above strongly suggests that it can exercise the ability to block the androgen receptor. Investigation later confirmed similar reproductive effects when anti-androgen exposure was carried out. This highlights the fact that while the mechanisms through which these compounds operate in any vertebrate system are dissimilar, yet the endpoints are same. DES can be reported, on that basis, to exhibit anti-androgenic activity. Hydroprogesterone and cyproterone acetate (CPA), its synthetic derivative, are reported to demonstrate both anti-androgenic and progestational activities (Huang et al., 1985; Brinkmann et al., 1983). *In vivo* exposure of CPA, progesterone and anti-progestin RU486 has been shown to induce nuclear transport, AR DNA binding and transcriptional activity due to competitive AR inhibition binding (Kemppainen et al., 1992). Cyproterone acetate belongs to the dydrogesterone (17-OH progesterone) class of anti-androgenic progestin family (Sitruk-Ware, 2008). Cyproterone acetate exerts its anti-androgenic activity by either engaging in competitive inhibition with AR or by disrupting the conversion agent (enzyme) of testosterone to dihydrotestosterone (DHT) (Sitruk-Ware, 2008). Other clinical anti-androgens in regular use today consist of flutamide, finasteride, nilutamide, spironolactone, hydroxyflutamide, valproate, osaterone acetate, danazol and ketoconazole (Imperato-McGinley et al., 1992; Death et al., 2005; Gunes and Fetil, 2000; Minato et al., 2005; Terouanne et al., 2002; Korner et al., 2004; Xu et al., 2006; Schurmeyer and Nieschlag, 1984) (Figure 1.9). Flutamide, nilutamide and bicalutamide are another category of clinical anti-androgens which operate in similar manner as CPA. Despite their regular use, their concentrations in the environment are not reported.

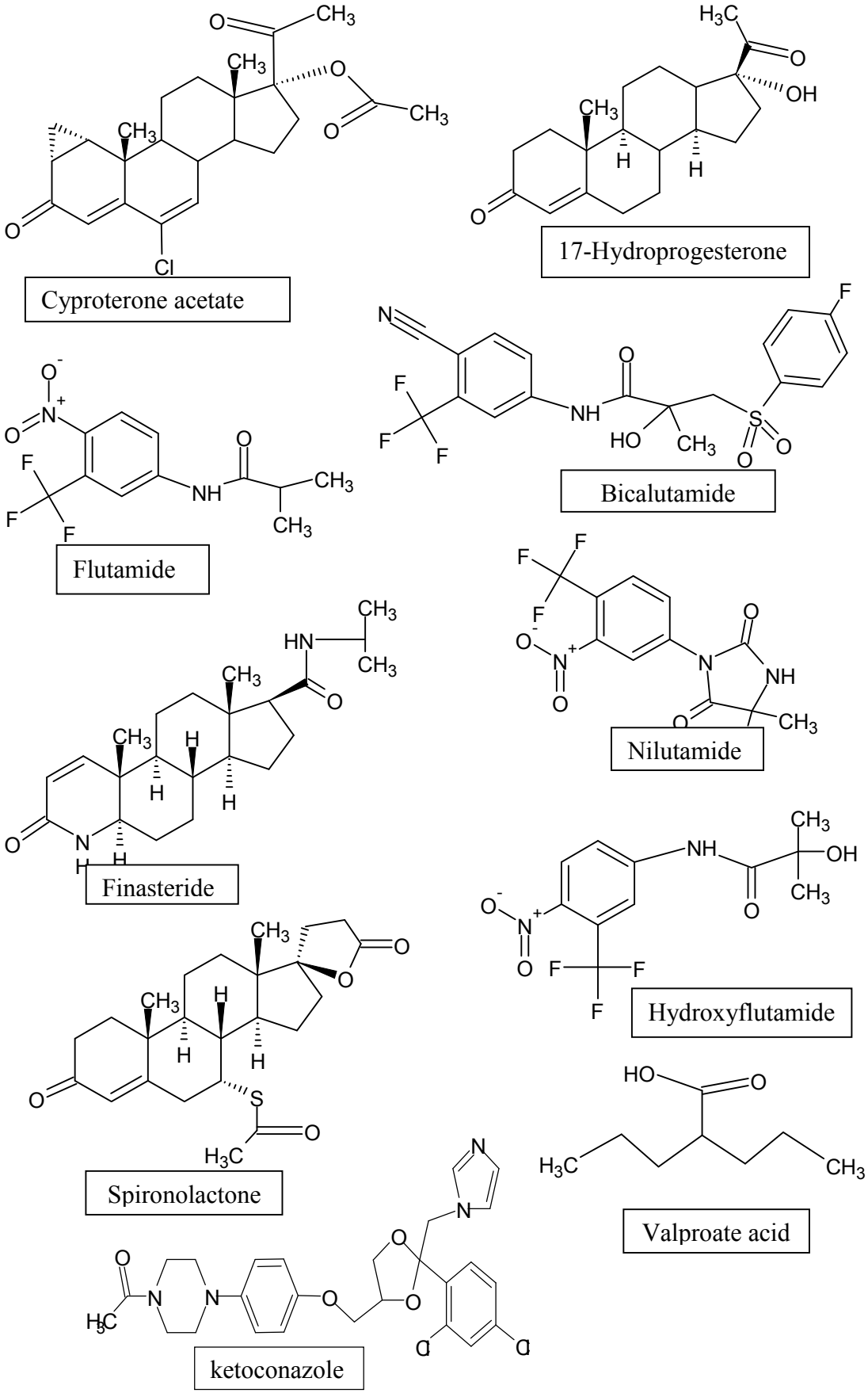


Figure 1.9: Structures of some clinical anti-androgens

1.5.4.4 Industrial Anti-androgens

Anti-androgenic effects of industrial chemicals are increasingly being reported. This surge is due, in part, to the growing production of nonsteroidal chemicals, many of which have not been characterised. Examples of chemicals identified with anti-androgenic activity include a range of phthalates such as dibutyphthalate (DBP), diethylhexylphthalate (DEHP) and butylbenzylphthalate (BBP) (Wilson et al., 2004) and polybrominated diphenyl ethers (PBDEs) such as DE-71 and DE-100 (Stoker et al., 2005). Phthalates are ubiquitous environmental contaminants many of which have been reported to possess anti-androgenic activity *in vitro*. Although, *in vivo* studies indicate that phthalates are non-bioaccummulative, in this instance the parent compounds are metabolised into active hydrolytic monoesters (or oxidative metabolites via enzymatic oxidation of the alkyl chain when high molecular weight phthalates are involved) which may induce abnormal reproductive disorders (Silva et al., 2003; Gray et al., 1999; McIntyre et al., 2001; Mylchreest et al., 1998; Mylchreest et al., 1999). Male offsprings of pregnant animals exposed to DBP and DEHP were reported to induce cryptorchidism, hypospadias and testicular weight loss (Mylchreest et al., 1998). Similarly, exposure of laboratory animals to PBDEs have produced abnormal reproductive health effects some of which are delayed puberty in both sexes of rodents, weight loss during pregnancy, decreased sperm concentration, alteration in the ovary cells, decreased testicular size, exhibition of gender-specific sexual-related behaviours and some birth defects (Akutsu et al., 2008; Chao et al., 2007; Lilienthal et al., 2006; Meeker et al., 2009; Schreder et al., 2006; Stoker et al., 2005; Yang et al., 2009). Other anti-androgenic industrial chemicals detected in the environment or identified through laboratory analysis include bisphenol A (Sohoni and Sumpter, 1998), bisphenol F (Satoh et al., 2004), TCDD, polychlorinated biphenyls (PCBs), ethane-1,2-dimethane sulphonate (EDS) (Wolf et al., 1999; Gray et al., 2001), some polyhalogenated aromatic hydrocarbons (Calle et al., 2002; Sharpe and Irvine, 2004) and polycyclic musks (e.g. tonalide, phantolide, celestolide) and UV filters (e.g. homosalate, 3-benzylidene camphor, benzophenone-3 and 4-methylbenzylidene camphor) (Ma et al., 2003; Schreurs et al., 2005) (Figure 1.10).

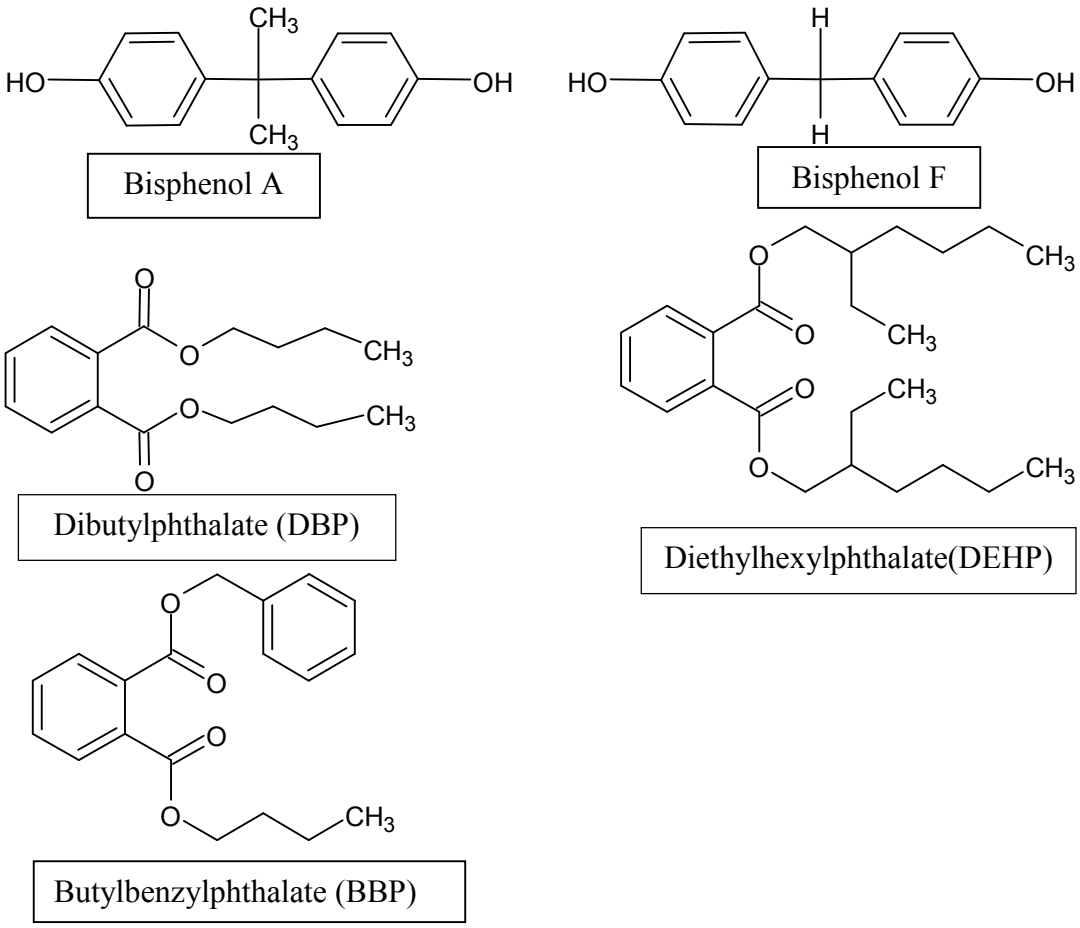


Figure 1.10: Diagrams of some industrial anti-androgens identified in the environment.

1.6. Anti-androgens in Wastewater Effluents as Potential Causal Agents of Intersex in Fish Living in UK Rivers and Lagoons.

Anthropogenic chemicals present in aquatic environments can interfere with the endocrine system of the living organisms dwelling in them to the extent of modulating biological responses that could result in irreversible physiological and reproductive abnormalities. In the late 1970s and early 1980s, anglers in the United Kingdom observed the preponderance of abnormal incidence of intersexuality (i.e. simultaneous occurrence of male and female gonads) in roach (*Rutilus rutilus*), a gonochoristic fish, caught in the River Lee downstream of a sewage treatment plant. Studies have also shown that it is possible for some fish species to develop intersexuality, among other reproductive abnormalities, when exposed to chemicals in wastewater effluents via water samples from aquatic environments (Jobling et al., 1998; Allen et al., 1999b; Harshbarger et al., 2000; Hashimoto et al., 2000; Vigano et al., 2001; van Aerle et al., 2001; Sole et al., 2003). Some of such chemicals identified in wastewater effluents possess estrogenic properties and they include ethynylestradiol, an active compound in birth control pills, bisphenol-A, octylphenol, alkylphenol ethoxylates (APEs) and nonylphenol (Korner et al., 2000). The identities of many other compounds in wastewater effluents are still yet unknown (*ibid*.). It has been discovered that some estrogenic compounds can induce vitellogenin at low concentrations and intersexuality at high concentrations. Some studies have shown that exposure of young medaka to 17β-estradiol at concentration range of 0.01-1.66µg/L for a month would lead to sex reversal while exposure of its eggs and fry hatchlings to a concentration as high as 15µg/L of 17β-estradiol could result in the formation of ovo-testis (Nimrod and Benson, 1998; Koger et al, 2000). Similarly, laboratory exposure of some juvenile fish species to androgens at a range of concentrations can result in the formation of vitellogenin just as its long-term exposure can also lead to the formation of intersex. Laboratory exposure of juvenile zebrafish at concentrations less than 1µg/L 17-methyltestosterone can lead to formation of intersexuality. The modality, by androgens, for inducing intersex in fish has been associated with its (androgens) aromatisation to estrogen in the course of which the concentration of estrogen in the fish builds up to a trigger threshold. For pulp and mill wastewater effluents, aromatisation of androgenic chemicals can lead to induction of vitellogenin in juvenile zebrafish, just as it can also induce intersex (Orn et al., 2006). It is not yet clear whether environmental estrogens are solely responsible for inducing intersex in fish. Laboratory studies have shown that anti-androgens can also

induce intersexuality as estrogens would (Urbatzka et al., 2007). Exposure of some fish species to anti-androgens (e.g.nonylphenol) could also lead to the formation of ovo-testis. Exposure of Japanese medaka (*Oryzias latipes*) to nonylphenol can induce intersex (Gray et al., 2007). Although, nonylphenol has estrogenic effect, it has also been shown to possess anti-androgenic property which underscores the assertion that some estrogens are equally anti-androgens (Sohoni and Sumpter, 1998).

1.7. Wastewater Treatment Plants as Potential Sources of Environmental Anti-androgenic xenobiotics.

Wastewater Treatment Plants (WwTPs) are collection and treatment centres for a wide range of environmental chemicals entering into the aquatic environments. Wastewater contributions from households, industries and commercial settlements are reported to contain high concentrations of chemicals which are hazardous to the well-being of the living environmental organisms. In order to prevent (or minimise) the potential risks that would be posed by their presence, wastewaters are made to undergo physical, chemical and biological treatment processes (Svenson et al., 2003). During these stepwise mechanical treatment processes (classified into primary, secondary and tertiary) (Figure 1.11), various chemicals present in the waste samples are either totally or partially removed (Svenson et al., 2003). The first process involves the use of coarse grits to remove objects which are greater than 3mm in diameter. Further removal of particulates is carried out with sand traps before running the filtrate into pre-settling basins where aeration is applied, and solid organic materials and nutrients are removed. Through the process of adsorption, a wide range of lipophilic compounds are also removed. This complete removal mechanism described above is referred to as primary treatment.

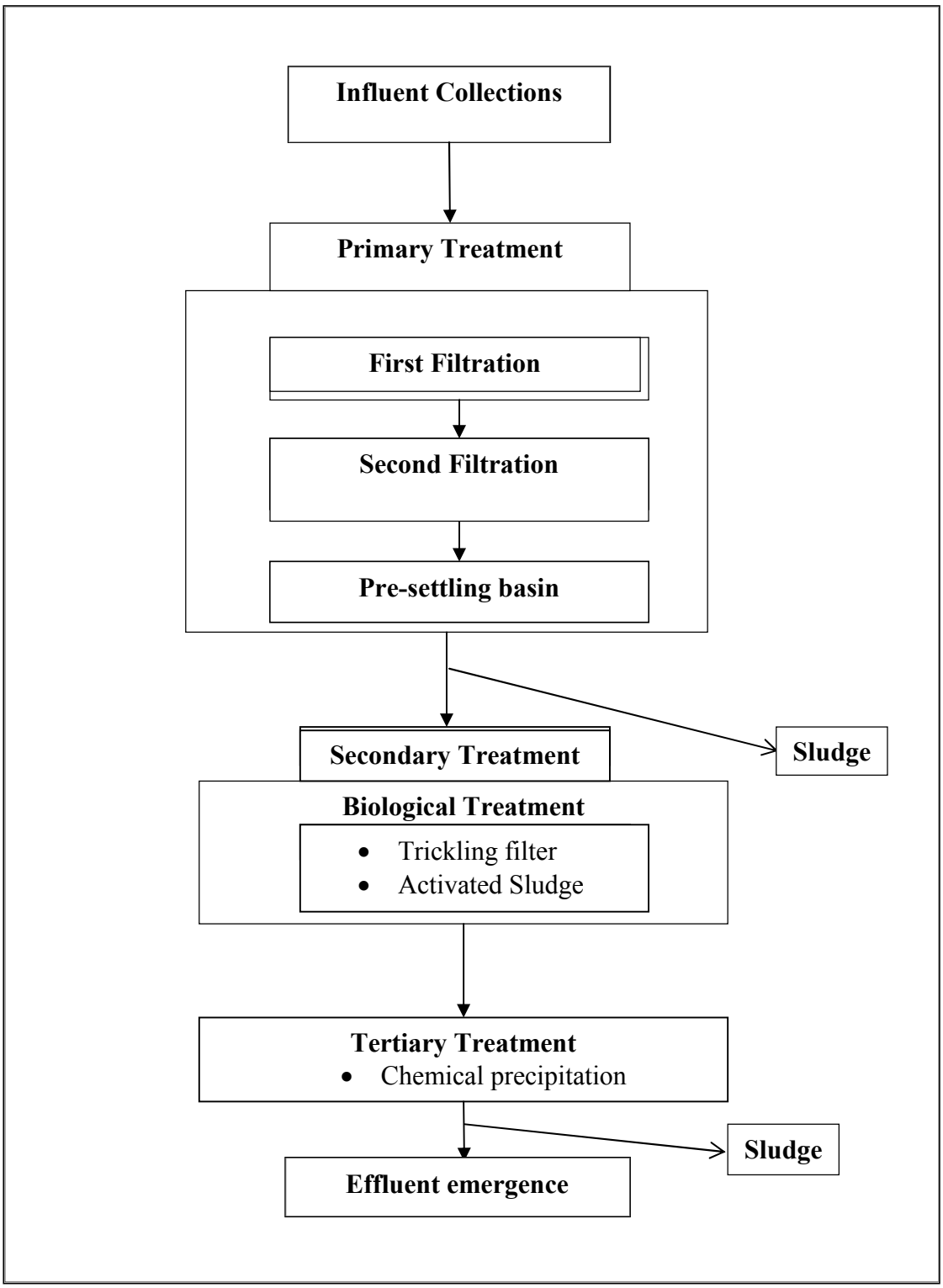


Figure 1.11: A diagram showing the primary, secondary and tertiary stages of wastewater treatment plants.

After leaving the pre-settling basin, the wastewater is pumped into the secondary treatment chamber where the mixture of bacteria and other sewage suspended matters are prepared. This treatment method, otherwise known as biological treatment method,

may proceed in more than one step. Using the activated sludge alone or in combination with trickling filters (or biobeds) and biological nitrogen removal means, bacteria, nitrogen and anoxic substances are removed. The tertiary stage requires the use of chemicals to remove phosphorus, particulates and other dissolved organic matter through the process known as coagulation or chemical precipitation. The coagulants are precipitated and discharged as sludge. The settling of the coagulants may be facilitated using polyelectrolytes which fused the small coagulant units into heavier ones. Commonly used precipitating chemicals include aluminium sulphate, lime and ferric chloride (Svenson et al., 2003). Sometimes a direct precipitation of these contaminants with chemicals may be undertaken after the first and second filtration (*ibid.*). In which case, the total microbial removal may be partly compromised. During this removal process, the sludge (which represents the waste substances removed) is treated and may be used as agricultural fertiliser. Otherwise, the sludge may be digested either anaerobically or aerobically or converted to compost manure.

Generally, a series of physical, chemical and biological activities are discovered to occur at the WwTPs during treatment processes through which chemicals and other unwanted components are removed. These, in summary, include flocculation (Kim et al., 2002; Ternes et al., 2002), sedimentation (Zhang and Emary, 1999; Strenn et al., 2003), coagulation (Adams et al., 2002), precipitation (Strenn et al., 2003), biofilteration (Shon et al., 2006), sorption or adsorption into sludge (Petrović et al, 2001), vaporisation or volatilisation (Meakins et al., 1994; Birkett and Lester, 2003), photolysis (Gray and Sedlak, 2003; Liu et al., 2003; Mansell et al., 2004), biotransformation or microbial metabolism (biodegradation, aerobic and anaerobic degradation) (Holbrook et al., 2002; Johnson and Sumpter, 2001; Semple et al., 1999) and chemical transformation (chemical hydrolysis by oxidation and reduction, enolisation and isomerisation) (Huang et al., 2001; Sedlak and Pinkson, 2001).

Studies have shown overtime that the final effluents which are released into receiving rivers and lagoons contain a complex mixture of chemicals indicating that some compounds are not completely eliminated during the treatment process. Published studies indicate that a wide range of some compounds found in effluent wastewaters possess estrogenic, anti-estrogenic and androgenic activities. A few of such reports have also indicated the occurrence of compounds having anti-androgenic activities. The diversity of the wastewaters treated is better appreciated based on the nature of the community from where supplies originate. By the same relationship, the nature of

compounds present in wastewater effluents is largely dependent on the origin of their sources. It is expected that a more industrialised community will produce wastewaters with higher industrial contribution than less industrial communities. However, due to the pathway of this research, focus will be directed to wastewaters with minimal industrial contribution and mainly domestic input.

1.8 Bioassays for Screening Endocrine Disruption Chemicals in Environmental Samples.

Bioassays are biomolecular preparations or mixture of cellular formulations that can be used to activate and evaluate effect-based measureable end-points. Through formation of a new biomolecule, activation or inhibition of chemical reactions, induction of immunological responses or any variable parameter, screening of bioactive chemicals in environmental samples are achieved. The relevance of these biological assays includes detection, measurement and evaluation of biological activity, potency and toxicity of chemical compounds being considered for pharmaceuticals, or those derived from effluent wastes, contaminated soil and sediments. Two main categories of bioassays predominantly used are *in vitro* and *in vivo* bioassays.

1.8.1 *In vivo* Bioassays

Although *in vitro* bioassays are the most frequently used means of screening the endocrine chemicals especially due to the relative ease of use, cost effectiveness, reproducibility, sensitivity and accuracy, *in vivo* bioassays is still considered as the best evaluation approach. Tests of chemicals for endocrine effects are carried out in the system of living animals where true reaction of such an animals to the activity can be effectively measured. Common *in vivo* assays used are Hershberger, three-spined stickleback kidney cell culture assay and Tier 1 screening assay (Gray, 1998; Jolly et al., 2006; Katsiadaki et al., 2002, 2006). Given that the volume of environmental samples prepared for analysis at any specific time could be enormous, it is practically uneconomical to adopt *in vivo* method. For that reason, discussion on bioassays will be limited to *in vitro* bioanalysis.

1.8.2 *In vitro* Bioassays

In vitro bioassays are a broad range of rapid, cost-effective bioanalytical techniques developed based on high sensitivity, specificity and optimally throughput biological scheme and used to identify, quantify and screen for the biological effects of endocrine disruptors from various environmental samples (Fertuck, 2002; Zacharewski, 1997). Three major *in vitro* bioassays are currently used to screen androgen and anti-androgens. They include: receptor competitive binding assays (or transcription activation assays), cell proliferation Screen Assays and Reporter Gene Assays. Receptor binding bioassays are test standards developed to bind to a specific receptor sites of target compounds. The non-radiolabelled ligand (the test compound) engaged in competitive binding with the established radiolabelled ligand (hormone) for androgen receptor site (Lambright et al., 2000) situated on the cellular or nuclear region of the target cell or tissue during incubation (Lambright et al., 2000; Wong et al., 1995). The problem associated with this type of assay is its failure to distinguish between androgenic and anti-androgenic activity of test compounds which explains why it is poorly selective. Androgen cell proliferation screen assay explores the ability of endogenous androgen to stimulate cell proliferation and induce hypertrophy in male secondary sex organs (Sonnenschein and Soto, 1998). The proliferation process is activated when cell lines interact with the test compounds. During the interaction, the test samples replace receptor binding on the labelled hormone conjugate leading to the occurrence of bioactivity. For anti-androgenic compounds, the cell proliferation process is inhibited (Sonnenschein and Soto, 1998). A wide range of cell lines is readily available for this type of assay, which includes mammalian cells. Examples of this assay are MCF-7 and A-SCREEN. The major drawback is the inter-laboratory (and intra-laboratory) variability but can be employed productively, efficiently and effectively to distinguish androgenic and anti-androgenic activity due to its selectivity.

Endocrine disruptors have also been successfully evaluated with receptor gene bioassays where yeast or cell lines are used to measure the degree of transcriptional activity generated in response to hormone stimulation. The yeast or cell lines which are developed from either mammalian cell lines (e.g. MCF-7, COS-1, CHO, AR-CALUX, HEH293) or yeast strains (e.g. *Saccharomyces cerevisiae*) are transfected with plasmids encoding the hormone receptor and the androgen-dependent receptor gene (Figure 1.12). The receptor gene is coupled with a promoter (e.g. β-galactosidase or luciferase)

which consists of hormone response elements (HREs) via which the receptor activation process is facilitated (Sohoni and Sumpter, 1998; Roy et al., 2004).

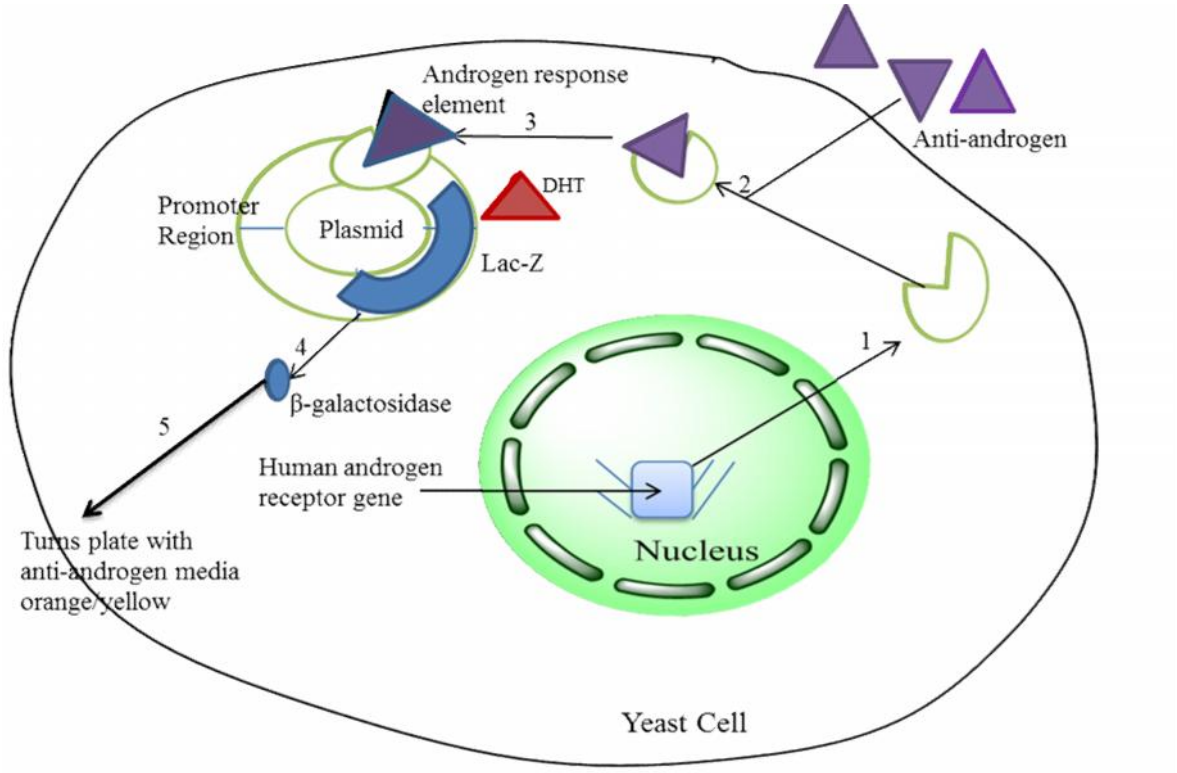


Figure 1.12: Diagram showing the (i) typical structural configuration of a genetically modified yeast strain in anti-androgen yeast screen (AYAS) assay, (ii) anti-androgenic binding pathways (1-3) and the (iii) activation of gene expression (4-5). The human androgen receptor (hAR) gene is incorporated into the yeast genome and expresses cytosolic hAR. In the presence of an androgen ligand such as DHT, it binds to an androgen receptor element (1) occurring within the promoter on the inserted plasmid. The anti-androgen complex (3) formed between the anti-androgen molecule and the human receptor gene (2) inhibits DHT, the natural steroid agonist, and the generation and expression of *Lac-Z*, (4) the reporter gene through which enzyme β–galactosidase is produced. Non-secretion of β–galactosidase into the medium containing the anti-androgen will cause a chromogenic response from red to yellow colour to occur.

Sample-receptor complex is produced from the active binding brought about by chemical interaction of the potential androgen sample and the receptor present on the

yeast strains. The HRE of the reporter gene is activated when the sample-receptor complex binds to it following which the enzymes such as luciferace, β-galactosidase and chloramphenicol acetate transferase, which are suitably measured with luminometry or spectrophotometry, are produced (Sohoni and Sumpter, 1998; Roy et al., 2004). Generally, reporter gene bioassays are easy to culture and manipulate genetically. In addition, they possess the ability to metabolise the test samples. They produce non-uniform permeability when yeast cell walls are exposed to test compounds which cause them to have different set of AR co-regulators better than the mammalian cells. They have been used for screening of androgens and lately anti-androgens (*ibid*.).

1.9 Aims and Objectives.

The aims of this research work are to investigate, identify and quantify anthropogenic chemicals that can induce androgen-receptor antagonism, and to measure the relative potency of these compounds in predominantly domestic wastewater influents and effluents sourced from Horsham Wastewater Treatment Plant, South East of England. This investigation encompasses the following objectives:

1. To investigate the profiles of androgen-receptor antagonists in a domestic wastewater (from Horsham wastewater treatment works) using the *in vitro* androgen recombinant receptor transcription yeast screen (AYAS).
2. To identify the structures of androgen receptor antagonist chemicals present in effluent and influent samples from Horsham wastewater treatment works using Toxicity and Identification Evaluation (TIE) techniques.
3. To measure the relative potency of anti-androgens identified in the wastewater samples.

Literature Review

1.0 Background

Chemical communication is the fundamental process by which all living organisms exchange vital information within their internal systemic components, with other organisms and with their immediate environment (Cheek et al., 1998; Fox, 2004). This process is basically accomplished through three major communication mechanisms: endocrine, immune and nervous systems. Although their modes of action are dissimilar, the endocrine and immune communication systems are co-ordinated through the blood (Pardridge, 1981). The presence of some man-made chemicals and their by-products in the global environment can pose a serious risk to public health when they interfere with any of these communication mechanisms (Arcand-Hoy and Benson, 2001). Given that the endocrine communication system is responsible for the regulation of metabolic processes, homeostasis, reproduction and basic developmental mechanisms (Kime, 1998), it is thus possible for some chemicals that possess hormone-like behaviours to disrupt its normal regulatory activities. Within the progressive attempts aimed at meeting the rising challenges and burden of the human needs (health, social, food and economic demands), production and use of consumer products such as pharmaceuticals, cosmetic products, laundry and washing detergents, agronomic and synthetic foodstuffs and other human consumables have increased, and their potential for environmental contamination has also heightened. As a consequence, exposure to cycles of potentially harmful chemicals can bring about endocrine modulation and genetic alterations, which may lead to dangerous health effects in humans, mammals and other living organisms. This revelation has added to the speculation that environmental chemicals may contribute to the growing cases of certain global health problems.

1.1 Endocrine System

The endocrine system is responsible for co-ordinating a wide range of complex biological processes in the body. It controls the physiological activities of the body by secreting hormones through the collection of endocrine glands and non-endocrine organs in the body. Some of these hormone-secreting bodies include the hypothalamus (which is situated above the brainstem and under the cerebrum), the pituitary glands (that are attached to the underside of the brain by stem stalks) and the various other

organs such as thyroid, kidney, pancreas, ovary and testis (Figure 1.0). Hormones are chemical messengers that communicate useful coded information to the target sites where chemical ‘messages’ are utilised to bring about necessary changes. For example, hormones such as gonadotropin-releasing hormones (GnRH), thyrotropin-releasing hormones (TRH) and corticotropin-releasing hormones (CRH) are secreted by the neurons in the hypothalamus and transported via the blood to encourage the pituitary glands to secrete some endocrine regulatory hormones. Hormones such as thyroid-stimulating hormone (TSH), follicle-stimulating hormone (FSH), luteinising hormone (LH), adrenocorticotropic hormone (ACTH), prolactin (PRL) and growth hormone are secreted by the anterior pituitary while antidiuretic hormone (ADH) and oxytocin (OXY) are produced by the posterior pituitary. At the particular target site where exchange of chemical messages are required, a number of functional receptors are situated either on the surface of the target cells on the plasma membrane or at the nuclear region. These facilitate the binding of hormones to the specific receptors through which chemical signalling takes place. Action of chemical communication via hormones could occur in a localised area of the body (autocrine and paracrine effects) and, sometimes, it extends to all over the body (endocrine effect) (Bigsby et al., 1999).

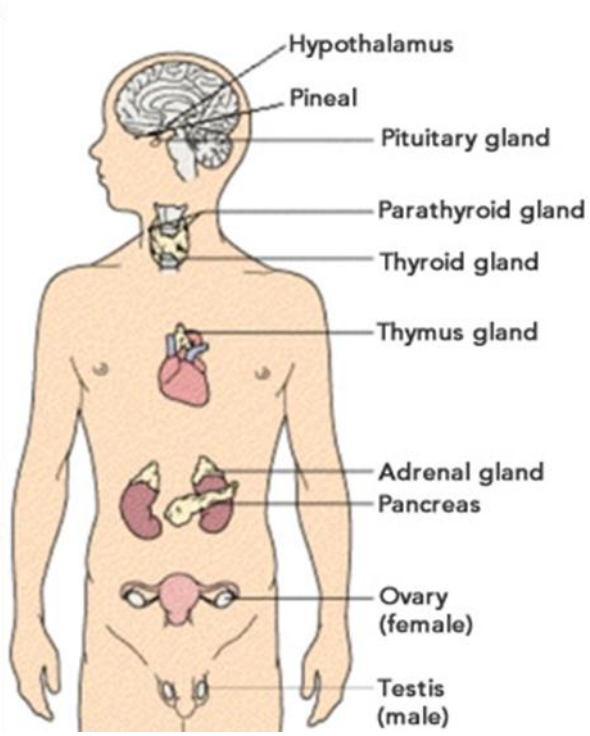


Figure 1.0: The diagram of human endocrine system showing the locations of various internal organs associated with hormone secretion in the body. Adapted from Endocrinology Medicine website, www.endoatsoim.com

1.2 Steroidal and Non-steroidal Hormones

Hormones occur as steroidal and nonsteroidal molecules with different structural forms which accounts for their diverse molecular weight. Their occurrence in different molecular weight as lipophilic or lipophobic compounds will explain why the activity of some hormones are localised to a particular region of the body. Generally, steroids are found in nature as fat-soluble, sparingly water-soluble, organic compounds (lipids) which have evolved as plant and animal hormones (Bishop and Koncz, 2002). Steroidal hormones are broadly classified into five subdivisions based on their physiological behaviours, which are: mineralocorticoids (which influence sodium retention through renal tubules), glucocorticoids (which regulate carbohydrate metabolism and inhibit the absorption of calcium in the intestine) and the three sex steroid hormones (estrogens, androgens and progestins) (Miller, 1988).

Non-steroidal hormones constitute the majority of hormone population in a category comprising proteins, peptides, amino acids and fatty acid derivatives (Hinuma

et al., 1998). Peptide hormones are water-soluble, relatively moderate molecular weight molecules (in comparison with protein) which are formed by varying chain-lengths of linear and/or ring amino acids (Neal, 2001). Some hybrid non-steroidal hormones, formed as a linkage between peptide hormones and carbohydrates, have also been identified. This class of hormones are known as glycoproteins (*ibid.*). Few common examples of glycoproteins are luteinising hormone (LH), follicle stimulating hormone (FSH), human chorionic gonadotropin (β-hcG) and thyroid-stimulating hormone (TSH) (*ibid.*). In this section, the functions of some steroid hormones especially sex steroid hormones and non-steroid hormones like GnRH, CRH and TRH are discussed.

1.2.1 Sex Steroid Hormones

The sex steroid hormones are synthesised by the endocrine system of most animal species and are responsible for the regulation of critical stages in the animals' life cycle which include gametogenesis, fertilisation, sexual development and reproduction (Gross et al., 2003). They can be divided into three major classes; namely: estrogens, androgens and progestins (or progestagens). Generally, steroid hormones have chemical structures composed of a three-dimensionally arranged four, fused carbon rings in a 6-6-6-5 ring structure with a ketonic, aldehydic or alcoholic functional group and terpenoid appendages in rare instances (Xu et al., 2003; Ying et al., 2002). This core nuclear arrangement has been described as cyclopentan-o-perhydrophenanthrene ring system, having 17 carbon atoms in total (Shen and Lin, 2006; Wynn, 1965; Ying et al., 2002). The ring nature varies from one steroid to another. Whereas the first six-carbon ring may be saturated (cyclohexane) or partly saturated in one structure, yet in another it would be fully unsaturated (benzene ring). The fourth ring is a cyclopentane, a saturated five-carbon ring (Xu et al., 2003) (see Figure 1.1).

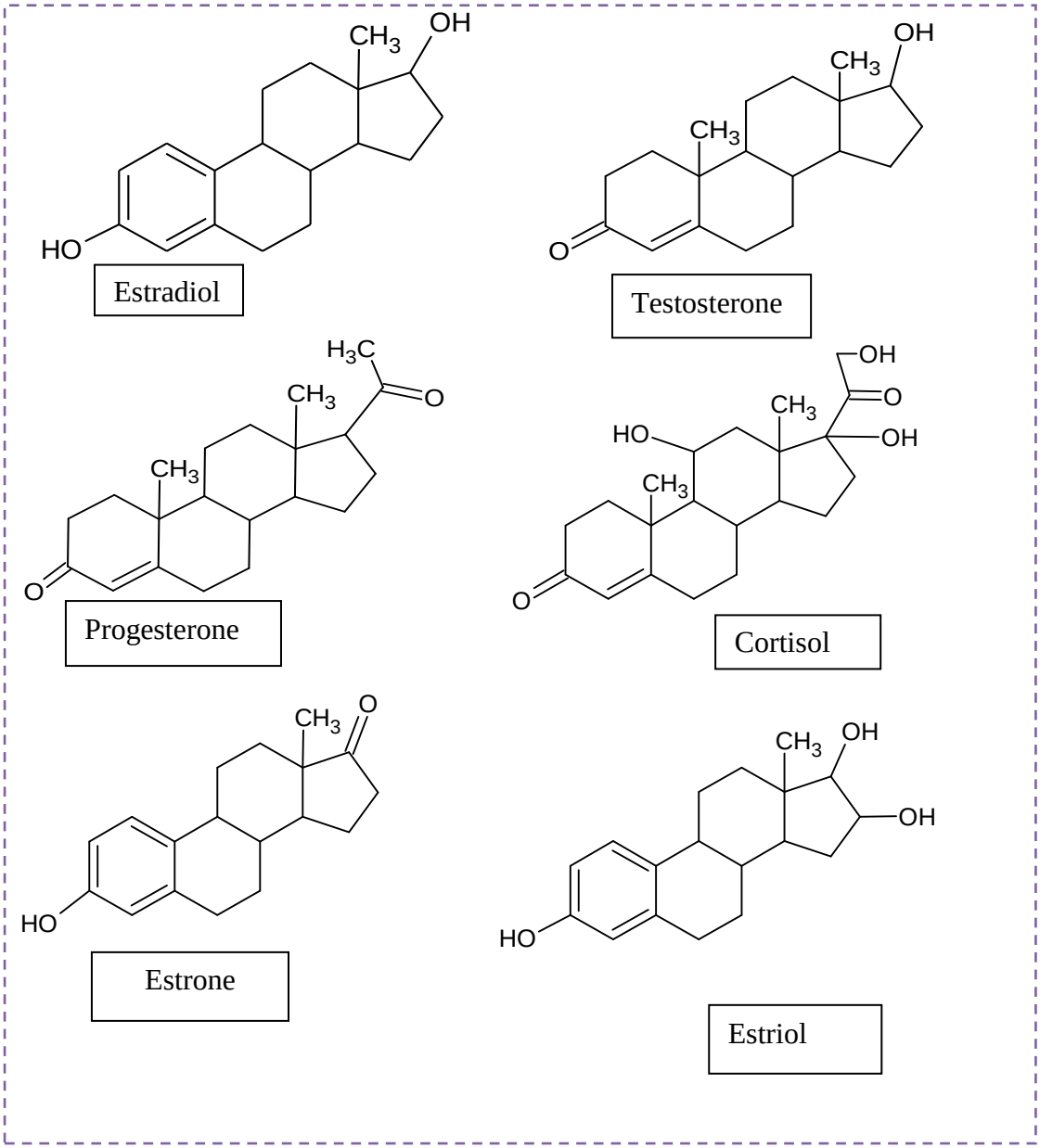


Figure 1.1: Chemical structures of common endogenous steroid hormones in the vertebrate endocrine system.

1.2.1.1 Estrogens

Estrogens are a group of steroid sex hormones which are responsible for the maintenance and regulation of growth, development, differentiation and function of reproductive organs, sexual characteristics and other reproductive processes in both mature wildlife and humans (Ciana et al., 2003). In addition, they control some important body organs such as the bones, brain and cardiovascular system (*ibid.*). Although, these hormones are found largely in females, they are present in male animals in trace amounts. In male animals, estrogen may regulate the commencement process of

spermatogenesis by facilitating spermatogonia multiplication and by enhancing the role of follicle stimulating hormone (FSH) in activating spermatogenesis (Ebling et al., 2000; Kula, 1988; Kula et al., 2001; Walczak-Jedrzejowsksa et al., 2005). They are composed of estrone, estradiol and estriol which are produced by the ovaries, adrenal glands and the fatty tissues. The first two hormones are formed in the ovaries while the last is produced in one of the reproductive tissues during luteal phase in the corpus luteum (Barlow and Logan, 1966). Estriol can also be formed through aromatisation of 16-OH-dehydroepiandrosterone sulphate (a DHEA derivative formed in the liver) during pregnancy in the placenta (Ryan, 1959).

1.2.1.2 Androgens

Androgens are a class of sex steroid hormones which are produced by both mature male and female animals. They control the development and maintenance of masculine characteristics, sperm induction and sexual differentiation as well as enhance virility and libido; in addition, they regulate water (through the synthesis of aldosterone hormone which is produced by adrenal cortex of the adrenal gland situated above the kidney) (Shigeoka et al., 1985; Milledge et al., 1983), nitrogen retention (Urban et al., 1995; Brodsky et al., 1996) and activate skeletal growth (Quigley et al., 1995). This class of hormones include androsterone, testosterone and dihydrotostesterone and they are secreted by the testis, ovaries, adrenal glands and placenta. Androgens are produced from enzymatic metabolism of cholesterol and steroid intermediates as shown in Figure 1.2.

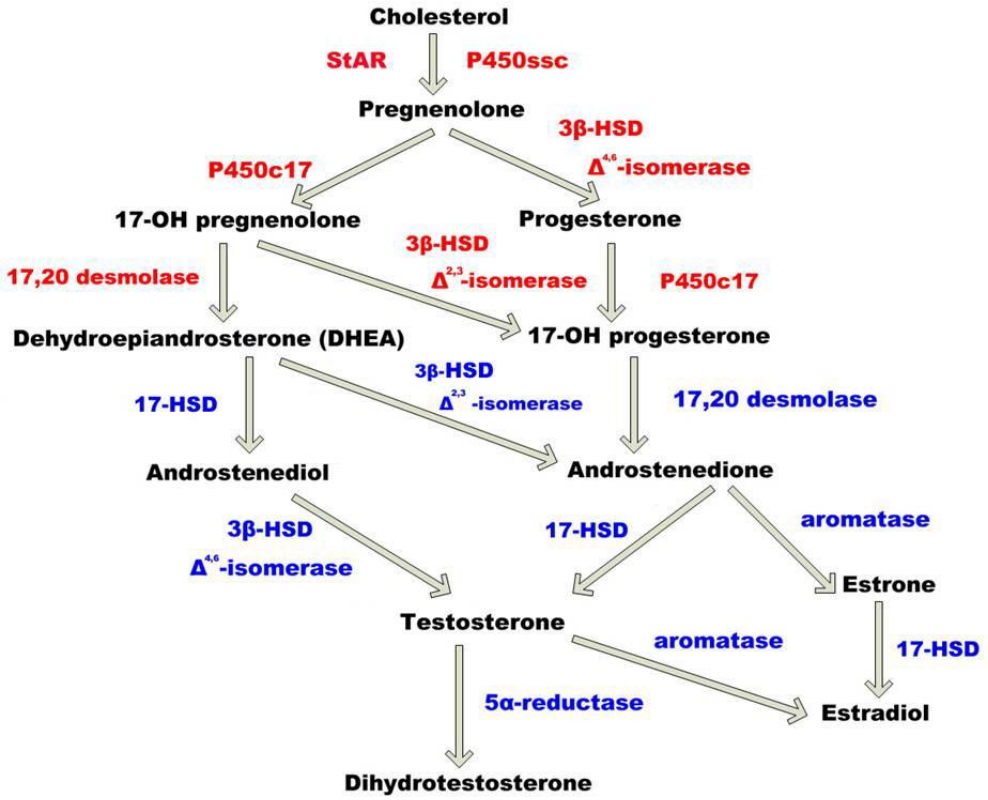


Figure 1.2: Biosynthetic pathways of some androgen hormones and their enzyme-mediated conversion products. The blue enzymes catalyse the formation and metabolism of androgen hormones including the aromatase. The red enzymes act on the pro-androgen hormone precursors and metabolites produced during steroidogenesis (adapted from, http://www.conf.ncku.edu.tw).

1.2.1.3 Progestins

Progestins are a class of steroid hormones derived from both natural and synthetic origins. They are used to sustain pregnancy to maturity (reduce preterm delivery), inhibit ovulation and menstrual cycle control, reduce the endometrial wall and stem the growth of prostate cancer (Sitruk-Ware 2004). The natural progestins (such as progesterone) are manufactured and secreted in the corpus luteum (ovary), the placenta and the adrenal cortex of female humans (Schindler et al., 2003). In the male humans, this hormone is secreted by the testes and the adrenal cortex (Simpkins et al., 2005). Comparatively, the level of progesterone in female humans is higher than that in male counterpart but this drops to the level found in the male during the follicular stage of the menstrual cycle (Chrousos et al., 2001). Apart from the natural progestins, a wide range of progestins are being manufactured on an industrial scale today for therapeutic use (e.g. manufacturing of contraceptives) (Falconer, 2006). The presence of

polychlorinated biphenyls (PCBs) in the body could facilitate the breakdown of progesterone in the liver (Colborn et al., 1997). For example, it has been documented that women who experience incessant miscarriages possess relatively higher level of PCBs in their bodies compared to those carrying normal pregnancies (Leoni et al., 1989; Saxena et al., 1981). The presence of PCBs could possibly lead to drastic reduction or non-availability of progesterone in the body system (*ibid*.). While the occurrence of PCBs may be correlated to the incessant miscarriages in pregnant women, it has not yet been proven that they are responsible for the phenomenon.

1.2.2 Non-steroidal Hormones

1.2.2.1 Gonadotropin-Releasing Hormone (GnRH)

Gonadotropin-releasing hormone (GnRH) is a decapeptide produced by the cell bodies of hypothalamic neurons present in the brain. It is released into the portal blood system and transported to the anterior pituitary to stimulate gonadotropin production. The gonadotropin FSH and LH are heteromeric glycoproteins which stimulate gonadal synthesis of sex steroid hormones (testosterone, estrogen and progesterone), which are required for spermatogenesis and oogenesis (Dalken et al., 2001). Both FSH and LH hormones have a similar α-subunit and a distinctive β-subunit (Conn, 1994). The secretion of GnRH occurs during the outset of puberty. Although the function of GnRH in the gonads (the testis, the ovary), the uterus (the placenta), the breast and the central nervous system is unknown, it has been detected in these organs (Grossman, 1998).

1.2.2.2 Corticotropin-Releasing Hormone (CRH)

Corticotropin-releasing hormone is a 41-amino acid peptide produced by the parvocellular neurons situated in the hypothalamic paraventricular nucleus (Vale et al., 1981). In addition to their presence in the hypothalamus, the CRH-secreting neurons are distributed across the central nervous system as well as the adrenal cortex, the spinal cord and the limbic regions (Brady et al., 1990; Merchenthaler et al., 1984; Swanson et al., 1983; Thompson et al., 1987). It influences the synthesis of adenocorticotropic hormone (ACTH) by activating the anterior pituitary cells. The ACTH secreted acts on the adrenal cortex and facilitates secretion of glucocorticoids (e.g. corticol in human and corticosterone in rat), the hormones which act in response to stress. The ACTH is directly responsible for regulating the effect of stress on immune system or inflammatory system (Berridge and Dunn, 1987). Although CRH is a widely known

moderator of endocrine and immune responses to stress, it is also responsible for a wide range of behaviour such as reproduction, arousal and feeding (De Souza, 1995; Dunn and Berridge, 1990). It is a key regulatory element required during human pregnancy and parturition (*ibid.*). As the period of gestation advances, the concentration of the CRH peptide in placenta increases correspondingly as that in the maternal plasma (Sasaki et al., 1987; Riley et al., 1991).

1.2.2.3 Thyrotropin-Releasing Hormone (TRH)

Thyrotropin-releasing hormone is another tropic tripeptide hormone which is produced by the paraventricular nucleus of the hypothalamic neurons. It is released at the median eminence to facilitate the secretion of thyrotrophic-stimulating hormone (TSH) at the anterior pituitary which regulates the release of triiodothyronine (T_3) and thyroxine (T_4) from the thyroid gland (Ghamari-Langroudi et al., 2010). The thyroid hormones (T_3 and T_4) secreted can also act as the negative feedback mechanism by controlling the activity of TRH-secreting neurons in the hypothalamus (*ibid.*).

1.2.3. Intracellular and Extracellular Hormone Receptors

Hormones are biological change agents that bind with specific hormone receptors to regulate some essential biological activities (such as growth, metabolism, puberty and reproduction) in the body. Extracellular messages carried by the hormones are converted to intracellular signals after forming a complex with the hormone receptors of the target cell. The nature of the receptors involved in such binding depends on the chemical structure of the hormone. Based on their cellular localisation, hormone receptors are broadly classified into two main categories, namely extracellular receptors otherwise known as cell-surface receptors (CRs) and intracellular receptors (IRs). In addition to the two major receptors, there are some receptors whose activating ligands are not known. This class of receptors is referred to as orphan receptors. CRs are glycoprotein structures located in the phospholipid bilayer of the cell membrane which explains the reason why they are referred to as cell-surface receptors. Structurally, they transverse the hydrophilic outer cell surface, the hydrophobic plasma membrane and hydrophilic inner framework of the cell cytoplasm. CRs are remarkably sensitive to different extracellular signalling molecules especially high molecular mass peptides that cannot diffuse naturally into the cell through the plasma. The commonly known

examples of CRs are G-protein coupled receptors (GPCRs), receptor tyrosine kinases (RTK), integrins and toll-like receptors.

Intracellular receptors are hormone receptors using hormone-binding to induce intracellular signals in a cellular system. Unlike the CRs, they are localised in either the inner cytoplasm or the nucleus of the cell. This class of hormone receptors has affinity for low molecular mass hormones which can easily diffuse through the plasma membrane to initiate binding in the cell. Examples of intracellular receptors include androgen receptor (AR), estrogen receptor (ER), progesterone receptor (PR), thyroid hormone receptor (THR), retinoic acid receptor (RAR), retinoid X receptor (RXR) and vitamin D receptor (VDR). Interestingly, some intracellular receptors can also bind and activate certain CRs. A member of the GPCRs family (GPR30) has recently been identified to demonstrate high binding affinity to estrogen in contrast to binding to the known cellular estrogen receptor (e.g. ERα and ERβ) (Revankar et al., 2005; Thomas et al., 2005). It was also discovered that the GPR30 binding produces multiple intracellular responses that are associated with growth, proliferation and differentiation (Filardo et al., 2000; Kanda et al., 2003; Kanda et al., 2004). This new finding is regarded as a possible significant step towards treating tumours which are not dependent on estrogen receptor agonism.

1.2.4 Mechanisms of Hormone Action

Hormones modulate gene expression via genomic and non-genomic signalling. While a genomic signalling is initiated by influencing the RNA and certain protein synthesis through receptor complex interaction with the hormone response element of the DNA, a non-genomic pathway bypasses these components by recruiting other signalling pathways (Vincent et al., 2008). Some non-steroid hormones, such as insulin, oxytocin and vasopressin, are known to elicit cellular expressions via non-genomic signalling but steroid hormones (e.g. estrogen, progesterone and androgen) can regulate cellular expressions via both genomic and non-genomic mechanisms (Losel et al., 2003; Simoncini and Genazzani, 2003; Vincent et al., 2008). In this Section, both transcriptional and non-transcriptional mechanisms of steroid and non-steroid hormone actions are briefly described.

When steroid hormones are released into the blood stream after secretion, they are transported to the target cells where the process of biological changes is set to occur. At these cells, they locate and bind to the functional intracellular receptors, known as

steroid receptors, which are present either in the cytoplasm (cytosol), when in free state or in the nucleus, when they occur as complex (see Figure 1.3; Ing and O'Malley, 1995; Jacobson et al., 1995; Kemppainen et al., 1992; Waller et al., 2000). Steroid hormone receptors are biological co-change agents with hormones which can facilitate internal communication and transformation. Steroid hormone receptors are made up of amino- and carboxyl-terminus, the DNA and ligand binding domains (LBD) (Beato, 1989). Generally, they exist in the cytoplasm as a monomer with perinuclear distribution as well as homodimers or heterodimers (Jacobson et al., 1995; Kemppainen et al., 1992; Waller et al., 2000). After the formation of steroid receptor complexes, the heat shock proteins (HSP) detached from the receptors while the receptor complexes formed diffuse through the cytoplasm and the nuclear pores as they translocate into the nucleus (Keller et al., 1996). In the nucleus, the receptor complexes bind to the human response elements (HREs) on the DNA. Prior to the binding, the receptor complexes undergo a series of conformational changes which modify them into a form that will interact with the hormone response elements (HREs) (see Figure 1.3; Beato et al., 1987; Beato, 1989; Mckenna et al., 1999; Mckenna and O'Malley, 2002). To initiate transactivation, the HREs attract coactivators or corepressors which induce the transcription of downstream DNA to messenger RNA. The necessary gene expressions are stimulated in the target cells by the protein formed through the RNA transcription (Ing and O'Malley, 1995). The choice of either coactivators or corepressors is determined by the nature of the ligand that bounds to the receptor. An agonist signalling could sometimes recruit coactivators which will lead to increased expression of one or more genes and the proteins which they encode. The process is referred to as gene up-regulation. For instance, increase in concentration of testosterone in the bloodstream induces production of more androgen receptor and hence activates gene up-regulation that would bring about skeletal muscle development in animals (e.g. cattle) (Squires, 2003). Conversely, decrease in certain gene expression and their encoding protein are induced when an agonist signalling occurs by recruiting corepressors. The phenomenon is known as gene down-regulation. The build-up of progesterone concentration in the uterine and endometrial regions leads to the reduction of progesterone receptors and hence causes gene down-regulation (Bazer, 1998). While some knowledge of the genetic behaviour of agonist signalling exists, little is known about genetic behaviour of antagonist signalling.

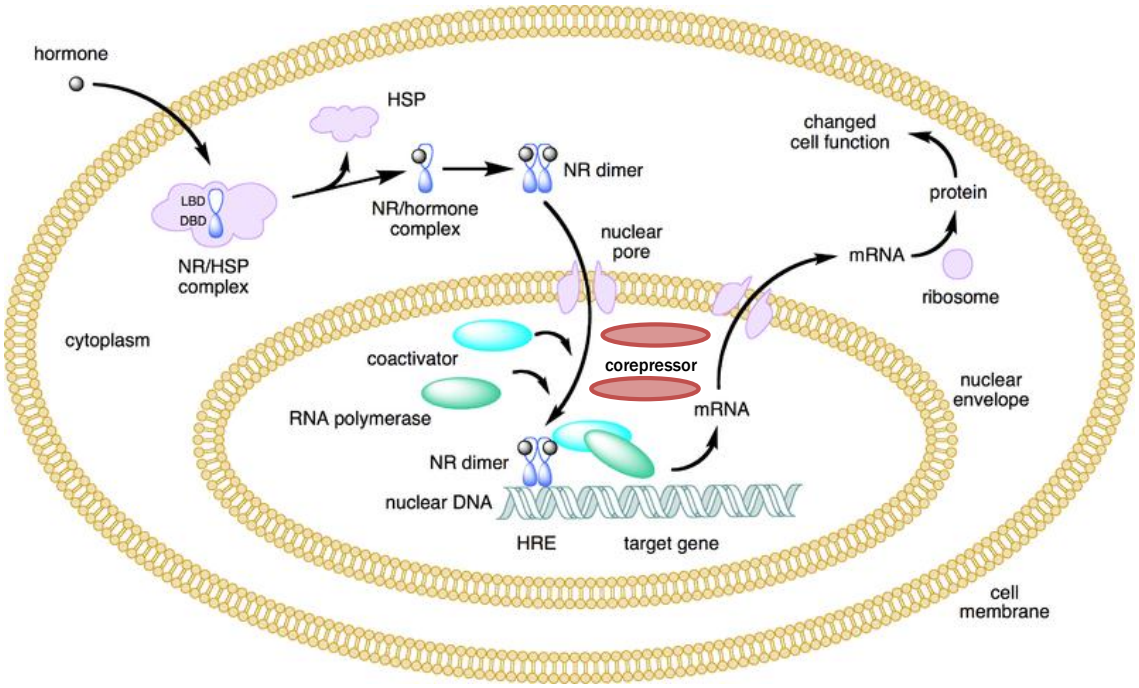


Figure 1.3: Structural configuration and nature of a typical nuclear (e.g. androgen) receptor: showing the location of nuclear receptor (NR) in the cytosol in absence of ligand, the dissociation of heat shock proteins (HSP), dimerization and nuclear translocation of activated receptor as well as the coactivator (or corepressor) recruitment, the DNA binding of the translocated receptor and the translation of downstream DNA to RNA which would effect change in the cell function (adapted from Wikimedia Commons, http://www.commons.wikimedia.org and reconstructed).

The mechanisms by which non-steroid hormones carry out their actions are somewhat complex compared to the modalities involved in steroid hormones. Given the relatively large size of their molecules, it is difficult for non-steroid hormones to diffuse through the cell wall in order to bind intracellular receptors. Rather, they interact with membrane receptors on the surface of the cell to form a complex which generates signal that alters the conformation of the receptor. G-protein coupled receptors (GPCRs) are just one example of a number of protein hormone receptors present in animal tissues. The receptor in such modified configuration facilitates coupling to the G-protein to form hormone-receptor-G protein complex (Birnbaumer and Birnbaumer, 1995). Non-activated G proteins are heterotrimeric guanine nucleotide-binding protein which has three subunits (Gα, Gβ and Gγ subunits) and the guanosine diphosphate (GDP) moiety (Chedrese, 2009). The direct binding of the receptor complex to the Gα-subunit of the

G-protein leads to the activation of G-protein and causes the dissociation of G protein into Gα and Gβγ subunits (Neer, 1995; Surya et al., 1998; Strader et al., 1994). Hitherto activation, the Gα of the G-protein is bound to the GDP as Gα-GDP but upon activation, it becomes Gα-GTP. The activation of G-protein is presumed to facilitate the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) (Neer, 1995; Surya et al., 1998). The activated G protein dissociates from the receptor to modulate the activity of the effector protein, which could be an ion channel or an enzyme (e.g. adenylyl cyclase, phospholipase C and guanylyl cyclase), to produce certain levels of second messengers (e.g. cyclic adenosine monophosphate (cAMP), diacylglycerol (DAG) and Inositol triphosphate (IP_3)) and transduction of signals (Chedrese, 2009).

However, given that Gα subunits carry some G-protein signalling modulating proteins, referred to as GTPase activating protein, GTP can be hydrolysed to GDP (Melmed and Conn, 2005). This same GTPase activating property is possessed by some effector proteins such as adenylyl cyclase (Melmed and Conn, 2005). Upon activation, these effector proteins will interact with Gα–GTP and convert it to Gα-GDP thus making the Gα inactive (Chedrese, 2009; Melmed and Conn, 2005). The Gα reassociates with Gβγ subunits of the G protein, fully ready to repeat the cycle (Chedrese, 2009). Hormones associated with these cell surface receptors (and second messengers) include TSH and catecholamine. As hitherto indicated (Section 1.2.3), CR signalling can also be modulated via other molecular mechanisms such as ligand-gated ion exchange (e.g. nicotinic acetylcholine receptors), receptor serine/threonine kinases (e.g. receptors of activins and inhibins), receptor tyrosine kinases (RTK), receptor guanylate cyclase (e.g. atrial natriuretic factor receptor), integrins and toll-like receptors beside using G-protein coupled receptors (Kronenberg et al., 2008).

1.3 Endocrine Communication Pathways, Feedback Mechanism and Reproduction End-points.

Endocrine communication involves concerted contributions and collaboration of the central nervous system, the hypothalamus, the pituitary and the target tissues. The central nervous system is a major division of the nervous system that controls and co-ordinates dissemination of information useful for regulating biological processes of the whole body. Given that the brain and the spinal cord constitute the central nervous system, it directly supervises the activities of the hypothalamus and the pituitary gland which co-ordinate and regulate the production of endocrine hormones. The

hypothalamus, the pituitary glands and the effector organ or tissue are frequently, conveniently addressed as a single entity because they work in co-operation to produce the desired effects. Thus, many triangular communication partnerships between the hypothalamus, the pituitary gland and any of the different effector organs and tissues in the body have been recognised but this study will be limited to three major ones associated with reproduction, immune system and growth. These are the hypothalamic-pituitary-gonadal (HPG), the hypothalamus-pituitary-adrenal (HPA) and the hypothalamus-pituitary-thyroidal (HPT) axes. It is widely held that for the reproductive system to function normally the activities of the HPG, the HPA and the HPT axes must be balanced out (Simon and Polan, 1994). The HPG axis constitutes one of the major endocrine signalling pathways in which the endocrine hormones are deployed for specific localised actions on the body's reproductive processes (see Figure 1.4). The activities of the hypothalamus, the pituitary and the endocrine effector organs/tissues are complementary. The preoptic region of the hypothalamus is composed of gonadotropin-releasing hormone (GnRH) neurons, which induces the secretion of both luteinizing hormone (LH) and follicle-stimulating hormone (FSH). They work in concert to provide both positive and negative feedbacks to the hypothalamus and the pituitary gland (Conn and Crowley, 1994; Shupnik, 1996). In females, both the FSH and LH function essentially to stimulate the ovaries to produce estrogen and inhibin (Finkelstein et al., 1991; Billiar et al., 2003). They are also responsible for regulating the menstrual and ovarian cycles. While estrogen is known to contribute effectively to the negative feedback mechanism leading to inhibition of GnRH production in the hypothalamus, the inhibin is responsible for constraining activin which induces the production of GnRH producing cells (Holdcraft and Braun, 2004; Drummond and Findlay, 1999). In males, the LH controls the production of testosterone by activating the interstitial cells situated in the testis and the FSH contributes to spermatogenesis (Luconi et al., 2002). As in female, gonadal androgens can regulate cell growth, sensitivity to GnRH and level of expression of the gonadotropins by feeding back directly on the pituitary gonadotropes (Melmed and Conn, 2005). Generally, feedbacks on the hypothalamus and pituitary sites are activated by the gonadal hormones at high concentrations in order to regulate the secretion and release of gonadotropin (Melmed and Conn, 2005) (See Figure 1.4).

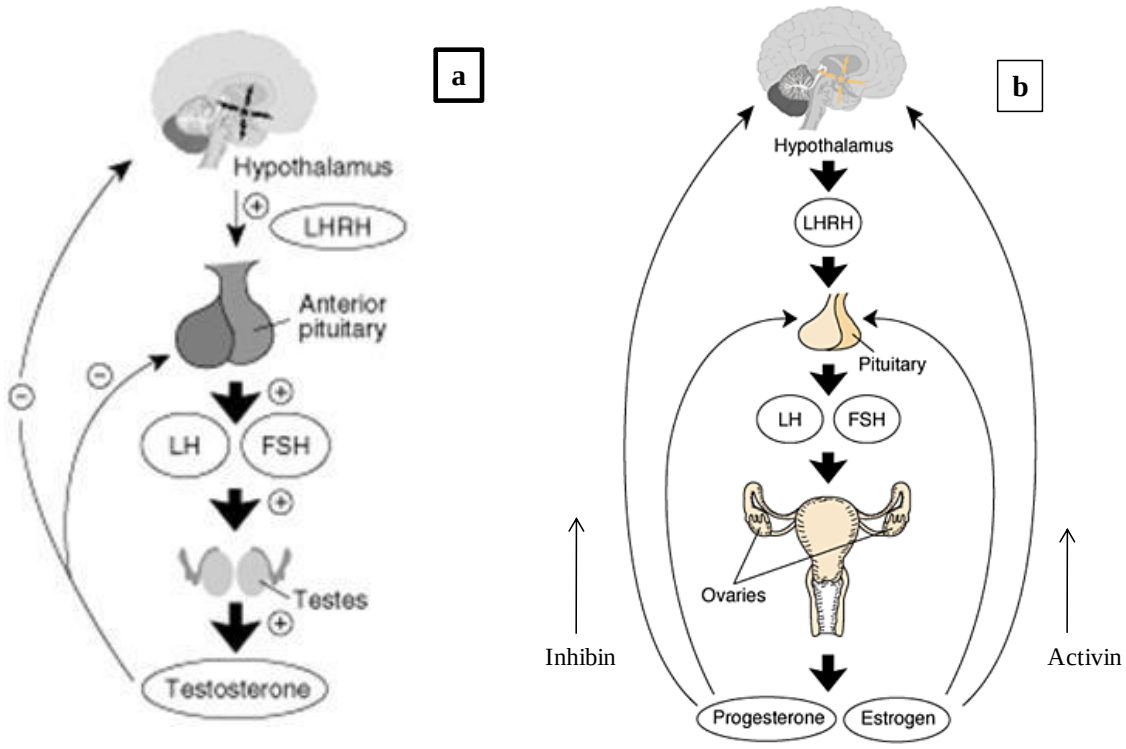


Figure 1.4: A diagram showing the transition of activity and feedback network (cross-talks) between the brain and hypothalamus-pituitary-gonad (HPG) axis during endocrine hormone regulatory activity in the body. In Figure 1.4(a), the + and – symbols in the diagram stand for and trace the positive and negative feedback pathways respectively in the male body. Figure 1.4(b) shows similar progression of activity and feedback mechanism in the female body (both diagrams were adapted from National Institute of Alcohol Abuse and Alcoholism publications, http://pubs.niaaa.nih.gov/publications). The feedback pathways are indicated by the upward arrows. In the two diagrams, the activin and inhibin are secreted by the gonads to regulate the feedback mechanism. The transition occurs via the action of luteinising hormone releasing hormone (LHRH) on the anterior pituitary through to the gonads.

The regulatory ability of the HPG axis could be influenced by the other two axes identified in this study. The HPA axis is a control pathway which links the hormone system and the central nervous system. It has also demonstrated the ability to influence the activity of HPG axis. Stress serves as a major mechanism by which the HPA axis is activated. Some HPA components via which such activation is induced include CRH, corticotropin, β-endorphin and glucocorticoid (Weinstock, 1997). CRH and GnRH are

two peptide hormones that are secreted in the hypothalamus which are indirectly responsible for adrenal and gonadal activities respectively (Rivest and Riviest, 1995). Moreover, a direct neural linkage between the two hormones has been reported (*ibid.*). In addition to HPA regulation, CRH has also been shown to regulate proopiomelanocortin-derived peptides, such as β-endorphin, which inhibit the secretion of GnRH in the hypothalamus (Chrousos et al., 1998). This leads to non-secretion of LH and FSH which are responsible for gonadal reproductive activity (Chen et al., 1992). Likewise, the adrenal glucocorticoid can influence the HPG axis via positive and negative feedback mechanisms at the level of the hypothalamus, pituitary, gonad and other tissues (Chatterjee and Chatterjee, 2009; Dubey and Plant, 1985). It has been reported that long-term elevation of glucocorticoid concentration can lead to the suppression of HPG functions and the immune system (Melmed and Conn, 2005). Glucocorticoids can feedback on the gonads to suppress gonadotropin synthesis (*ibid*.)

The HPT axis constitutes another vital feedback mechanism which can influence the HPG axis. The process (described schematically in Figure 1.5 and Section 1.2.2.3) leads to the formation of thyroid hormones (thyroxine, T_4 and triiodothyronine, T_3) and regulation of hormone equilibrium in the body. The thyroid hormones are secreted to regulate development, reproduction, metabolism, behaviour and growth in vertebrate animals (Gorbman et al., 1983). The thyroid hormones influence the ovaries indirectly by reducing the gonadotropin-releasing hormones (GnRH) in anestrous ewe (Clark, 1988). It is also likely responsible for the seasonal reproductive patterns in *Bos indicus* cattle and Welsh Mountain ewe including development of refractoriness at the end of the reproductive season (De Moraes et al., 1998; Follet and Potts, 1990). When thyroid hormones are in excess of requirement during regulatory activities, they form a negative feedback network which will influence the collective processes that facilitate their secretion in the thyroid glands (Figure 1.5). Such a negative feedback mechanism will down-regulate the activities of the hypothalamus and the pituitary, and in turn, affect the activities along the HPG axis. For example, the relationship of the HPT with reproductive activities in some vertebrate fish species has been established. Cyr and Eales (1996) showed, over some specific phases in the reproductive cycle of some teleosts, that there exists temporal relationship between the thyroidal and the gonadal states. It was widely reported from studies on a wide variety of teleost species that the increased thyroidal activity observed during early gonadal development is usually

sustained during reproduction (*ibid.*). Conversely, the activity in both the thyroid and gonad reduces during the spawning and post-spawning stages (*ibid.*).

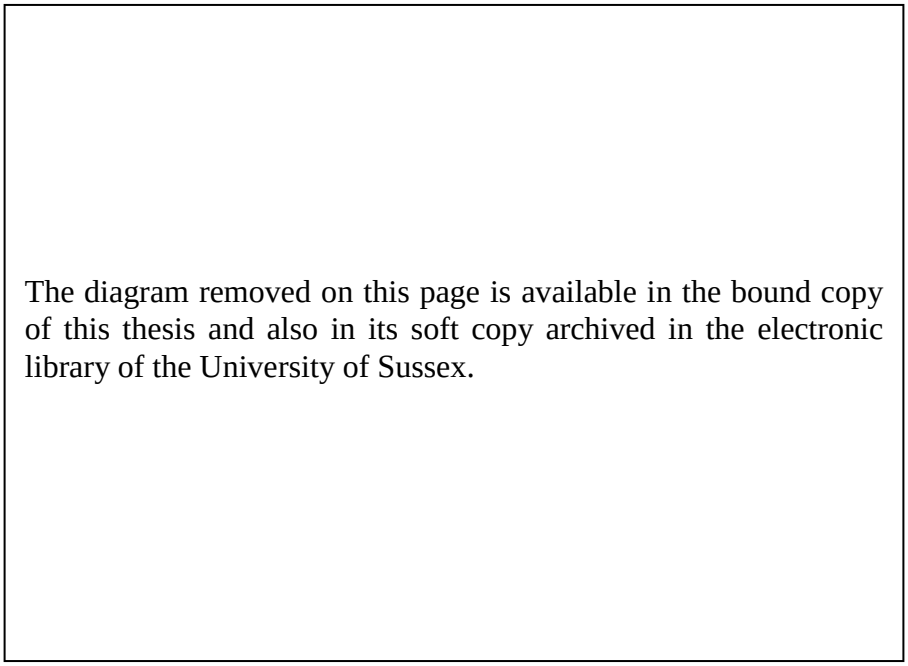


Figure 1.5: A diagram illustrating the positive and negative feedback mechanisms of the hypothalamus-pituitary-thyroid (HPT) axis between the brain, pituitary and thyroid organs of the body.

1.4 Endocrine Disrupting Chemicals

A wide variety of anthropogenic chemicals, and some exogenous natural chemicals, can influence the course of endocrine communication processes in humans and wildlife. This class of bioactive chemicals are referred to as endocrine disrupting chemicals, or simply as endocrine disruptors. An endocrine disruptor is broadly defined as any exogenous agent that interferes with the normal processes of production, secretion, transportation and receptor activation of natural hormones which are responsible for the maintenance of homeostasis, growth, reproduction, and other internal activities (Kavlock et al., 1996; USEPA, 1998). Endocrine disrupting chemicals are mostly lipophilic in nature, bioaccumulative and environmentally persistent while some are characterised with low vapour pressure (Colborn et al., 1995). At low

concentrations, endocrine chemicals have potentials of inducing biological effects. The likely reason for this is associated with the ability of these endocrine disrupting compounds to act via multiple mechanisms. The effects of endocrine disrupting chemicals and their biological end-points in living organisms may be irreversible. Therefore, there are possibilities that these biological end-points may be expressed during developmental and reproductive stages (Rhind, 2002). Controlled laboratory exposure of fetal rodents to p,p'-DDT has resulted in reproductive defects such as reduced and impaired fertility, decreased viability of the offspring, variation in hormonal level and sexual behaviour of adult males and occurrence of some developmental abnormalities such as cryptorchidism, hypospadias, disruption in the network of intercellular bridges conjoining the germ cells in the testes and anomalies in the acrosome, the nucleus and the shape of the sperms in these adults (Veeramachaneni, 2008). Effects such as increased anogenital distance (AGD), vagina agenesis, masculinisation of female offsprings and induced male-like accessory tissues have also been reported in female rats exposed *in utero* to β-trenbolone (Hotchkiss et al., 2007a; Wilson et al., 2002). It has also been reported that most environmental chemicals exhibiting endocrine disrupting characteristics have dissimilar chemical structures (*ibid.).*

1.4.1. Behavioural and Structural Diversity of Endocrine Disrupting Chemicals

Although most disrupting activities of environmental chemicals are presumed to occur via steroid receptor activation, it is also possible for these chemicals to alter the synthesis, transport and metabolism pathways of hormones. The steroid hormone production process is largely controlled by receptor-based feedback mechanisms and consists of networked pathways of precursors, enzymes and products. In addition to steroidogenesis (steroid hormone synthesis), endocrine chemicals can also alter the level of hormone excretion and steroid biotransformation mechanisms. In gastropod mollusks (e.g. dogwhelk, *Nucella lapillus*), tributyltin (TBT) can obstruct the formation of sulphate conjugates of testosterone and its related metabolites regarded as less potent and easily excreted forms of testosterone (Nelson et al., 1996; Ohno et al., 2005; Ohhira et al., 2006). TBT can also inhibit aromatase cytochrome P450 enzymes involved in biotransformation of androgens to estrogens. In most cases, environmental chemicals having endocrine disrupting ability are different in structure when compared to the structures of endogenous hormones modulating similar effects (McLachlan, 2001;

Mantovani, 2002). While ethynylestradiol (EE2) and estrogen possess structural similarities that could be receptor active, compounds such as nonylphenol (NP) and dibutylphthalate (DBP) possess structures that are relatively different from that of estrogen and yet are estrogen receptor(ER)-active (Sonnenschein and Soto, 1998). In addition, chiral compounds may exhibit different biological activities based on their enantiomeric forms (Garrison et al., 2000; Puttmann et al., 1989; Rodman et al., 1991; Ulrich et al., 2001; Shen et al., 2006). Studies of induction of ethoxyresorufin-O-deethylase (EROD) and benzphetamine Ndemethylase (BPDM), the cytochrome P450-dependent enzymes, by 2,2',3,4,6-pentachlorobiphenyl (PeCB), 2,2',3,4,4',6-hexachlorobiphenyl (HeCB), and 2,2',3,3',4,4',6,6'-octachlorobiphenyl (OCB) showed that their (-)-enantiomers are more potent than the (+)-enantiomers except for HeCB where the reverse is the case (Rodman et al., 1991). Therefore, it may be practically difficult to design models for predicting the steroid receptor activity of compounds based on their structures. Some of the diverse chemical structures are highlighted in Figure 1.6.

1.4.2: Mechanisms of Endocrine Disruption Action

There is a wide range of means by which bioactive xenobiotics could disrupt the normal endocrine activities. However, this review will be restricted to cellular-based endocrine disrupting mechanisms which may or may not involve steroid hormone-receptor sites and these include ligand receptor agonism, ligand receptor antagonism, biosynthetic interruption, hormone transport interference and hormone metabolism.

1.4.2.1 Steroid Hormone-Receptor Agonism and Antagonism

Hormones produce effect-directed responses after successfully binding to the targeted hormone receptor site. Hormone receptor sites are generally made of flexible, broad domains with unique size and structure where interactions with a wide range of hormone-like chemicals can take place. This tolerance behaviour of steroid hormone receptors (Cooper and Kavlok, 1997) explains why xenobiotics would be able to disrupt the activities of the endocrine system. Steroid hormone-receptor interaction can be classified broadly into four categories based on the nature and type of ligand, and the eventual hormone-receptor complexes formed: direct-acting agonists, direct-acting antagonists, indirect-acting agonists and indirect-acting antagonists (Cooper and Kavlock, 1997). Akin to endocrine hormones, bioactive xenobiotics can activate the

hormone receptor sites to produce similar effects caused by endogenous hormones. The principle associated with the receptor binding of endogenous hormone is the same as that involving a direct-acting agonism except for the biochemical response. The level of response induced corresponds to the potency of such compounds and the effectiveness (success) of such binding. It must be stated that binding effectiveness accomplished by a xenobiotic is determined by how closely fit in size and shape the ligand is to the receptor in the ligand-receptor complex. Xenobiotics with higher potency are expected to produce stronger physiological response than endogenous hormones of the same concentration. Examples of xenobiotics known to possess hormone receptor agonism include estrogen agonists diethylstilbestrol (DES), nonylphenol (NP), octylphenol (OP), nonylphenol polyethoxylates (NPE), genistein, bisphenol-A (BPA), paraben and tamoxifen (Fisher et al., 1999; Gray et al., 2001a; Snyder et al., 1999) and androgen agonists 17α-and 17β-trenbolone, PCB-169, androstenedione and androstadienedione (Ankley et al., 2003; Denton et al., 1985; Gray et al., 1999; Hewitt et al., 2000; Hotchkiss et al., 2007a,b). The structures of some estrogen agonists, estrogen antagonists and mixed agonist-antagonist are shown in Figure 1.6.

However, some receptor binding will not elicit response. This receptor-binding effect is referred to as direct-acting antagonism and can be via competitive or non-competitive antagonism. In the former, the hormone antagonists compete with agonists for hormone binding sites. If the antagonist-receptor binding was successful, it will be practically impossible for the binding to transform to signal. Chemical compounds that can exhibit this form of behaviour are tamoxifen (a breast cancer drug), linuron, cyproterone acetate, vinclozolin, dibutylphthalates (DBP), diethylhexylphthalate (DEHP) and nafoxidine (Baker, 2001; Clark et al., 1973; Mantovani, 2002). In non-competitive antagonist binding, the hormone antagonist can work in two possible ways: inhibition of structural alteration at the receptor site which will cause ligand binding to occur or inhibition of post-binding interactions with the HRE of the DNA that would normally produce a response to the ligand. Vinclozolin derivatives (M1 and M2) have been reported to demonstrate the potentials of impeding androgen-dependent gene expression activation by effectively blocking androgen-induced steroid-steroid receptor complexes (AR) binding to the HRE on the DNA (Kelce et al., 1994; Wong et al., 1995). Some synthetic chemicals can express dual behavioural responses in different biochemical environments. Such chemicals can be classified into partial agonist-partial antagonist and mixed agonist-antagonist. The action of the latter produces different

binding competence in different environmental conditions but sometimes the former can act under such different environmental conditions too. The former acts as a partial agonist by binding to the target receptors as a surrogate ligand in absence of the endogenous hormone. Most often, such binding occurs at concentrations comparatively lower than the effective concentrations of the endogenous ligand and the biological responses produced are usually very weak (De Castro et al., 1991). However, as partial antagonist, the exogenous ligand competitively binds the receptors at elevated concentrations compared to that required for binding when it was acting as partial agonist as well as that required by endogenous ligand (Ayd, 2000). When the endogenous hormone is involved, the binding interaction mostly favours the exogenous ligand due to its comparative advantage (i.e. high concentration). The biological response induced in the process falls below that produced if endogenous ligands were to act alone. In succinct description, partial agonist-partial antagonist compounds usually produce agonistic response that increases as the concentration of the compound increases at the receptor until it reaches a plateau where further increase in concentration will no longer raise the level of the response (De Castro et al., 1999). Any further increase in concentration will only elicit antagonistic response at that same receptor (*ibid.*). Sometimes, the responses displayed by compounds having partial agonist-partial antagonist characteristics are induced by the ionic strength of the environment they operate in. Common examples of compounds acting relative to this environmental condition are olanzapine and clozapine (Bymaster and Falcone, 2000). Mixed agonist-antagonist phenomenon is expressed by tamoxifen which acts as anti-estrogen in the breast tissues but also exhibits agonist behaviour in the bone and uterine environments (Lewis-Wambi and Jordan, 2005; Fawell et al., 1990; Figure 1.6). Raloxifene is another mixed agonist-antagonist compound which is highly effective as an antagonist in the reproductive tissues as well as an agonist in the bone (Draper et al., 1996; Gustafsson, 1998; Jordan, 1998). An example of a partial agonist-partial antagonist is 2-arylpyrazolo[4,3-c] quinolin-3-ones, a derivative of benzenediazepines (Yokoyama et al., 1982).

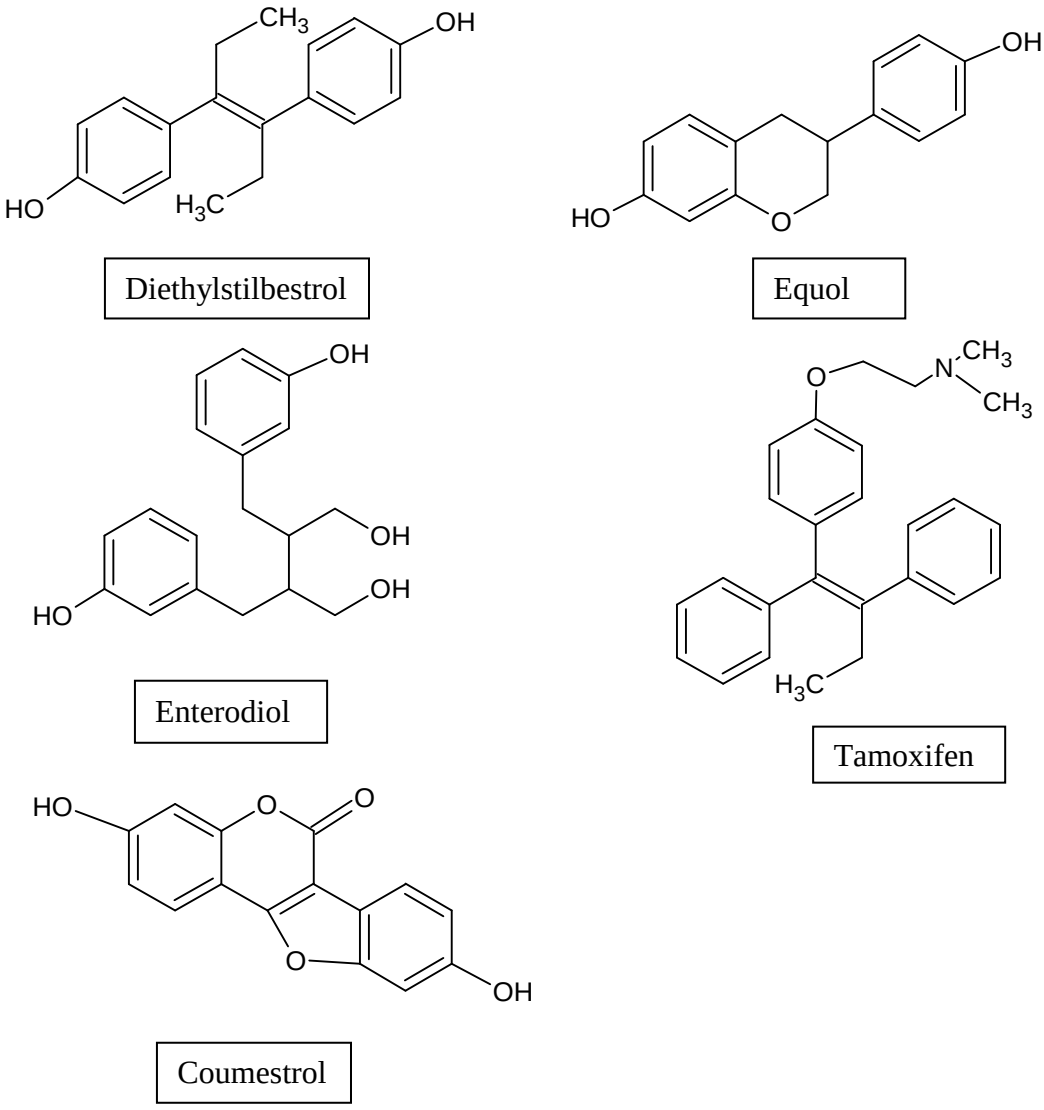


Figure 1.6: Structures of some endocrine disrupting chemicals behaving as estrogen receptor-binding agonist (diethylstilboestrol, enterodiol and coumestrol) and estrogen antagonist (equol). Tamoxifen acts as estrogen agonist and antagonist (or estrogen mixed agonist-antagonist).

1.4.2.2 Disruption of Steroidogenesis

Hormone biosynthesis and metabolism are significant molecular processes relevant to regulation and maintenance of cellular activities. With cholesterol as the starting materials, a wide array of hormones can be produced by the action of enzymes either directly on cholesterol or indirectly via other intermediates of steroid hormone biosynthesis (see Figure 1.2). It is possible for xenobiotics to interfere with any of these vital enzymatic pathways to induce endocrine-related health problems (Sanderson et al., 2006). The broad range of enzymes involved in steroidogenesis is classified into four

basic categories: aromatase, demolases, hydroxylases and hydroxysteroid dehydrogenases. The biosynthetic pathways in Figure 1.2 reveal the many potential disruption points where enzymatic functions may be altered during steroidogenesis. A known enzymatic disruption pathway that has been reported in wildlife occurred in dogwhelk (Bettin et al., 1996; Oehlmann et al., 1996). Bettin and colleagues (1996) showed that TBT can inhibit P450 aromatase enzyme (known to be responsible for the conversion of testosterone to estradiol) in dogwhelks when exposed to it. The study also indicated that the aromatase inhibition by TBT must have led to the accumulation of testosterone which is responsible for the formation of penis (imposex) in female dogwhelks (*ibid*.). Ketoconazole, an antifungal antibiotic, is another compound that can inhibit certain mammalian cytochrome P450-dependent enzyme such as $C_{17,20}$-lyase and 17-hydroxylase (which are responsible for biosynthesis of androgens in the animal) at elevated concentration (Vanden Bossche et al., 1987). It can also inhibit the production of cortisol in the human adrenal glands (Engelhardt et al., 1985; Mantovani, 2002).

1.4.2.3 Interference with Hormone Transport

Steroid hormones are lipophilic biological molecules which account for why they are sparingly soluble in water. They are transported in free or bound state to their binding destination via the blood plasma. Special hormone carrier proteins such as albumins and globulins are manufactured in the liver and the steroids are transported in conjugated form as glucoronides and sulphates or as the lipid-soluble hormones themselves. These globulin proteins occur as steroid hormone-binding globulin (SHBG) or testosterone-estrogen-binding globulin (TEBG) and they may bind to either testosterone or estrogen (Pertschuk et al., 1980; Roy et al., 2008). Since hormones are transported in small concentrations, any slight increase in globulin concentration is expected to reduce the chances of hormone availability for binding. Steroid hormones are temporarily stored in the blood carrier proteins where they are shielded from enzymatic activities which may lead to metabolism (*ibid.*). The carrier protein may release these hormones, from time-to-time whenever there is a short-fall in free-state concentration present in the blood. The role of the blood carrier proteins is to shield the hormones from enzymatic action (e.g. deactivating activity of the liver) and this contributes to why they are able to persist longer in the body. When hormones occur below the receptor binding concentration, there is a possibility that they may be less effective and hence have a shorter half-life *in vivo*. With the presence of circulating

xenobiotics in the body, the transport protein binding sites are subjected to competition with other chemicals and so may reduce binding of endogenous hormones to the carrier protein and disrupt the transport of the hormone. The implication of disrupting the hormone transportation mechanism is that the biological process of hormone complex formation which induces gene expression could be significantly impaired or affected. This may lead to serious health problem.

1.5 Endocrine Disruption Phenomena in the Environment

Every living organism requires the functionality of an effective chemical communication mechanism to regulate and facilitate its complex internal arrays of activities (MacLachlan 2001). These complex networks of biological activities include cellular growth, sexual differentiation, reproduction and biological development. In addition to normal intracellular regulation, the activities of organs and some specialised systems are also co-ordinated. In recent times, environmental agents of both natural and synthetic origins have come under intense investigation, especially concerning their role in disrupting chemical signalling and inducing adverse health effects in wildlife and humans. Interdisciplinary studies conducted to date have increasingly linked the growing endocrine-related health cases in wildlife to this chemical disruption phenomenon.

1.5.1 Evidence of Endocrine Disruption in Invertebrates.

A wide range of biological effects associated with the exposure of some species of invertebrate phyla to endocrine disrupting chemicals has been identified. The most comprehensive account of these environmental disturbances is summarised in the exposure of oyster (*Crassostrea gigas*), female marine gastropods (molluscs) and dog-whelk snail (*Nucella lapillus*) to organotin compounds (TBT). These compounds were used as biocides in marine antifouling paints for ship hulls, boats and aquaculture pen nets but also used in wood preservatives, agricultural pesticides, textiles and as UV stabilizers in some plastics (Oehlmann et al., 1998). A wide spread occurrence of imposex phenomenon, also known as pseudohermaphroditism, has been reported globally in marine gastropod species. Molluscs account for the majority of marine invertebrates found to be sensitive to TBT exposure. The term imposex defines the reproductive defect where the male reproductive organs (penis and vas deferens) are superimposed on the female marine species (Bryan et al., 1986; Horiguchi, 2006).

Additional effects linked to imposex include increased concentration of testosterone in the tissue of marine snail (Spooner et al., 1991). About 150 species of gonochoristic prosobranch gastropods worldwide are found to exhibit this reproductive abnormality (*ibid.*; Matthiessen and Gibbs, 1998). At low concentration (1-2ng/l), female dogwhelk exposed to TBT developed a blockage of oviduct, otherwise known as sterility (Bryan et al., 1986). Further elevation in concentration (3-8ng/l) led to diminished juvenile recruitment, induced masculinisation, reproductive difficulties, population reduction and extinction (Matthiessen and 1998; Fox, 1992). These morphological abnormalities are thought to be the consequence of inhibiting the aromatase (enzyme), which is responsible for the conversion of testosterone to 17β-estradiol (Oehlmann et al., 1996). The increased concentrations of testosterone induced biological effects which resulted in this masculinisation phenomenon (Matthiessen and Gibbs, 1998). With competing arguments suggesting a wide range of other possible modes of action of TBT such as its action as a retinoid X receptor agonist (Nishikawa et al., 2004), interruption of neuropeptide signaling pathways and modification of testosterone metabolism, the most likely mechanism of action based on causal-effect evidence comes from aromatase (CYP19) inhibition of testosterone metabolism. In oyster (*Crassostrea gigas*), the consequences of TBT include shell deformation and decimation of adult population as reproductive failure increased. Following worldwide confirmation of the long-term toxicological implication associated with continued use of TBT, the International Maritime Organisation (IMO) treaty in 2001 ratified the imposition of a partial and total worldwide ban on its use on boats and ships which indeed took effect from 2003 and 2008 respectively (Antizar-Ladislao, 2008).

Similar to TBT, triphenyltin, a compound used in similar capacity as TBT in the production of certain antifouling paint, has shown the potential of inducing imposex phenomenon in some species of marine snail. It is significant to state that triphenyltin (TPT) usage is not restricted to the production of antifouling paint alone (Crompton, 1998). In the 1960s, TPT was the main constituent of triphenyltinhydroxide and triphenyltinacetate used as fungicides to treat potato blight, leaf spot and powdery mildew (Keijzer and Loch, 1995). Schulte et al. (2000) reported imposex in female freshwater ramshorn snail (*Marisa cornuarietis*), an ampillariidae-mesogatropod, exposed to TPT. Some male sexual abnormalities associated with TPT have been reported to include azoospermia (spermatogenesis impairment) and partial infertility. These experimental discoveries underscore the endocrine disrupting impact of TPT

across both sexes of freshwater ramshorn snail (*Marisa cornuarietis*) (*ibid.*). While no significant negative effects were recorded in female netted whelk (*Hinia reticulate*), dogwhelk (*Nucella lapillus*) showed abnormal sexual features highlighting impairment of oogenesis and spermatogenesis when exposed to TPT.

Another biological effect of endocrine disruption in invertebrates is intersexuality. Intersex reproductive abnormality was reported as the phenotypic interference of sex determining organs occurring between the gonad and genital tracts (Oehlmann et al., 1994; Bauer et al., 1995). Intersex males and females retained phenotypic identity but are differentiated by the presence of additional reproductive features specific to their opposite sex (Ford et al., 2004). While the female intersexed species possess one or two penis or vas deferens (genitalia), the male species carry rudimentary oostegites, the female brood plates (*ibid.*). It is reported that periwinkles (*Littorina littorea*) developed intersexuality effects when exposed to TBT, a phenomenon that contrast with the outcome of the previous exposures (Matthiessen et al, 1995). Intersex phenomenon has been reported in crustaceans (*harpacticoid copepods*) exposed to sewage effluents along the east coast of Edinburgh, Scotland (Moore and Stevenson, 1994). Although the mechanisms surrounding the phenomenon is unclear, factors responsible for intersex vary and may include age and the species (De Bock and Greco, 2010), parasitism (Ford et al., 2004), temperature (Vazquez et al., 2004), bacterial infection (Rigaud and Juchault, 1998), genetic control (Lebederf, 1939), environmental sex determination (Dunn et al., 1996; Dunn et al., 2005), protandrous hermaphroditism (Yaldwyn, 1966), exogenous chemicals (Olmstead and Leblanc, 2007) and pollution (Moore and Stevenson, 1991; Ford et al., 2004a). The following copepods were discovered to exhibit intersexuality: *Paramphiascella hyperborea* and *Stenhelia gibba* and it was also evident in two species of *Halectinosoma* (*Similidistinctum* and *finmarchicum*). Intersex contrasts with imposex in their reproductive morphology. While imposex phenomenon can be described as complete superimposition of the female reproductive organs with the penis or the vas deferens (the male reproductive organs), intersex phenomenon is characterised with deepening development of abnormal pallial oviduct by the female reproductive organs.

1.5.2 Evidence of Endocrine Disruption in Vertebrates.

1.5.2.1 Reptiles:

Accidental spillage of a mixture of chemicals containing diclofol, DDT, DDE and sulphuric acid, into Lake Apopka in Florida induced a wide range of reproductive and developmental abnormalities in juvenile alligators (*Alligator mississippiensis*) living in the lake (USEPA, 1979). In addition to the spill, the lake is known to receive agricultural wastewaters which have further caused the level of its contamination over time to be complicated (Guillette et al., 1994). Three years post-spillage review of the alligator densities in the lake showed 90% reduction in the population of juvenile alligators in the lake (Guillette et al., 1995; Jenning et al., 1988). This noticeable decline in the alligator population has been attributed to reproductive failure modulated by DDT and its metabolites, DDE and DDD. Woodward et al. (1993) reported reduced hatching success from 80% to about 20% of the eggs taken from alligators harvested from Lake Apopka. The juvenile male alligators that matured from these hatchlings were discovered to have developed small penis size. High mortality rate of hatchlings (about 50%) occurred after 14 days when the concentration of bioaccumulated pesticides and their metabolites recorded in the eggs doubled (*ibid.*). Abnormal morphological effects attributed to these endocrine disrupting chemicals in both adult male and female alligators include demasculinisation of male alligator (where increased length of testicular phalluses was developed), malformation of cell structures in seminiferous tubules, ovarian defects in the female alligators (*ibid*.). Plasma hormone concentration studies of the gonad region have reportedly confirmed elevation of estradiol-testosterone ratio (E2/T2) by a factor of 2 in female alligators and quadrupled factor from 0.5 in the male reptiles under the influence of DDT and their metabolites stressing the biological implication of xenoestrogens *in vivo* (Milnes et al., 2002; Guillette et al., 1994). Laboratory studies revealed that o, p'-DDE which is suspected to be responsible for most hormonal effects (estradiol) in alligator could effect sex differentiation when eggs coated with chemical was incubated in the hatchery (Hileman, 1994). At normal male hatching temperature, DDE coated eggs produced a mixture of male, female and intersex in ratio 2:1:2 against 100% male recorded by DDE-free eggs (*ibid*.).

Another known example of environmental occurrence of endocrine induced chemical effects detected in Lake Apopka occurred in red-eared slider turtle (*Trachemys scripta*) population. Investigation conducted on the eggs, juvenile and adults of this turtle species in Lake Apopka revealed some developmental and reproductive

abnormalities which have been attributed to exposure to chemical contaminants in the lake. Seven years review of the turtle densities after the spillage revealed an alteration in the hormonal balance and the trend of androgen synthesis. Most male sexed turtles are progressively demasculinised while hatchlings either result in abnormal male or intersex according to histopathological studies (Guillette et al., 1994). Studies have shown that two hydroxylated PCB(2',4',6'-trichloro-4-biphenylol and 2',3',4',5'-tetra-4-biphenylol), which may be detected as metabolites of PCBs in the marine environment, could activate estrogenic characteristics in eggs of red-eared male turtles that are pre-dosed (coated) with these compounds when incubated at male-producing temperature (Bergeron et al., 1994). Their endocrine disrupting characteristic could induce sex reversal in male but the end-results in female turtles are unknown (*ibid.*).

1.5.2.2 Amphibians:

The declining profile of amphibians in the wild has raised questions about the role of endocrine disrupting chemicals in the reduction of their population. Currently, the available evidence implicating xenobiotic chemicals for the extinction of some wildlife species is so far limited. Exposure of some amphibian species {e.g. northern leopard frogs (*Rana pipiens*), green frogs (*Rana clamitans*) and mink frogs (*Rana septentironalis*)} to agricultural chemicals has resulted in some developmental deformities in addition to some physiological and immunological abnormalities and propensities such as alteration in developmental hormones, increased susceptibility to diseases, missing or supernumerary limbs, bony limb-like projections, digit and muscle defects as well as those associated with central nervous system (Ankley and Giesy, 1998). Chemical-induced developmental and reproductive malformations have further suggested that chemical exposure may be responsible for the decline of some amphibian populations. Metamorphosis is a crucial developmental process by which eggs of a wildlife species are transformed to the adult wildlife. Each of these stages is mediated by the endocrine system through the endogenous hormones. Although the role of endogenous hormones on the formation and transformation of larvae is not known, it is suspected that sex steroid hormones can hinder larval development in some amphibian species (Gray and Janssens, 1990; Hayes, 1997; Richards and Nace, 1978). Long-term exposure of amphibians to estrogen and estrogen-like chemicals can modulate sex differentiation during metamorphosis (Bevan et al., 2003; Christensen et al., 2005; Goto et al., 2006; Mackenzie et al., 2003). Studies of cricket frog (*Acris crepitans*) taken from

a PCBs- and polychlorinated dibenzofuran (PCDF)-contaminated site in Illinois, USA revealed that male frog species showed sex ratio alteration. The additional reproductive abnormalities reported include formation of testicular oocytes, incomplete metamorphosis, and feminisation. Common morphological abnormalities documented in female frogs comprise female-biased sex ratio and gonadal malformation (Jofre and Karasov, 2007). Similar malformations have been reported in Northern leopard frogs (*Rana pipiens*) captured in Ontario, USA.

1.5.2.3 Aves (Birds):

The best known examples of endocrine disrupting effects of environmental chemicals in wild birds pertain to egg shell thinning, supernormal clutches and sex differentiation. Widespread DDT and DDE pollution across the Pacific coast of the United States of America between the 1950s and 1970s has been linked with some developmental and reproductive abnormalities. Roughly 2 million kilograms of industrial DDT and its metabolites were released into the coastline via the sewers leading to bioaccumulation and bioconcentration of their particulates in the tissues of fish, sea birds and sea lions. The effect-based ecological response to these contaminants led to breeding failure in double-crested cormorants (*Phalacrocorax auritus*), brown Pelicans (*Pelecanus occidentalis*) of Anacapa Island, common egrets (*Casmerodius albus*) in California, sparrowhawks (*Accipiter nisus*) of South Scotland, fish-eating Caspian and Foster's terns (*Stern caspia and Stern forsteri*), white-tailed eagles (*Haliaeetus albicilla*) of Schleswig Hostein, herring gulls (*Larus argentatus*) of Lake Ontario and shoreline-nesting bald eagles (*Haliaeetus leucocephalus*) in the Great Lake Basin. These and some other chlorinated pesticides were linked to the general decline in the population of wild birds (Fry and Toone, 1981; Palmiter and Mulvhill, 1978). The step-wise formation process of medullary bone (which is recognised as the primary source of calcium during formation of eggs and eggshells) and calcium metabolism are regulated by the sex steroid hormone, estrogen. It is possible for bioaccumulated DDT and its metabolites in the tissues to act against the transportation of calcium across eggshell gland mucosa given that some chlorinated pesticides have been reported to have estrogenic characteristic. This calcium transportation inhibition leads to eggshell thinning in predatory and non-predatory birds (Taylor and Harrison, 1999; Risebrough et al., 1968). In addition to eggshell thinning, seen to be the prevalent symptom in most exposed bird species, sex differentiation (skewed sex ratios) such as feminisation and

intersexuality, have been reported to occur in some breeding populations of some wild birds. Such bird species include Japanese quails (Bryan et al., 1989), Western gulls and Herring gulls (Fry et al., 1981). Further long-term effects of morphological abnormalities reported in wild birds include development of malformed ovarian tissues and oviducts in male embryos (Fry et al., 1981), hematology (Bryan et al., 1989), developmental abnormalities, such as feather malformation and growth retardation, and mortality (Safe et al., 2000).

Another illustrative evidence of endocrine disruption reported in wild birds is the incidence of supernormal clutches, where the average number per clutch (nest) rose from three to between four and six (Dawson, 2000; Kovacs and Ryder, 1985; Ryder and Somppi, 1979). Due to biased sex ratio in the population density (which resulted in the shortage of male population density), it is observed that two female members could come together (in what has been termed female-female pairing effect) to lay eggs in common nest and share the responsibility of rotational incubation which was usually undertaken with the male (Hunt and Hunt, 1977; Kovacs and Ryder, 1985). This phenomenon was triggered by the high level of o, p'-DDT contaminants which induced feminisation of male Western gull embryos found in the heavily contaminated Santa Barbara Island (Fry and Toone, 1981). Supernormal clutches in member birds was caused by skewed sex ratio in the population. Another explanation tenable for this behaviour, which has not been investigated, is the masculinisation of female Western gulls' behaviour. The effect was no longer noticed after a worldwide ban was imposed on the production and use of DDT as pesticide.

1.5.2.4 Pisces (Fish):

Among the five classes of vertebrate animals, fish ranks as one of the most widely studied to illustrate endocrine disrupting effects of environmental chemicals. This is because the aquatic environment, which is their natural habitat, is regarded as "the ultimate sink" for natural and anthropogenic chemicals (Sumpter, 1998). Studies have also shown that wastewater effluents are one of the major sources of chemical contaminants of the aquatic environment (Desbrow et al., 1998; Snyder et al., 1999; Baronti et al., 2000). There is a general view that severe endocrine disrupting consequences, which could threaten the reproductive health and population sustainability of fish, would not only spell their extinction but could also have adverse consequences on the population and health of humans, predatory animals and other

living organisms. Studies of the physiology and morphology of fish species caught downstream of some wastewater-receiving rivers and lagoons and some laboratory exposures showed diverse degrees of abnormalities (Kime, 1998; Tyler et al., 1998). Gonad analysis of caged and wild male roach (*Rutilus rutilus*) exposed to wastewater effluents during sexual differentiation revealed the induction of protein egg-yolk, known as vitellogenin, a phospholipoprotein manufactured in the liver of egg-laying female vertebrates (Folmar et al., 1996; Larsson et al., 1999; Purdom et al., 1994; Rodgers-Gray et al., 2000, 2001). This egg-yolk formation has been defined as feminisation of the male fish. Similarly, formation of vitellogenin has been reported in juvenile male rainbow trout (*Oncorhynchus mykiss*), Japanese medaka (*Oryzias latipes*), fathead minnows (*Pimaphales promelas*), zebrafish (*Danio rerio*) and cyprinid fish species exposed to laboratory dosage of estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) (Brion et al., 2001; Harries et al., 2000; Orn et al., 2006; Purdon et al., 1994; Routledge et al., 1998; Thorpe et al., 2000, 2003). The presence of nonylphenol, a metabolite of nonylphenol ethoxylate surfactants, in wastewater effluents induces the egg yolk formation and inhibits testicular growth (Harries et al., 1995). However, other variables such as water temperature, the migratory habits of fish, the nature and dosage of EDC exposed to (including the diverse past EDCs exposure which can leverage the fresh exposure threshold) can also contribute to the level of vitellogenic response (Purdom et al., 1994; Kirby et al., 2004; Pait and Nelson, 2003; Panter et al., 2002).

In addition to feminisation effect in male fish, formation of cysteine-rich glycoprotein called spiggin in female fish species, as yet another reproductive abnormality, has been reported. At the biochemical level during the breeding period, the male stickleback fish develops kidney hypertrophy under the influence of androgen thereby causing the epithelial cells of the kidney to produce hydrophobic protein named spiggin (Jakobsson et al., 1999; Sanchez et al., 2008). This 230kDa protein is released into the urinary bladder where it is constituted as a structural thread (or glue) that is used for the purpose of nest-building preparatory to the egg-laying process by female fish (Jakobsson et al., 1999). Conventionally, kidneys of female sticklebacks are not known to produce spiggin until recently when the spiggin-forming genes in female stickleback were found in dormant (latent) form (Sanchez et al., 2008). As in male stickleback, these dormant genes in the female can be induced by androgen receptor agonists to produce spiggins. It is realised that spiggin formation via androgen induction

in young and immature female stickleback fish could serve as a reliable end-point which can be used to screen (xeno)-androgens and anti-androgens in the laboratory and the field (Allen et al., 2002; Bjorkblom et al., 2007; Katsiadaki et al., 2002, 2006; Jones et al., 2001) .

In Florida, USA, female mosquito fish (*Gambusia affinis*) harvested in rivers downstream of pulp and paper mills were observed to exhibit masculinisation phenomenon which affected its behavioural pattern too (Howell et al., 1980). In addition, the female mosquito fish was reported to develop elongated anal fins which are a replica of male gonopodium (*ibid*.). The males caught in the same rivers were also reported to show hypermasculinisation which makes them develop a more hyperaggressive mating behaviour than the normal males (*ibid.*). Other abnormalities identified include reduced sex hormone level, delayed sexual maturity, reduced and abnormal sexual organs, reproductive difficulties, reduced fecundity and intersexuality (Gray et al., 1999; Hirai et al., 2006; Lange et al., 2001; Robinson et al., 2007). Although, the environmental chemicals responsible for these reproductive effects were not immediately known as studies indicated that such chemicals have hormonal characteristics similar to endogenous androgens.

A wide range of scientific studies undertaken have identified some of the xenobiotic chemicals involved in some of these reproductive endpoint abnormalities to include PCBs (mostly components or metabolites of known pesticides e.g. DDT, DDE and chlordane), natural estrogens (e.g. 17β-estradiol, phytoestrogen) and other unknown chemicals (Allen et al., 1999; Sumpter and Jobling, 1995). Significantly, laboratory studies have further advanced the knowledge on endocrine disrupting effects of environmental chemicals as most of the observed effects have been replicated. Incidences of vitellogenin production and gonadosomatic index development in juvenile Atlantic cod (*Gadus morhua*) and flounder (*Platichthys flesus*) have been demonstrated in the laboratory (Allen et al., 1997; Ilylland et al., 1997).

1.5.2.5 Mammals:

A spectrum of well-established evidences of endocrine disrupting chemicals in mammals has been reported. Most examples documented were based on the reproductive endpoints and population reduction in terrestrial and aquatic mammals. For instance, Florida panthers (*Felis concolor coryi*), grey seal (*Halichoerus grypus*), common seal (*Phoca vitulina*), Baltic ringed seal (*Phoca hispicla botnica*), bottlenose

dolphin (*Tursiops truncatus*), striped dolphins (*Stenella coeruleoalba*) and beluga whales (*Delphinapterus leucas*) exhibit a wide range of developmental abnormalities and reproductive dysfunction. Following progressive declines in their populations, investigation revealed that a high level of organochlorines was found in the tissues of the Florida panthers (Facemire et al., 1995). The organochlorines detected are suspected to originate from raccoon (*Procyon lotor*) food items contaminated by mercury and pesticides during production (*ibid.*). Florida panthers (*Felis concolor coryi*) have also shown reproductive and morphological abnormalities such as low ejaculation volume, diminished sperm concentration, sperm characterised with high morphological anomaly, cryptorchidism and male infertility (Facemire et al., 1995; Roelke, 1990). Declining trends of grey and ringed seals' population in the Baltic Sea has likewise been linked to endocrine disruptive effects of environmental chemicals (Taylor and Harrison, 1999). Increased concentration of PCBs, DDT and its metabolites in the tissues of seals has also been detected, some of which have developed abnormalities such as testicular steroidogenetic defects, immune dysfunction and infertility linked with these endocrine disrupting chemicals. Similar abnormalities occurred when seals harvested in the heavily polluted Wadden Sea were analysed (*ibid.*). Additional evidences documented from this experimental analysis include high incidence of fatality and reduced concentrations of estradiol resulting in imbalances in this endogenous hormone. For example, polar bears (*Ursus maritimus*) in Svalbard, Norway recorded a drastic decline in female population following chemically-induced hormonal imbalance that nearly wiped out the female bear population from age sixteen upward (Derocher et al., 2003). Occurrence of pseudo-hermaphrodites, a phenomenon synonymous to females developing small penis in front of their vagina, has been documented in female polar bears (Wiig et al., 1998). The elevated PCB concentration in polar bears may be the potential causal agent responsible for dwindling younger polar bear population in Svalbard although there is no enough evidence yet to suggest that these are the key agents (*ibid.*).

1.5.2.6 Evidence of Endocrine Disrupting Phenomena in Human.

The best known evidence of endocrine disruption in human beings involved use of diethylstilbestrol (DES). DES is a popular anabolic agent widely used in the USA to improve the economic values of livestock. However, DES was administered to pregnant women between late 1940s and early 1970s to prevent spontaneous abortion and this led

to a myriad of irreversible reproductive abnormalities, many of which affected the unborn baby directly (Marselos and Tomatis, 1992). The outcome of long-term *in utero* studies of DES exposure, which has widened through meat and dairy-related products, has been characterised. Similar to laboratory animals, risks of human exposure to DES include suppression of lactation, testicular malformation, underdevelopment or absence of vas deferens, gynecomastia (development of abnormally large breast tissue in men), retention of Mullerian ducts, deteriorated sperm quality and formation of epididymal cysts in matured male progeny (McLachlan et al., 2001; Steinberger and Lloyd, 1985). Structural malformations such as cryptorchidism, hypoplastic testis and penis and meatal stenosis (narrowing urethra tube leading to the penis opening) have been similarly reported in children male born after their parents' exposure to DES (Gill et al., 1977; Bibbo et al., 1977; Gill et al., 1979; Stillman et al., 1982). Female children that emerged after *in utero* exposure in their parents' womb have been shown to develop reproductive and developmental malformations. Some of such effects include subfertility and virilisation (Kunz et al., 2004), vaginal adenocarcinoma, premature birth, pseudo precocious puberty (Felner and White, 2000), vaginal adenosis, elevated serum testosterone, structural abnormalities in the uterus, ectopic pregnancy, hood and polyps of cervical and vaginal organs, anatomical masculinisation and malformation of cervical canal, reduced libido, sexual impotence and tumours of reproductive organs in adult women (Steinberger and Lloyd, 1985; Newbold, 1995; Grajewski et al., 1996; Whelan et al., 1996).

1.5.3 Environmental Sources of Exposure to Endocrine Disruptors

A wide range of synthetic and natural chemicals which are capable of modulating endocrine-related reproductive and developmental health problems have been detected in the environment. Synthetic chemicals find their way into the environment via accidental and deliberate human and non-human activities from several point and non-point sources. Some of these sources identified include agricultural, industrial, commercial and residential outlets. For instance, pesticide applications (to improve crop yields in plantations), industrial by-products discharges, landfill leachates, combustion process, sewage sludge and domestic wastes discharges are commonly known means of chemical sources in the environment. The fact that endocrine disruptors possess a wide range of physicochemical properties will suggest the possibility of exhibiting different modes of behaviour in the environment. For instance,

most EDCs are known to be lipophilic but have variable chemical and biological transformation pathways in the environment which are dependent on their structures and other chemical properties. Moreover, catabolic processes such as chemical degradation often are altered by environmental factors (e.g. aerobic and anaerobic conditions, elevated temperature and photocatalysis, and hydrolysis) to change the form and, sometimes, the state of these chemical compounds. However, some of these compounds are relatively stable as they do not undergo any degradation. For this reason, they tend to bioaccumulate in the environment, maintain a high shelf-life and long enduring transportation away from their sources. These are expected to impact on the general mechanism of distribution. Human and wildlife exposure to endocrine disrupting chemicals can take place through contact with food, water, air, personal care products (consumer products), soil and aquatic sediments. These exposure sources are broadly classified into two: indoor and outdoor sources. Generally, endocrine disruptors enter into the blood streams of human and wildlife through ingestion, inhalation and direct access across the cell membrane via skin and gill contacts.

1.5.4 Anti-androgens in the Environment.

The word ‘anti-androgen’ can be defined, in a short phrase, as “action taken against androgen”. Androgens are useful hormones secreted by the gonads in conjunction with the pituitary glands to induce chains of biochemical processes in the body of vertebrate animals for the purpose of reproduction and reproductive activities. The word ‘androgen’ originated from the combination of two Greek words, “andros” (man) and “genein” (to produce). Androgens mediate biological responses through interaction with androgen receptor (AR) as described in Section 1.4.2. Anti-androgenic chemicals simply act by blocking the biological process of complex formation and/or transcription, which are strategic for activating biological responses. Exogenous anti-androgens are also referred to as xenoanti-androgens and they describe the endocrine disrupting chemicals that are responsible for interrupting these internal biologically strategic processes.

Therefore, broadly expressed, anti-androgens are any group of androgen (receptor) antagonists, hormone synthesis inhibitors, hormone metabolism interrupters or hormone transport modulators capable of obstructing partially or totally the biological responsibilities of any natural or synthetic chemical compounds (androgens), which act on appropriate target cells in the body system to promote and activate the

production, development and maintenance of masculine sex organs and sundry secondary sexual characteristics in vertebrates.

Anti-androgens existing in the environment originate generally from natural and synthetic sources as steroid and non-steroid compounds. Most known naturally occurring anti-androgens are derived from plant sources and are substantially non-steroid in form (Section 1.5.4.1). The knowledge of natural steroid and non-steroid anti-androgens from animal sources is currently not available. A wide range of exogenous anti-androgens, occurring in steroidal and non-steroidal forms, have been reported among the myriads of anthropogenic chemicals found in the environment. Presently, the only known synthetic anti-androgenic compound having steroidal property is cyproterone acetate (CPA)(Figure 1.9). From this analysis, it may be appropriate to say that most synthetic anti-androgens known are non-steroidal.

Nonsteroidal synthetic anti-androgens (NSA) are synthesised for myriads of human and non-human use, many of which cut across agriculture, medicine, environment, industries and domestic life.

1.5.4.1. Plant and Fungal Anti-androgens

Anti-androgens that occur in nature are limited and most of those reported so far originate from plant sources. Some of the exogenous anti-androgenic compounds reported in nature include permixon (an extract derived from an American dwarf palm tree, *Serenoa repens B*) (Wilt et al., 1998), a non-steroidal CpdA [2-(4-acetoxyphenyl)-2-chloro-*N* methylethylammonium chloride -a stable derivative of the African shrub, *Salsola tuberculatiformis* Botsch, prepared from less stable hydroxyphenyl aziridine] (Tanner et al., 2003) and isolates of three leaves -beech, oak and reed extracts (Hermelink et al., 2010) (see Figure 1.7). Also, estrogenic effects sometime reported in poultry and livestock have been linked with feeds contaminated with Zearalenone, a fungal mycotoxin produced by the action of *Fusarium sp* on carbohydrate feeds (Manfred, 2001; Meronuck et al., 1970; Roine et al., 1971). Studies have shown that zearalenone and its metabolites (zearalanone, zearalanol and zearalenol) have anti-androgenic potency almost equivalent to flutamide (Paris et al., 2005). This discovery has shown that some estrogens can act as also anti-androgens at receptor level. Although, the AR binding mechanism is currently poorly understood, zearalenone has been reported to demonstrate hepatic ER binding (Powell-Jones et al., 1981). There are *in vivo* data indicating that *in utero* exposure could lead to sex differentiation and

serious disruption of reproductive competence (*ibid.*). Equol (7-hydroxy-3[4'-hydroxyphenyl]-chroman) [see Figure 1.6] has also been investigated as a biologically active compound with anti-androgenic property (Lund et al., 2004). Equol is a chief component of daidzein, a phytoestrogenic derivative of soyabeans. It is found that equol has poor AR affinity but bind selectively, with high affinity, to 5α-dihydrotestosterone (DHT) to deny DHT from AR binding opportunity (*ibid.*). Binding affinity of equol to DHT is partly accountable for lean epididymal and prostate weight after exposure (*ibid.*).

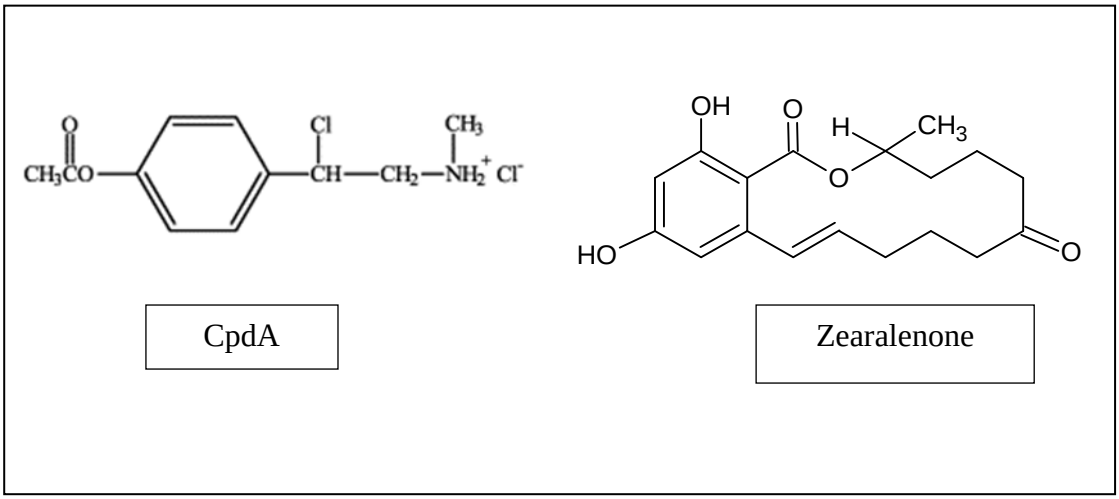


Figure 1.7: The chemical structures of 2-(4-acetoxyphenyl)-2-chloro-*N*-methyl-ethylammonium chloride (CpdA) and Zearalenone.

1.5.4.2 Agricultural Anti-androgens

A wide range of chemicals that are associated with agricultural practices have been reported to possess anti-androgenic properties. Effect-based studies of the anti-androgenic activities of some of these agro-allied chemicals under *in vivo* and *in vitro* experimental conditions have been conducted. Gray and co-workers (1994) discovered that, at cell receptor level, some pesticides possess anti-androgenic characteristics *in vitro* or *ex vitro* and, in some, with a high degree of potency. The majority of these pesticides have been discovered in the environment in their original or metabolised form and their mechanism of anti-androgenic action is expressed via aromatase inhibition. For most environmental compounds that modulate anti-androgenic effects, aromatase induction clears away the competitive binding hurdles posed by the endogenous androgen hormones at the target receptors and makes passage to the receptors free for the chemical anti-androgens to bind to. Classical examples of anti-

androgens in the environment include insecticides DDT and its metabolite-DDE (Kelce et al., 1995a,b), fenitrothoin, chlorpyrifos-methyl (CPM) (Kang et al., 2004), toxaphene and metachlor (its metabolite, HPTE), fungicide vinclozolin and metabolites M1 and M2, procymidone (Kelce et al, 1994; Ostby et al., 1999; Gray et al., 2001), prochloraz (Gray et al., 2001) and herbicide linuron (Lambright et al., 2000; McIntyre et al., 2000; Vinggaard et al., 2005) (Figure 1.8). *In utero* exposure of some male terrestrial mammals (e.g. rat, dog, sheep and pig) to vinclozolin and p, p'-DDE induces reduced anitogenital distance (AGD), reduced weight of sex accessory glands, hypospadias, ectopic and undescended testes, vaginal pouch and retained nipples (Gray et al., 2001; Wolf et al., 1999; Kelce et al., 1995a, b). No knowledge of phenotypic effect has so far been reported in the females of all the laboratory animals exposed to procymidone, p,p'-DDE and vinclozolin. Identified effects of developmental exposure to each of the three compounds include testicular cancer (Shakkebaek, 1972), erectile dysfunction and infertility in rats (Brien et al., 2000). Given that anti-androgenic response of vinclozolin is very weak relative to its metabolites (M1 and M2), the potency is comparatively low. Although, linuron and methoxychlor also exhibit AR antagonism *in vivo* and *in vitro*, and induce reproductive-based effects similar to vinclozolin, procymidone and p,p'-DDE, the fact that their bioactive concentration are higher implies low potency.

Most of these agricultural anti-androgens have been detected in the environment either in their original or metabolised form. Due to their non-persistence in nature, residues of a wide range of pesticides have been discovered in water, air, soil and food samples. Organochlorine chemicals such as DDT, linuron, procymidone, metachlor, vinclozolin and methoxychlor are widely used on agricultural farmlands to control broad range of pests. Although DDT is either completely banned or restricted in some countries across the globe, its residues are still being recovered in previously exposed soil layers years afterward. For instance, DDT detected in Tianjin soil samples analysed in China occurred in the range of 628.1-2840.5ng/g decades after its application was banned (Gong et al., 2002). In Argentina where DDT usage has been restricted, traces of it at levels of 0.0204-2.123ppm have been reported in dairy by-products such as butter (Lenardon et al., 1994). Similar investigation in Spain has also confirmed the occurrence of DDT in trace quantity in pasteurised milk (Martinez et al., 1997). Investigation carried out in Ayeduasi, KNUST and K-Poly in Kumasi, Ghana has also reported the occurrence of DDT in yoghurt at concentrations of 8.96µg/kg, 4.09µg/kg and 7.52µg/kg respectively (Darko and Acquaah, 2008). In addition, studies further

indicate that traces of DDT were detected in fresh milk at a concentration of 12.53µg/kg in KNUST samples and in cheese sampled from Aboabo, Tafo and Asawasi at concentrations of 14.02µg/kg, 298.57µg/kg and 42.17µg/kg respectively (Darko and Acquaah, 2008). In an experimental study undertaken thirty-days after procymidone application to four supervised leek fields in China, an average range of 0.033-0.17mg/kg and 0.020-1.75mg/kg of its residues, corresponding to 74.9-100% and 82.5-92.5%, were detected in leeks and soil samples respectively (Chen et al., 2010). Procymidone residues have been discovered in wine grapes and vegetables harvested from exposed farmlands (Urruty et al., 1997; Rodriguez et al., 2002). Linuron is a herbicide that is used extensively for cultivating tuber and vegetative crops such as onions, carrots and potatoes (Sanchez-Camazano et al., 2000). About 70% of linuron was reported to leach into the soil when four natural soil samples taken from the Province of Salamanca, Spain were investigated (*ibid.*). There was a significant variation in the result outcome when the organic content of the soil samples was modified indicating that the level of linuron retention in the soil changes with different soil profiles (*ibid.*). Prochloraz is a fungicide for controlling eyespot disease and powdery mildew in cereals and fungal diseases in vegetables and fruits (Birchmore et al., 1977; Lafuente and Tadeo, 1985; Kapteyn et al., 1992). A multiresidue analysis carried out on pesticide-contaminated soil samples taken from Sao Paolo state, Brazil reported a recovery range of 54-73% of prochloraz between 55-73^{0}C temperatures. During the analysis, recoveries of linuron and vinclozolin were also measured at the respective range of 55-70% and 69-73% under the same temperature conditions (Risatto et al., 2005). Prochloraz-formylurea and prochloraz-urea are two metabolites of prochloraz that have been detected at different concentrations of various subsoil layers (Hollrigl-Rosta et al., 1999). The level of prochloraz degradation is a function of the texture, pH and the temperature of the soil (*ibid.*).

Analytical studies of horticultural soil samples taken in five different locations in northern Portugal showed the occurrence of chlorpyrifos (a derivative of chlorpyrifos-methyl) in 33.8µg/kg respectively (Goncalves and Alpendurada, 2005). While chlorpyrifos-methyl has been shown to possess anti-androgenic activity, the biological activity of chlorpyrifos is yet to be known. In Saudi Arabia, residues of toxaphene were detected in subsoil samples of contaminated farmlands (30-60cm) at concentration between 0.006-0.162mg/kg (Al-Wabel et al., 2011). Another commonly known anti-androgenic organochlorine pesticide is methoxychlor. It is used in isolation

or in combination with other chemicals to control cereal and fruit pests especially during storage and planting seasons (ATSDR, 1994). It is also used on agricultural fields planted with vegetable, soyabeans and nuts to control insect pests (*ibid.*). Its mode of action is known to be similar to that of DDT. Traces of methoxychlor residue have been detected in food stuff, water and soil. Due to its less persistence, methoxychlor is rarely found in the environment but could be detected near the disposal, dispensal or the manufacturing premises. In soil, methoxychlor has an anaerobic half-life of 30 days and an aerobic half-life over 100 days (Muir and Yarechewski, 1984). Fenitrothion is a non-persistent organophosphorus insecticide that has been identified to exhibit anti-androgenic property. It is commonly used to control insects associated with cereals, vegetables, stored grains and cotton on agricultural fields (Tamura et al., 2001). It undergoes biodegradation under aerobic condition to produce toxic nitrophenolic metabolites in soil and water (Mikami et al., 1985). One major metabolite generated during its aerobic biodegradation, which only occur via artificial means, is 3-methyl-4-nitrophenol (MNP) (Keith and Telliard, 1979). Due to the possibility of producing myriads of toxic metabolites aerobically, extensive use of fenitrothion has become the likely channel that could constitute environmental health concerns in humans and wildlife (Tago et al., 2006). The structures of these agricultural anti-androgenic compounds are provided in Figure 1.8.

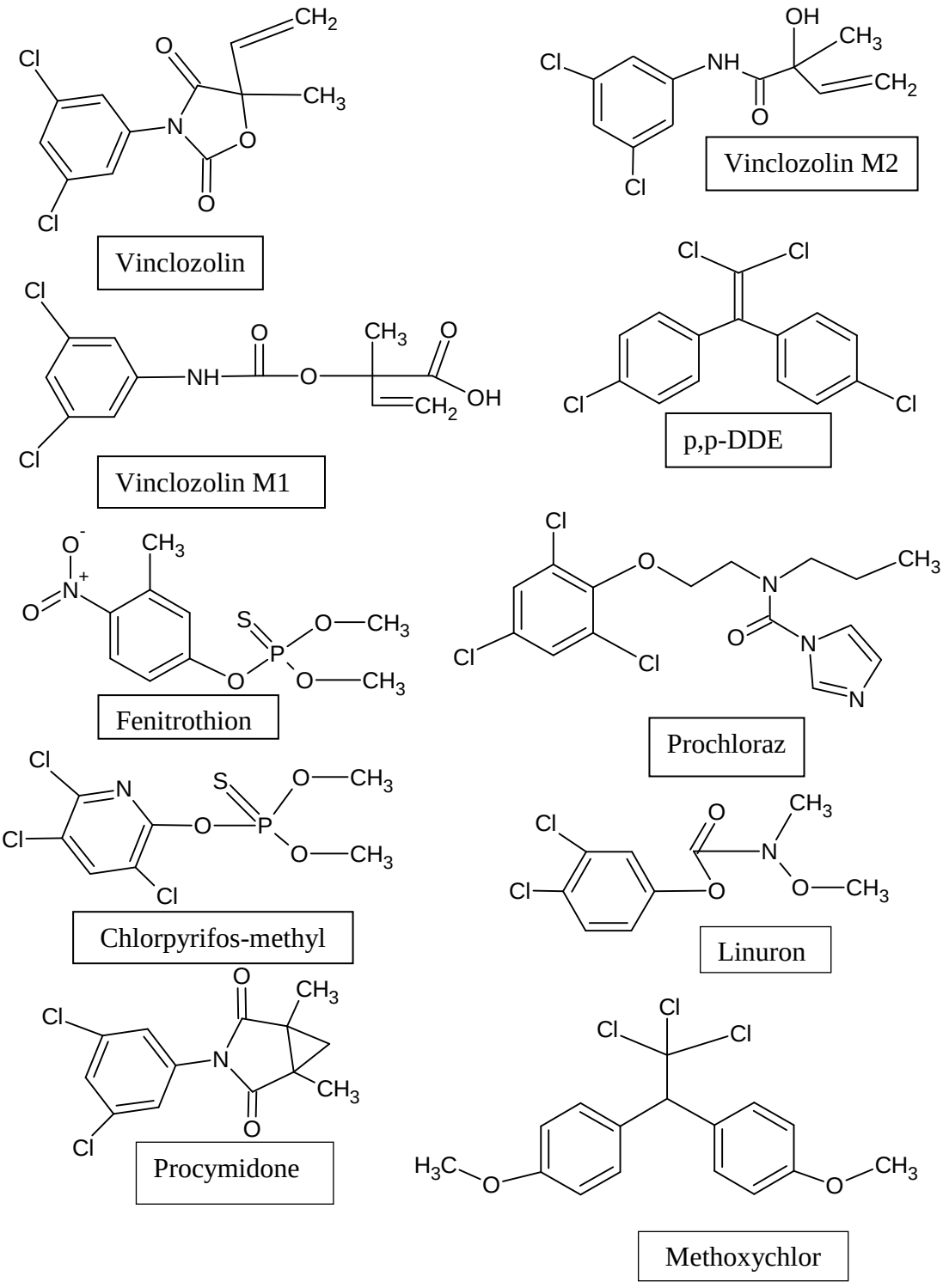


Figure 1.8: A diagram showing examples of agricultural anti-androgens found in the environment.

1.5.4.3 Clinical Anti-androgens

In utero administration of DES to pregnant women, as estrogenic supplement, for three decades in the USA led to a wide range of phenotypic end-points that indicated compromise in health conditions. Cases of cryptorchidism, hypospadias, and diminished volume and quality of sperm in adult laboratory animals have been reported (Imperato-McGinley et al., 1992; van der Schoot, 1992; Silversides et al., 1995; Sharpe et al., 2000). For the fact that an estrogen could induce all the effects highlighted above strongly suggests that it can exercise the ability to block the androgen receptor. Investigation later confirmed similar reproductive effects when anti-androgen exposure was carried out. This highlights the fact that while the mechanisms through which these compounds operate in any vertebrate system are dissimilar, yet the endpoints are same. DES can be reported, on that basis, to exhibit anti-androgenic activity. Hydroprogesterone and cyproterone acetate (CPA), its synthetic derivative, are reported to demonstrate both anti-androgenic and progestational activities (Huang et al., 1985; Brinkmann et al., 1983). *In vivo* exposure of CPA, progesterone and anti-progestin RU486 has been shown to induce nuclear transport, AR DNA binding and transcriptional activity due to competitive AR inhibition binding (Kemppainen et al., 1992). Cyproterone acetate belongs to the dydrogesterone (17-OH progesterone) class of anti-androgenic progestin family (Sitruk-Ware, 2008). Cyproterone acetate exerts its anti-androgenic activity by either engaging in competitive inhibition with AR or by disrupting the conversion agent (enzyme) of testosterone to dihydrotestosterone (DHT) (Sitruk-Ware, 2008). Other clinical anti-androgens in regular use today consist of flutamide, finasteride, nilutamide, spironolactone, hydroxyflutamide, valproate, osaterone acetate, danazol and ketoconazole (Imperato-McGinley et al., 1992; Death et al., 2005; Gunes and Fetil, 2000; Minato et al., 2005; Terouanne et al., 2002; Korner et al., 2004; Xu et al., 2006; Schurmeyer and Nieschlag, 1984) (Figure 1.9). Flutamide, nilutamide and bicalutamide are another category of clinical anti-androgens which operate in similar manner as CPA. Despite their regular use, their concentrations in the environment are not reported.

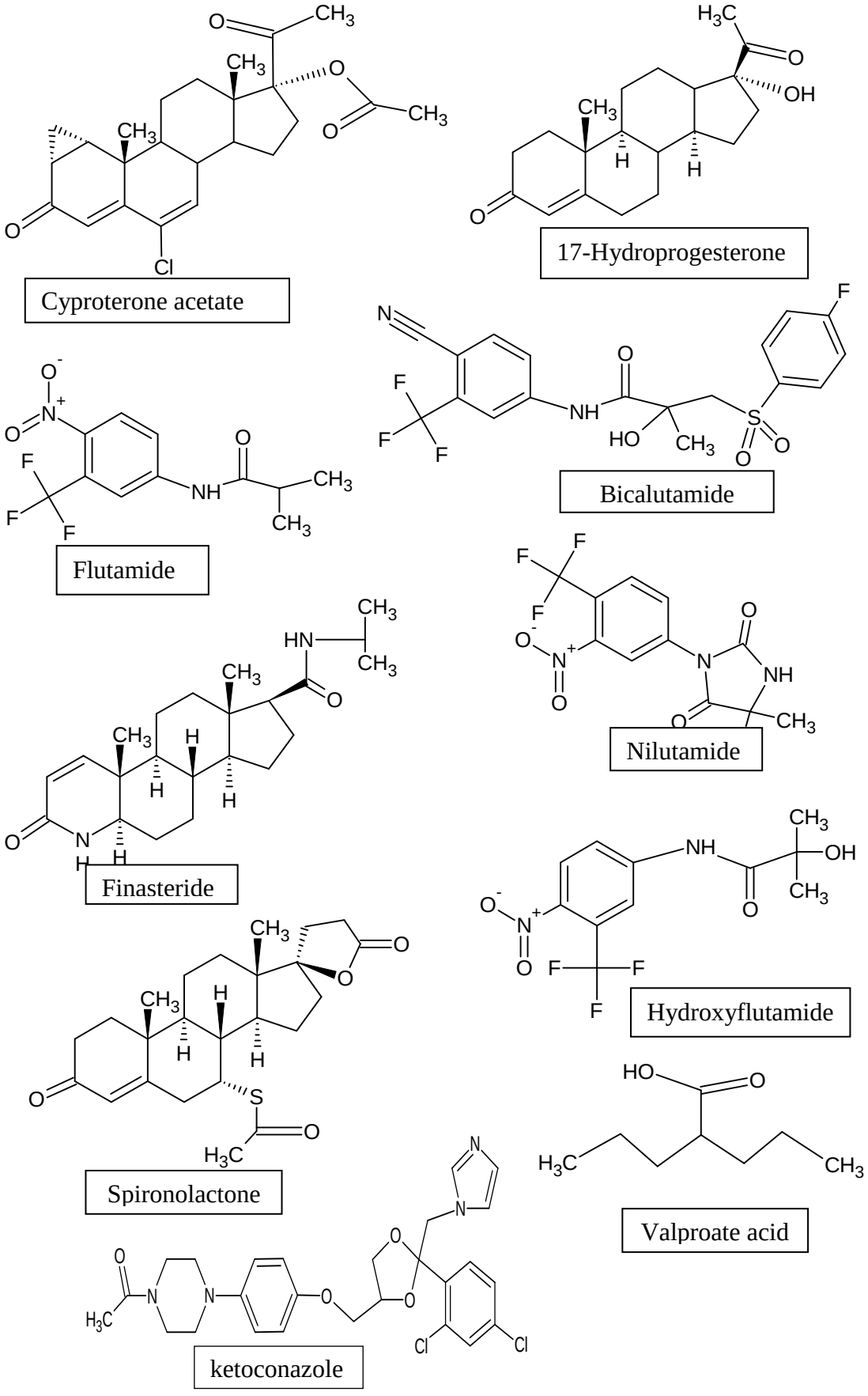
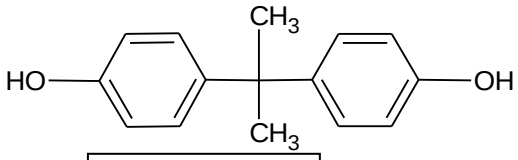


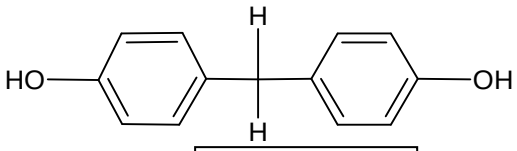
Figure 1.9: Structures of some clinical anti-androgens

1.5.4.4 Industrial Anti-androgens

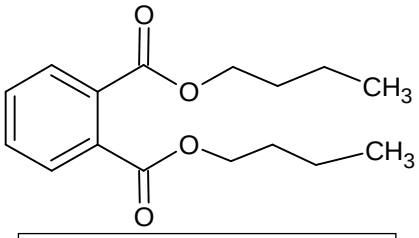
Anti-androgenic effects of industrial chemicals are increasingly being reported. This surge is due, in part, to the growing production of nonsteroidal chemicals, many of which have not been characterised. Examples of chemicals identified with anti-androgenic activity include a range of phthalates such as dibutyphthalate (DBP), diethylhexylphthalate (DEHP) and butylbenzylphthalate (BBP) (Wilson et al., 2004) and polybrominated diphenyl ethers (PBDEs) such as DE-71 and DE-100 (Stoker et al., 2005). Phthalates are ubiquitous environmental contaminants many of which have been reported to possess anti-androgenic activity *in vitro*. Although, *in vivo* studies indicate that phthalates are non-bioaccummulative, in this instance the parent compounds are metabolised into active hydrolytic monoesters (or oxidative metabolites via enzymatic oxidation of the alkyl chain when high molecular weight phthalates are involved) which may induce abnormal reproductive disorders (Silva et al., 2003; Gray et al., 1999; McIntyre et al., 2001; Mylchreest et al., 1998; Mylchreest et al., 1999). Male offsprings of pregnant animals exposed to DBP and DEHP were reported to induce cryptorchidism, hypospadias and testicular weight loss (Mylchreest et al., 1998). Similarly, exposure of laboratory animals to PBDEs have produced abnormal reproductive health effects some of which are delayed puberty in both sexes of rodents, weight loss during pregnancy, decreased sperm concentration, alteration in the ovary cells, decreased testicular size, exhibition of gender-specific sexual-related behaviours and some birth defects (Akutsu et al., 2008; Chao et al., 2007; Lilienthal et al., 2006; Meeker et al., 2009; Schreder et al., 2006; Stoker et al., 2005; Yang et al., 2009). Other anti-androgenic industrial chemicals detected in the environment or identified through laboratory analysis include bisphenol A (Sohoni and Sumpter, 1998), bisphenol F (Satoh et al., 2004), TCDD, polychlorinated biphenyls (PCBs), ethane-1,2-dimethane sulphonate (EDS) (Wolf et al., 1999; Gray et al., 2001), some polyhalogenated aromatic hydrocarbons (Calle et al., 2002; Sharpe and Irvine, 2004) and polycyclic musks (e.g. tonalide, phantolide, celestolide) and UV filters (e.g. homosalate, 3-benzylidene camphor, benzophenone-3 and 4-methylbenzylidene camphor) (Ma et al., 2003; Schreurs et al., 2005) (Figure 1.10).



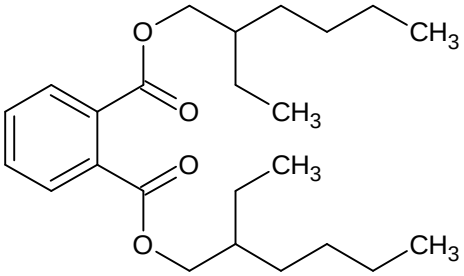
Bisphenol A



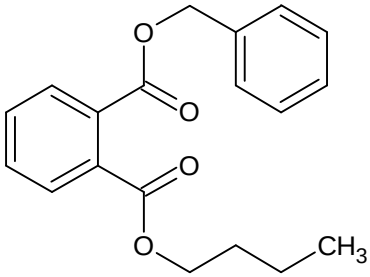
Bisphenol F



Dibutylphthalate (DBP)



Diethylhexylphthalate(DEHP)



Butylbenzylphthalate (BBP)

Figure 1.10: Diagrams of some industrial anti-androgens identified in the environment.

1.6. Anti-androgens in Wastewater Effluents as Potential Causal Agents of Intersex in Fish Living in UK Rivers and Lagoons.

Anthropogenic chemicals present in aquatic environments can interfere with the endocrine system of the living organisms dwelling in them to the extent of modulating biological responses that could result in irreversible physiological and reproductive abnormalities. In the late 1970s and early 1980s, anglers in the United Kingdom observed the preponderance of abnormal incidence of intersexuality (i.e. simultaneous occurrence of male and female gonads) in roach (*Rutilus rutilus*), a gonochoristic fish, caught in the River Lee downstream of a sewage treatment plant. Studies have also shown that it is possible for some fish species to develop intersexuality, among other reproductive abnormalities, when exposed to chemicals in wastewater effluents via water samples from aquatic environments (Jobling et al., 1998; Allen et al., 1999b; Harshbarger et al., 2000; Hashimoto et al., 2000; Vigano et al., 2001; van Aerle et al., 2001; Sole et al., 2003). Some of such chemicals identified in wastewater effluents possess estrogenic properties and they include ethynylestradiol, an active compound in birth control pills, bisphenol-A, octylphenol, alkylphenol ethoxylates (APEs) and nonylphenol (Korner et al., 2000). The identities of many other compounds in wastewater effluents are still yet unknown (*ibid*.). It has been discovered that some estrogenic compounds can induce vitellogenin at low concentrations and intersexuality at high concentrations. Some studies have shown that exposure of young medaka to 17β-estradiol at concentration range of 0.01-1.66µg/L for a month would lead to sex reversal while exposure of its eggs and fry hatchlings to a concentration as high as 15µg/L of 17β-estradiol could result in the formation of ovo-testis (Nimrod and Benson, 1998; Koger et al, 2000). Similarly, laboratory exposure of some juvenile fish species to androgens at a range of concentrations can result in the formation of vitellogenin just as its long-term exposure can also lead to the formation of intersex. Laboratory exposure of juvenile zebrafish at concentrations less than 1µg/L 17-methyltestosterone can lead to formation of intersexuality. The modality, by androgens, for inducing intersex in fish has been associated with its (androgens) aromatisation to estrogen in the course of which the concentration of estrogen in the fish builds up to a trigger threshold. For pulp and mill wastewater effluents, aromatisation of androgenic chemicals can lead to induction of vitellogenin in juvenile zebrafish, just as it can also induce intersex (Orn et al., 2006). It is not yet clear whether environmental estrogens are solely responsible for inducing intersex in fish. Laboratory studies have shown that anti-androgens can also

induce intersexuality as estrogens would (Urbatzka et al., 2007). Exposure of some fish species to anti-androgens (e.g.nonylphenol) could also lead to the formation of ovo-testis. Exposure of Japanese medaka (*Oryzias latipes*) to nonylphenol can induce intersex (Gray et al., 2007). Although, nonylphenol has estrogenic effect, it has also been shown to possess anti-androgenic property which underscores the assertion that some estrogens are equally anti-androgens (Sohoni and Sumpter, 1998).

1.7. Wastewater Treatment Plants as Potential Sources of Environmental Anti-androgenic xenobiotics.

Wastewater Treatment Plants (WwTPs) are collection and treatment centres for a wide range of environmental chemicals entering into the aquatic environments. Wastewater contributions from households, industries and commercial settlements are reported to contain high concentrations of chemicals which are hazardous to the well-being of the living environmental organisms. In order to prevent (or minimise) the potential risks that would be posed by their presence, wastewaters are made to undergo physical, chemical and biological treatment processes (Svenson et al., 2003). During these stepwise mechanical treatment processes (classified into primary, secondary and tertiary) (Figure 1.11), various chemicals present in the waste samples are either totally or partially removed (Svenson et al., 2003). The first process involves the use of coarse grits to remove objects which are greater than 3mm in diameter. Further removal of particulates is carried out with sand traps before running the filtrate into pre-settling basins where aeration is applied, and solid organic materials and nutrients are removed. Through the process of adsorption, a wide range of lipophilic compounds are also removed. This complete removal mechanism described above is referred to as primary treatment.

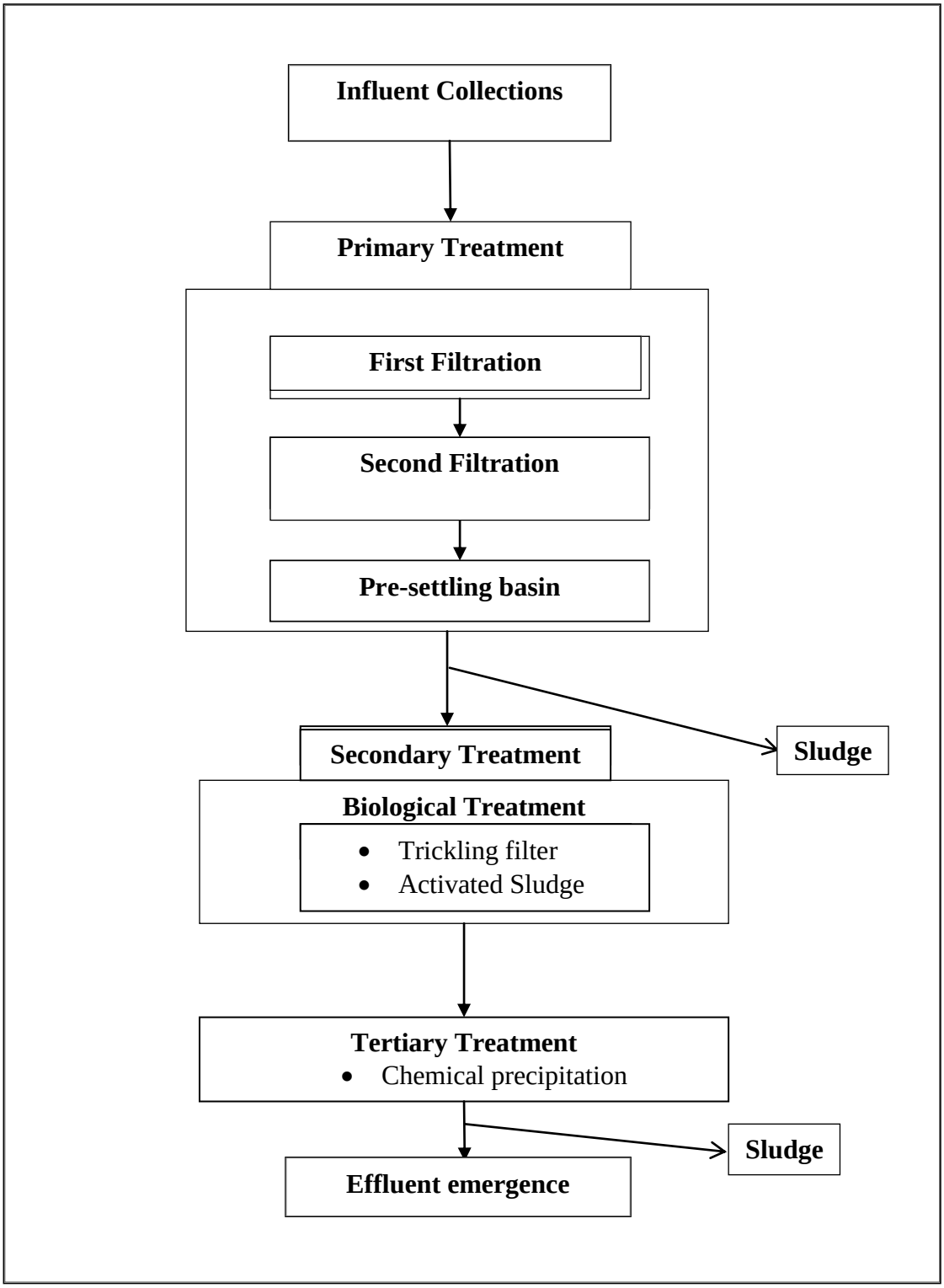


Figure 1.11: A diagram showing the primary, secondary and tertiary stages of wastewater treatment plants.

After leaving the pre-settling basin, the wastewater is pumped into the secondary treatment chamber where the mixture of bacteria and other sewage suspended matters are prepared. This treatment method, otherwise known as biological treatment method,

may proceed in more than one step. Using the activated sludge alone or in combination with trickling filters (or biobeds) and biological nitrogen removal means, bacteria, nitrogen and anoxic substances are removed. The tertiary stage requires the use of chemicals to remove phosphorus, particulates and other dissolved organic matter through the process known as coagulation or chemical precipitation. The coagulants are precipitated and discharged as sludge. The settling of the coagulants may be facilitated using polyelectrolytes which fused the small coagulant units into heavier ones. Commonly used precipitating chemicals include aluminium sulphate, lime and ferric chloride (Svenson et al., 2003). Sometimes a direct precipitation of these contaminants with chemicals may be undertaken after the first and second filtration (*ibid.*). In which case, the total microbial removal may be partly compromised. During this removal process, the sludge (which represents the waste substances removed) is treated and may be used as agricultural fertiliser. Otherwise, the sludge may be digested either anaerobically or aerobically or converted to compost manure.

Generally, a series of physical, chemical and biological activities are discovered to occur at the WwTPs during treatment processes through which chemicals and other unwanted components are removed. These, in summary, include flocculation (Kim et al., 2002; Ternes et al., 2002), sedimentation (Zhang and Emary, 1999; Strenn et al., 2003), coagulation (Adams et al., 2002), precipitation (Strenn et al., 2003), biofilteration (Shon et al., 2006), sorption or adsorption into sludge (Petrović et al, 2001), vaporisation or volatilisation (Meakins et al., 1994; Birkett and Lester, 2003), photolysis (Gray and Sedlak, 2003; Liu et al., 2003; Mansell et al., 2004), biotransformation or microbial metabolism (biodegradation, aerobic and anaerobic degradation) (Holbrook et al., 2002; Johnson and Sumpter, 2001; Semple et al., 1999) and chemical transformation (chemical hydrolysis by oxidation and reduction, enolisation and isomerisation) (Huang et al., 2001; Sedlak and Pinkson, 2001).

Studies have shown overtime that the final effluents which are released into receiving rivers and lagoons contain a complex mixture of chemicals indicating that some compounds are not completely eliminated during the treatment process. Published studies indicate that a wide range of some compounds found in effluent wastewaters possess estrogenic, anti-estrogenic and androgenic activities. A few of such reports have also indicated the occurrence of compounds having anti-androgenic activities. The diversity of the wastewaters treated is better appreciated based on the nature of the community from where supplies originate. By the same relationship, the nature of

compounds present in wastewater effluents is largely dependent on the origin of their sources. It is expected that a more industrialised community will produce wastewaters with higher industrial contribution than less industrial communities. However, due to the pathway of this research, focus will be directed to wastewaters with minimal industrial contribution and mainly domestic input.

1.8 Bioassays for Screening Endocrine Disruption Chemicals in Environmental Samples.

Bioassays are biomolecular preparations or mixture of cellular formulations that can be used to activate and evaluate effect-based measureable end-points. Through formation of a new biomolecule, activation or inhibition of chemical reactions, induction of immunological responses or any variable parameter, screening of bioactive chemicals in environmental samples are achieved. The relevance of these biological assays includes detection, measurement and evaluation of biological activity, potency and toxicity of chemical compounds being considered for pharmaceuticals, or those derived from effluent wastes, contaminated soil and sediments. Two main categories of bioassays predominantly used are *in vitro* and *in vivo* bioassays.

1.8.1 *In vivo* Bioassays

Although *in vitro* bioassays are the most frequently used means of screening the endocrine chemicals especially due to the relative ease of use, cost effectiveness, reproducibility, sensitivity and accuracy, *in vivo* bioassays is still considered as the best evaluation approach. Tests of chemicals for endocrine effects are carried out in the system of living animals where true reaction of such an animals to the activity can be effectively measured. Common *in vivo* assays used are Hershberger, three-spined stickleback kidney cell culture assay and Tier 1 screening assay (Gray, 1998; Jolly et al., 2006; Katsiadaki et al., 2002, 2006). Given that the volume of environmental samples prepared for analysis at any specific time could be enormous, it is practically uneconomical to adopt *in vivo* method. For that reason, discussion on bioassays will be limited to *in vitro* bioanalysis.

1.8.2 *In vitro* Bioassays

In vitro bioassays are a broad range of rapid, cost-effective bioanalytical techniques developed based on high sensitivity, specificity and optimally throughput biological scheme and used to identify, quantify and screen for the biological effects of endocrine disruptors from various environmental samples (Fertuck, 2002; Zacharewski, 1997). Three major *in vitro* bioassays are currently used to screen androgen and anti-androgens. They include: receptor competitive binding assays (or transcription activation assays), cell proliferation Screen Assays and Reporter Gene Assays. Receptor binding bioassays are test standards developed to bind to a specific receptor sites of target compounds. The non-radiolabelled ligand (the test compound) engaged in competitive binding with the established radiolabelled ligand (hormone) for androgen receptor site (Lambright et al., 2000) situated on the cellular or nuclear region of the target cell or tissue during incubation (Lambright et al., 2000; Wong et al., 1995). The problem associated with this type of assay is its failure to distinguish between androgenic and anti-androgenic activity of test compounds which explains why it is poorly selective. Androgen cell proliferation screen assay explores the ability of endogenous androgen to stimulate cell proliferation and induce hypertrophy in male secondary sex organs (Sonnenschein and Soto, 1998). The proliferation process is activated when cell lines interact with the test compounds. During the interaction, the test samples replace receptor binding on the labelled hormone conjugate leading to the occurrence of bioactivity. For anti-androgenic compounds, the cell proliferation process is inhibited (Sonnenschein and Soto, 1998). A wide range of cell lines is readily available for this type of assay, which includes mammalian cells. Examples of this assay are MCF-7 and A-SCREEN. The major drawback is the inter-laboratory (and intra-laboratory) variability but can be employed productively, efficiently and effectively to distinguish androgenic and anti-androgenic activity due to its selectivity.

Endocrine disruptors have also been successfully evaluated with receptor gene bioassays where yeast or cell lines are used to measure the degree of transcriptional activity generated in response to hormone stimulation. The yeast or cell lines which are developed from either mammalian cell lines (e.g. MCF-7, COS-1, CHO, AR-CALUX, HEH293) or yeast strains (e.g. *Saccharomyces cerevisiae*) are transfected with plasmids encoding the hormone receptor and the androgen-dependent receptor gene (Figure 1.12). The receptor gene is coupled with a promoter (e.g. β-galactosidase or luciferase)

which consists of hormone response elements (HREs) via which the receptor activation process is facilitated (Sohoni and Sumpter, 1998; Roy et al., 2004).

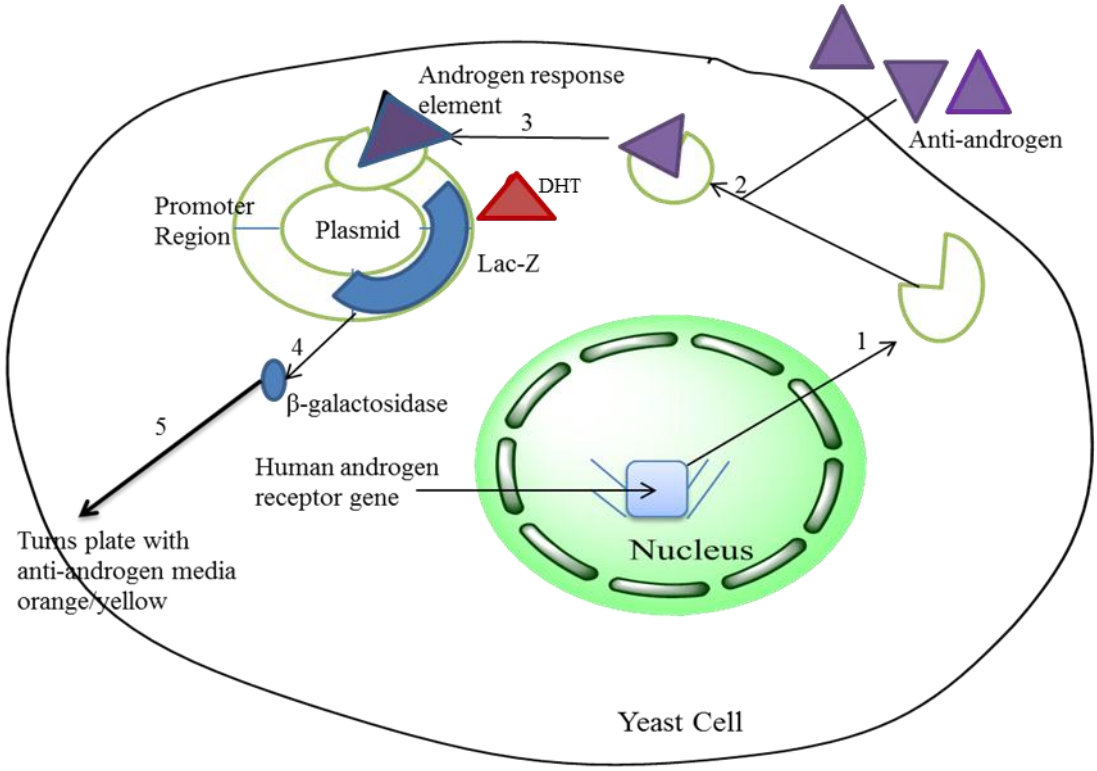


Figure 1.12: Diagram showing the (i) typical structural configuration of a genetically modified yeast strain in anti-androgen yeast screen (AYAS) assay, (ii) anti-androgenic binding pathways (1-3) and the (iii) activation of gene expression (4-5). The human androgen receptor (hAR) gene is incorporated into the yeast genome and expresses cytosolic hAR. In the presence of an androgen ligand such as DHT, it binds to an androgen receptor element (1) occurring within the promoter on the inserted plasmid. The anti-androgen complex (3) formed between the anti-androgen molecule and the human receptor gene (2) inhibits DHT, the natural steroid agonist, and the generation and expression of *Lac-Z*, (4) the reporter gene through which enzyme β–galactosidase is produced. Non-secretion of β–galactosidase into the medium containing the anti-androgen will cause a chromogenic response from red to yellow colour to occur.

Sample-receptor complex is produced from the active binding brought about by chemical interaction of the potential androgen sample and the receptor present on the

yeast strains. The HRE of the reporter gene is activated when the sample-receptor complex binds to it following which the enzymes such as luciferace, β-galactosidase and chloramphenicol acetate transferase, which are suitably measured with luminometry or spectrophotometry, are produced (Sohoni and Sumpter, 1998; Roy et al., 2004). Generally, reporter gene bioassays are easy to culture and manipulate genetically. In addition, they possess the ability to metabolise the test samples. They produce non-uniform permeability when yeast cell walls are exposed to test compounds which cause them to have different set of AR co-regulators better than the mammalian cells. They have been used for screening of androgens and lately anti-androgens (*ibid.*).

1.9 Aims and Objectives.

The aims of this research work are to investigate, identify and quantify anthropogenic chemicals that can induce androgen-receptor antagonism, and to measure the relative potency of these compounds in predominantly domestic wastewater influents and effluents sourced from Horsham Wastewater Treatment Plant, South East of England. This investigation encompasses the following objectives:

1. To investigate the profiles of androgen-receptor antagonists in a domestic wastewater (from Horsham wastewater treatment works) using the *in vitro* androgen recombinant receptor transcription yeast screen (AYAS).
2. To identify the structures of androgen receptor antagonist chemicals present in effluent and influent samples from Horsham wastewater treatment works using Toxicity and Identification Evaluation (TIE) techniques.
3. To measure the relative potency of anti-androgens identified in the wastewater samples.

CHAPTER TWO

Preliminary Studies on Characterisation of Anti-androgenic Activity Present in Wastewater from Sewage Treatment Works.

2.0 Introduction

Wastewater effluents are repositories of natural and synthetic chemicals as well as metabolites that can modulate endocrine activities and cause endocrine-related adverse health conditions. The review in Chapter One has shown that many natural and man-made chemicals which are present in wastewater treatment plant (WwTP) effluents can cause developmental and reproductive health abnormalities. Potential endocrine disrupting compounds identified in the previous studies of wastewater effluents include nonylphenol, phthalates, bisphenol A, alkylphenol polyethoxylates (Gimeno et al., 1997) as well as steroids such as androstenedione (Thomas et al., 2002), 17β-estradiol, 17α-ethynylestradiol, estrone and estriol (Desbrow et al., 1998; Snyder et al., 1999). However, the identity of many xenobiotics in WwTP effluents (Steven et al., 2003) as well as those new chemicals emerging in the environment (as a result of a steady annual rise in production and use of high volume consumer products) are yet unknown (Younes et al., 1999). Numerous studies undertaken on EDCs in WwTP effluents had identified compounds that mostly have estrogenic activities and, to a lesser extent, some anti-estrogenic activities, but some studies have also identified steroidal androgens and also some xenoestrogens, such as nonylphenols, which possess anti-androgen receptor activity too (Sohoni and Sumpter, 1998).

It has been shown that exposure of some wildlife and humans to endocrine disruptors can induce sex differentiation (Toppari et al., 1996; Vos et al., 2000; Chapter One). Exposure studies of some fish species to samples of WwTP effluents, and wastewater-receiving rivers, in United Kingdom have revealed cases of intersexuality suspected to be induced by endocrine disrupting chemicals (Allen et al., 1999; Harries et al., 1996; Jobling et al., 1998; Purdom et al., 1994). Male juvenile fish exposed to natural and synthetic estrogens can also develop intersex (Metcalfe et al., 2001). There is the possibility also that the presence of anti-androgenic chemicals in WwTP effluents could contribute to the demasculinisation and the development of ovo-testis in male fish exposed to WwTP effluents (Parks et al., 2000; Borch et al., 2004; Wilson et al., 2004).

Kelce and Wilson (1997) reported that exposure of fetal male rats to anti-androgens can disrupt male sex differentiation. Anti-androgens are generally known to alter androgen signalling through different modes of action which include blocking production, transportation and metabolism of androgen hormones (Katsiadaki et al., 2006; see Chapter One) as well as disrupting signalling of FSH and LH (Massaad et al., 2002). At the receptor level, a wide range of chemical compounds such as flutamide, vinclozolin, bisphenol-A, butyl benzyl benzoate (BBB), procymidon, linuron, o,p-DDE, p,p-DDE and other environmental chemicals had been tested *in vitro* (and some *in vivo*) in the laboratory and shown to have anti-androgenic activities (Cook et al., 1993; Gray et al., 1994; Kelce et al., 1994; Kelce and Wilson, 1997; Monosson et al., 1999; Ostby et al., 1999; Sohoni and Sumpter, 1998;Vinggaard et al., 1999). While some compounds can act purely as anti-androgen agonist at the receptor level, some others can act as both agonist and antagonist (Massaad et al., 2002). Some estrogenic receptor agonists, such as bisphenol-A, butyl benzyl phthalates and o,p-DDE, also show anti-androgenic characteristics (Sohoni and Sumpter, 1998).

Anti-androgens are generally considered to be less common in the environment. Recent studies have detected anti-androgens in final effluents of WwTPs surveyed in United Kingdom, many of which were investigated using *in vitro* yeast-based recombinant assay (AYAS) (Johnson et al., 2007). Although a number of *in vivo* assays are available to detect anti-androgenic activity (AA) of chemical compounds in wastewater samples (see Chapter One), the use of such assays is uneconomical and time-consuming given that a lot of test animals will be required to screen a massive quantity of sample fractions. In the studies conducted by Johnson and co-workers (2007) on effluent wastewater samples sourced from twenty-five WwTPs, a potency range of anti-androgenic activity between 2.13 and 228µgFeq/L was reported after analysis of April-May batch of samples. The July-August batch figures were higher with potency ranging from 90.7 to 1230.8µgFeq/L. Similar studies carried out on water samples of the river Lambro, a wastewater receiving river, show an anti-androgenic potency between 81.43 and 844.22µgFeq/L (Urbatzka et al., 2007). Kirk (2002) also reported anti-androgenic activity at a concentration range of 55-377µgFeq/L in tertiary effluents using *in vitro* assays. Generally, *in vitro* yeast recombinant assays have been successfully used to screen a wide range of receptor-based anti-androgenic compounds and other endocrine disrupting chemicals in wastewater and other complex

environmental samples. This is simply because it is economical, simple to set up, quick, stable, easy to use and record (Tanaka et al., 2001). For all these reasons, this assay was considered suitable to screen for synthetic anti-androgens present in influent and effluent samples of the domestic wastewaters on which this study will be based.

For this work, a combination of biological and chemical analytical techniques are necessary to provide a complete evaluation approach effective to capture all human androgenic receptor antagonist activity in the wastewater samples. In this study also, the influent and effluent samples were purified with hydrophilic-and-hydrophobic-balanced cartridges based on a previously published method (Evans, 2008). The anti-androgenic activity of the eluates was estimated with *in vitro* yeast-based assay demonstrated to be effective in identifying anti-androgenic chemicals in a number of chemical mixtures (Sohoni and Sumpter, 1998). In this study, however, this approach was used to screen for a broad range of novel, target and non-target anti-androgenic compounds in domestic wastewaters. The bioassay assessment technique was complemented by instrumentation analysis to enhance the quality of information that will be derived from this study. HPLC was used to fractionate the sample extracts and the anti-androgenic activity of the fractions were analysed and estimated using *in vitro* yeast-based TIE assay. This approach had been used previously to estimate activity of effluents (Bailey et al., 1995) and to identify estrogenic compounds in effluent samples (Desbrow et al., 1998).

The aims of this study in Chapter Two were:

1. To extract and quantify anti-androgenic activity present in the influent and effluent of a WwTP receiving waste from a predominantly domestic wastewater source.

2. To use HPLC methodology to compare the profiles of the anti-androgenic chemicals present in the extracts of the WwTP influent and effluent samples prepared from the first aim above.

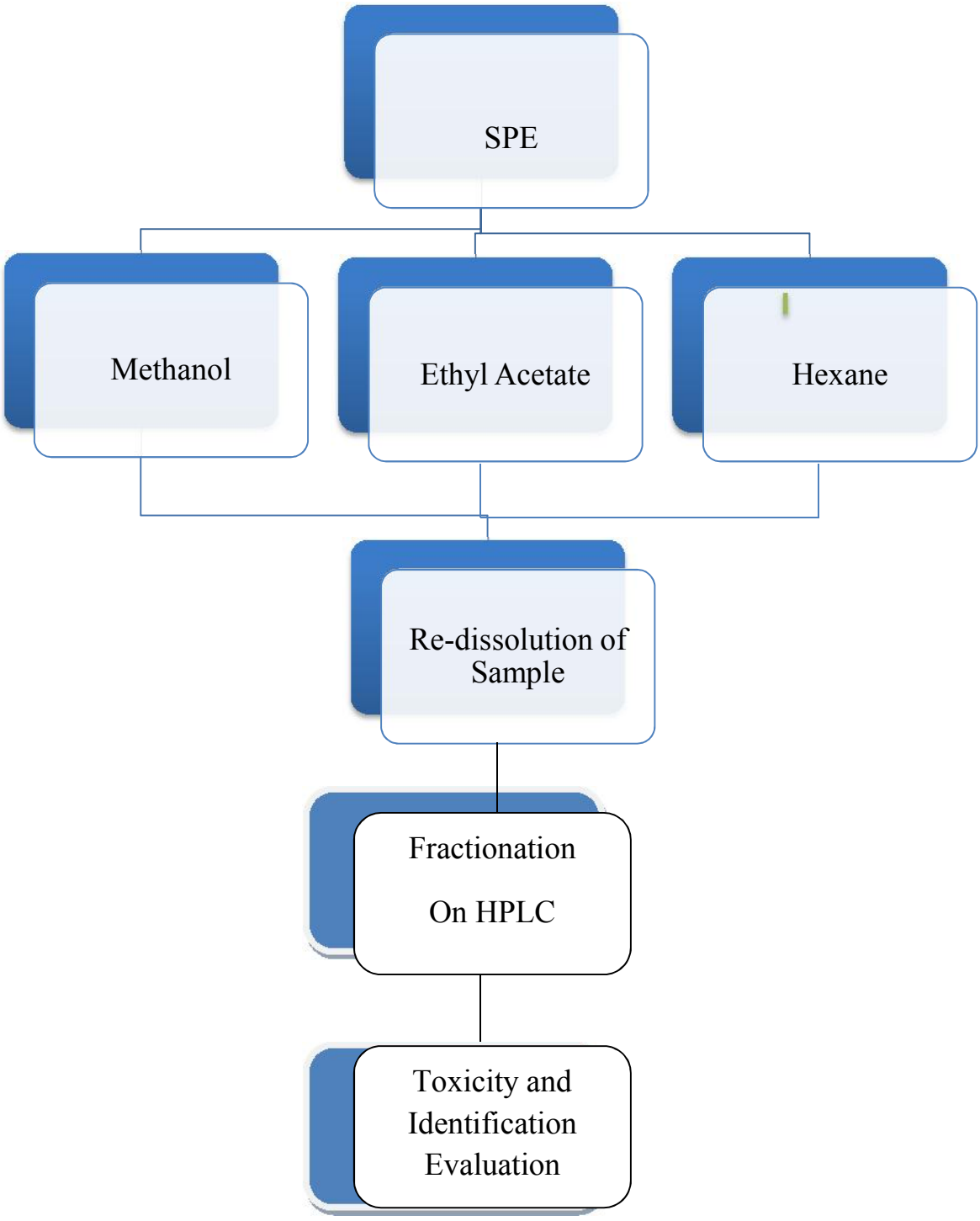
2.1 Materials and Methods

2.1.1 Materials

20cc (1g) OASIS® HLB (polydivinylbenzene-co-N-vinylpyrrolidine) cartridges used for purification and concentration of the wastewater samples were purchased from Waters, Milford, MA, USA. Glass distilled grades of hexane, ethyl acetate and HPLC grade water are common laboratory reagents purchased from Rathburn Chemicals Ltd., Walkerburn, Scotland. Acetic acid (≥99% pure) is a regular laboratory reagent which was purchased from Sigma-Aldrich, Steinheim, Germany. All amino acids (L-leucine, L-histidine, L-adenine, L-arginine-hydrochloride, L-methionine, L-tyrosine, L-isoleucine, L-lysine-hydrochloride, L-phenylalanine, L-glutamic acid, L-valine and L-serine) used to make growth media were purchased from ICN, Aurora, Ohio, USA and the other growth materials (iron(III)tetrasulphate(vi),potassium(III)phosphate(vi)(monobasic), magnesium(II)sulphate(vi)heptahydrate, flutamide, 5α-dihydrotestosterone) were purchased from Sigma, Poole, Dorset, United Kingdom. Chlorophenol red-β-D-galactopyranoside (CPRG) was purchased from Boehringer, Lewes, East Sussex, United Kingdom. Deionised water was distilled from Elga UHQ Distiller System made by Elga Systems, Buckinghamshire, United Kingdom. Microtitre plates, polystyrene and polypropylene, were purchased from Nunclon Surface, Roskilde, Denmark and Greiner Bio-One Ltd., Stonehouse, Gloucestershire, United Kingdom respectively. The hAR-incorporated recombinant yeast cells were provided by courtesy of Professor Sumpter, Brunel University, Uxbridge, United Kingdom.

2.1.2 Methods

2.12.1 Scheme of Analytical Procedure for Profiling of Anti-Androgenic Chemicals in Wastewater Treatment Plants Samples.



2.1.2.2 Collection of Wastewater Samples

Six Winchester bottles (2.5L capacity each) of wastewater samples were collected with an aluminium bucket using a grab sampling technique. The wastewater samples comprised three Winchesters of influent taken from the central inlet reservoir and three Winchesters of effluent sourced at the exit point reservoir. Prior to sampling, the aluminium bucket and the Winchester bottles were rinsed twice with 150mL of methanol and 500mL of target samples sequentially. In order to minimise bacterial degradation during sample transportation to the laboratory, 100mL of methanol was added to every 2.5L of samples collected. Wastewater samples for this experiment were collected on the 3rd of February, 2007 at Horsham Wastewater Treatment Works (WwTW) located in West Sussex county of United Kingdom. Horsham Wastewater Treatment Works are connected to five local districts serving a population of about one-quarter of a million in addition to wastewater deliveries from other sources. The wastewater received at Horsham WwTP consists of over 95% domestic contributions delivered at an average flow rate of 16,500m^3 per day.

2.2. Extraction, Purification and Storage of Samples

The wastewater samples were purified using solid phase extraction method used by Evans (2008). In the method, 20cc (1g) OASIS HLB cartridges (Waters, Milford, MA, USA) were plugged with glass cotton wool, mounted on the SPE vacuum manifold (Sigma-Aldrich Company Ltd, Poole, Dorset, United Kingdom) and connected to a vacuum pump via a conical waste flask. Glasswool was introduced to crudely filter solid material in order to prevent the cartridges' sorbent from being blocked by particulates and suspended solids in the wastewaters. The cartridges were conditioned with 5mL of hexane, 5mL of ethyl acetate, 10mL of methanol and 10mL of acetic acid in water (10% v/v). The conditioning of the cartridges creates an optimal sorbent environment such that the hydrophobic and lipophilic chains are exposed to solvent thereby increasing their effective surface area for retention of target analytes and eliminate impurities. For this experiment, fourteen such cartridges were prepared out of which four cartridges were used to process the influent, another four for the effluent and the remaining six were prepared for the control. A quadruplicate of each sample (influent, effluent) was prepared by adding 5mL of acetic acid-water solution (1:99, v/v) to each 500mL sample preparation. These sample preparations were loaded into the

respective cartridges at flow-rates of 5 mL/min (influent) and 10 mL/min (effluent). The loaded cartridges were rinsed with 10mL of aqueous methanol (methanol: water, 5:95, v/v) to remove final traces of impurities. Elution of the loaded cartridges was carried out with twice 5mL methanol, 5mL ethyl acetate and 5mL hexane respectively to extract the broad polarity analytes that are sorbed by the cartridges. The first and the second elutions were combined to make 10mL of each solvent extract. Each of the three eluted extracts (methanol, ethyl acetate and hexane eluates) was evaporated to dryness in a speed vacuum concentrator and resuspended in ethanol (300μL). An aliquot (10μL) of the resuspended sample was assayed on *in vitro* yeast recombinant androgen receptor assay in order to determine the total anti-androgenic activity. The remaining test solution was stored in the freezer at -20^0C temperature for further analysis. In addition, two sets of work-up controls (produced with three cartridges each) were made up. The first set of work-up control cartridges were prepared as described above and were used to analyse 500mL of HPLC grade water spiked with 5mL 1% acetic acid (acetic acid: water, 1:99, v/v). The second set of work-up control cartridges were only prepared and pre-conditioned as described for influent and effluent above. Whilst the first work-up control was prepared to estimate the contributions of both the cartridges and water to the overall anti-androgenic activity of the samples, the second is meant to evaluate the contribution of cartridges only.

2.3 DNA Recombinant Yeast Transcription Screen Assay

In vitro yeast recombinant screens have been actively used to investigate and detect agonistic and antagonistic activities of natural and synthetic estrogenic and androgenic chemical compounds (Sohoni and Sumpter, 1998). In both cases, the estrogenic and androgenic receptor ligands are quantitatively estimated. It is on this premise that *in vitro* yeast assay has been employed to conduct investigation into the likely behaviour of chemical compounds in living organisms. In the yeast, a human androgen receptor gene which forms the androgen response elements is incorporated into the nucleus of the yeast genome. On initiating binding with androgen-like substances, the ligand-binding receptor in the yeast expresses Lac-Z (a reporter gene) on the yeast plasmid which, in turn, induces the secretion of β–galactosidase. The degree of conversion of the chromogenic chemical indicator, chlorophenol red-β-D-galactopyranoside (CPRG), from yellow to red by the enzyme β-galactosidase is proportional to the amount of androgen-like substance involved in the binding process.

2.3.1 Cleaning and Sterilisation Protocols of Yeast Glassware

Apparatus for recombinant yeast assay must be kept clean and sterilised to reduce the level of background noise which may occur due to apparatus contamination. To achieve this, all glassware used for the yeast assay was soaked in a detergent for about 12 hours before washing. After washing and thorough rinsing, they were drained of water and furnaced at 500^0 C for 2 hours and were allowed to cool down. Preparation of yeast-related solutions (minimum medium, yeast culture, yeast growth medium and microtitre plates) was carried out in the culture hood (Hera Safe, Heraeus GmbH, Hanau, Germany). Together with other yeast assay materials (pipette tips, cotton wool and aluminium foil), they were sterilised in an autoclave (Osprey 401, LTE Scientific Ltd., Oldham, UK) at 121^0 C for 30 minutes and were left to cool. Other materials that were not directly used for the yeast assay was cleaned in absolute ethanol and allowed to dry. Before use, the laminar flow cabinet (culture hood) was disinfected and sterilised with industrial ethanol while the necks and caps of all reagent bottles were flame-sterilised before and after pipetting from them.

2.3.2 Preparation Procedure of Minimum Media for Yeast Screen Assay

A 1 L of minimum medium was prepared by weighing the following reagents into 900mL of ultra-pure water dispensed from Elga UHQ System (Elgar System, Buckinghamshire, UK) into a baked conical flask (1L) placed on a magnetic stirrer hotplate (Weiss Gallenkamp, England, UK) and set at 60^0C: 13.61g potassium phosphate (monobasic), 1.98g ammonium(II) tetraoxosulphate(VI), 4.20g potassium hydroxide pellets, 0.20g magnesium(II) tetraoxosulphate(VI)(heptahydrate), 50mg L-leucine, 50mg L-histidine, 50mg L-adenine, 20mg L-arginine-hydrochloride, 20 mg L-methionine, 30mg L-tyrosine, 30mg L-isoleucine, 30mg L-lysine-hydrochloride, 25mg L-phenylalanine, 0.10g L-glutamic acid, 0.15g L-valine, 0.38g L-serine, 1mL iron(III) tetraoxosulphate(VI) solution{40mg $Fe_2(SO_4)_3$/50mL water}. 100mL of ultra-pure water was added to make-up the solution after all the reagents had dissolved and the medium was dispensed in 45mL aliquots into glass bottles for autoclaving. The autoclave was operated for 30minutes at 121^0C and the bottles were allowed to cool and were stored at room temperature.

2.3.3 Preparation of Yeast Culture and Yeast Growth Media for Yeast Assay

Yeast culture was prepared by the addition of the following to 45mL minimum medium transferred into an autoclaved conical flask: 5mL glucose solution, 1.25mL L-aspartic acid solution, 0.5mL vitamin solution, 0.4mL L-threonine solution, 125µL copper (II) sulphate(VI) solution. This solution was seeded with pre-vortexed 125µL yeast stock (AYAS yeast cells in glycerol suspension) from a cryogenic vial stored in the freezer at -20^0C. The conical flask was placed on an orbital shaker and incubated at 28^0C for 24hours. Yeast growth medium for AYAS yeast assay was prepared the same way by adding all the items above (except for yeast where 0.7mL taken from the conical flask on the orbital shaker), 0.5µL chlorophenol red-β-D-galactopyranoside (CPRG) and 15.68µL dihydrotestosterone (DHT, $3.2x10^{-9}$M). All these preparations were carried out in a culture hood.

2.3.4 Preparation of AYAS Assay

Yeast growth medium prepared above (Section 2.3.3) contained DHT (an androgen receptor agonist and a natural ligand) used as a positive control to detect human androgen receptor (hAR) antagonism by producing 65% sub-maximal hAR agonistic response (Sohoni and Sumpter, 1998). The influent and effluent extracts stored in the freezer were defrosted and vortexed ready for use. A 20µL aliquot of the standard (flutamide) and ethanol were pipetted correspondingly into the first and third rows of the optically transparent flat-bottomed microtitre plates. Flutamide is chosen as the standard for this analysis because it is a known anti-androgen that had been used in previous *in vitro* yeast studies. For flutamide, the sequence of half-dilution proceeded from the most concentrated left well to the less concentrated right. Given that ethanol was used to resuspend the wastewater extracts for assay, the ethanol control was added to the plate to monitor the background noise. The same volume of the test samples were pipetted next by the same half-dilution concentration sequence for all test samples. The plates were left in the laminar flow cabinet to allow all the solvents in the wells to dry out. A 200µL of the aliquot yeast growth medium (Section 2.3.3) was dispensed into each well using a multi-channel, syringe-incorporated pipette. The plates were sealed up with a tape, mixed well with a shaker and incubated at 32^0C in a ventilated incubator (LMS Systems, Sevenoaks, Kent, UK) for 24 hours and changed over to 24^0C ventilated incubator (LMS Systems, Sevenoaks, Kent, UK) for another 24 hours. The sensitivity of

the yeast assay was monitored both at absorbance of 540nm and 620nm after the second 24 hours of incubation with ELx800 BioTec spectrophotometer (BioTek Instruments, Winooski, Vermont, USA). This ensures that the turbidity measurement is appropriately corrected to reflect the true growth rate of the yeast. The calculation for this is expressed mathematically thus:

Corrected Absorbance Measurement (CAM) value = [Sample ABS540nm-{Sample ABS620nm-Ethanol BlankABS620nm}].

The flutamide concentration (the standard) decreases from the bottom of the plateau to the top whereas the anti-androgenic activity increases in that order. As the concentration of flutamide decreases and the DHT becomes accessible across the wells there are high chances that the human androgen receptor in the yeast will bind to the DHT and produce the β-galactosidase. This is revealed on the plate (Figure 2.1) as the colour changes from yellow to orange to red across the wells in the first row. A deep red colour in the assay indicated no anti-androgen activity due to production of β-galactosidase whilst wells containing flutamide or samples with anti-androgens appeared yellow-light orange indicating little or no production of β-galactosidase (see Figure 2.1).

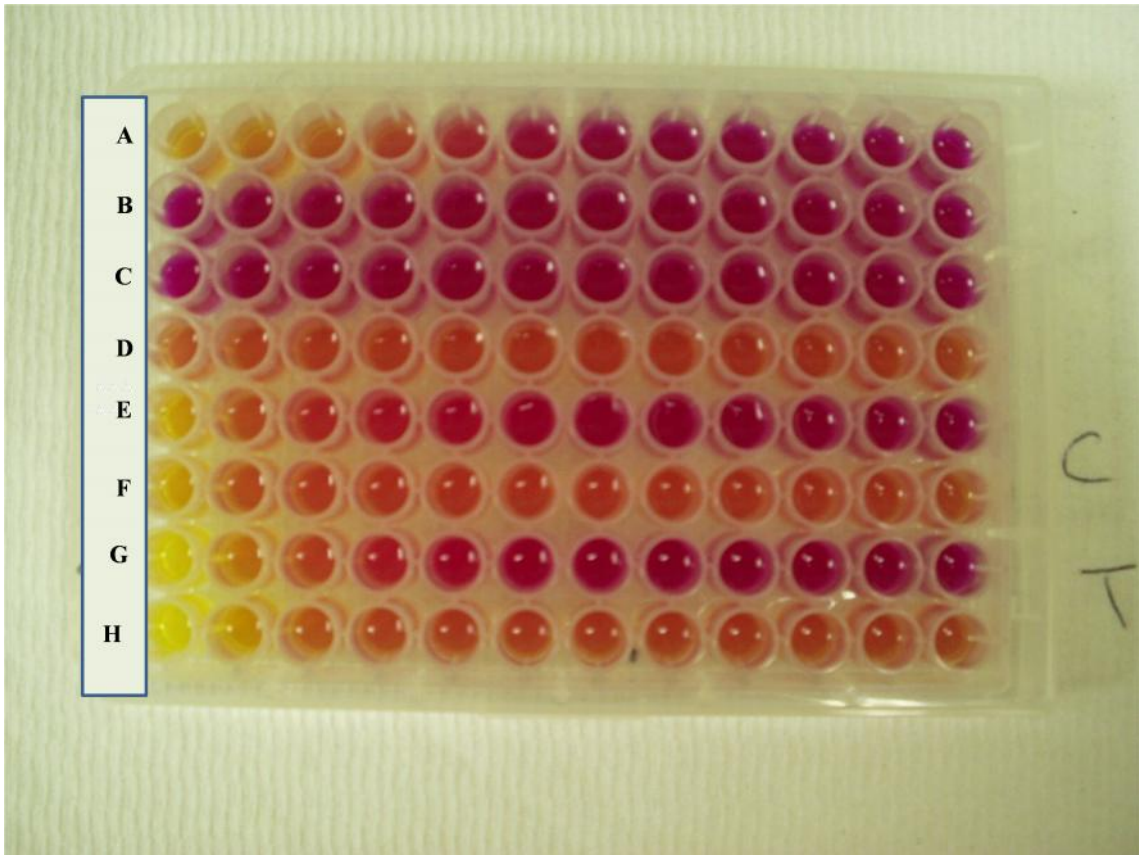


Figure 2.1: The photograph of an AYAS assay plate after 48 hours of temperature-regulated incubation. The light yellow and orange colours are indicators of positive response on the yeast medium. The deep clear yellow coloration may be indicating a response tending towards toxicity and could also be indicating cell lysis as the concentration of the test compounds approaches lethal dose. The samples plated on the assay are serially diluted two-fold in concentration from the left well (the most concentrated) to the right (the least concentrated) in both the test samples (Rows D-H) and flutamide standard (Row A) wells. The test samples which contain variable concentrations of triclosan and dichlorophene (2.5mg/L, 5mg/L and 7.5mg/L of triclosan are marked in rows D, F and H respectively and while 7.5mg/L and 15mg/L of dichlorophene are marked in rows E and G respectively) indicate different degree of responses on the yeast assay. Only Rows B and C, which are blank (media only) and ethanol solvent with media respectively, consistently indicate no AA on the assay as they are negative controls.

In accordance with Beer-Lambert law, the values of the absorbance generated from the plate corresponds proportionately to the degree of colour intensity in the wells. Inhibition on the assay was reflected by the yellow-light orange colour and corresponds

to the concentration of the anti-androgenic activity produced in the wells of the assay. The dose-response curve was plotted using the corrected values of absorbance to produce a reverse sigmoid curve (Figure 2.2 and 2.3). The flutamide standard curve (Figure 2.2) shows the normal positive response curve and graphical expression of the spectrophotometrically generated data from the yeast assay. The test samples (Figure 2.3) show similar positive response curve whenever they are active although with different shapes.

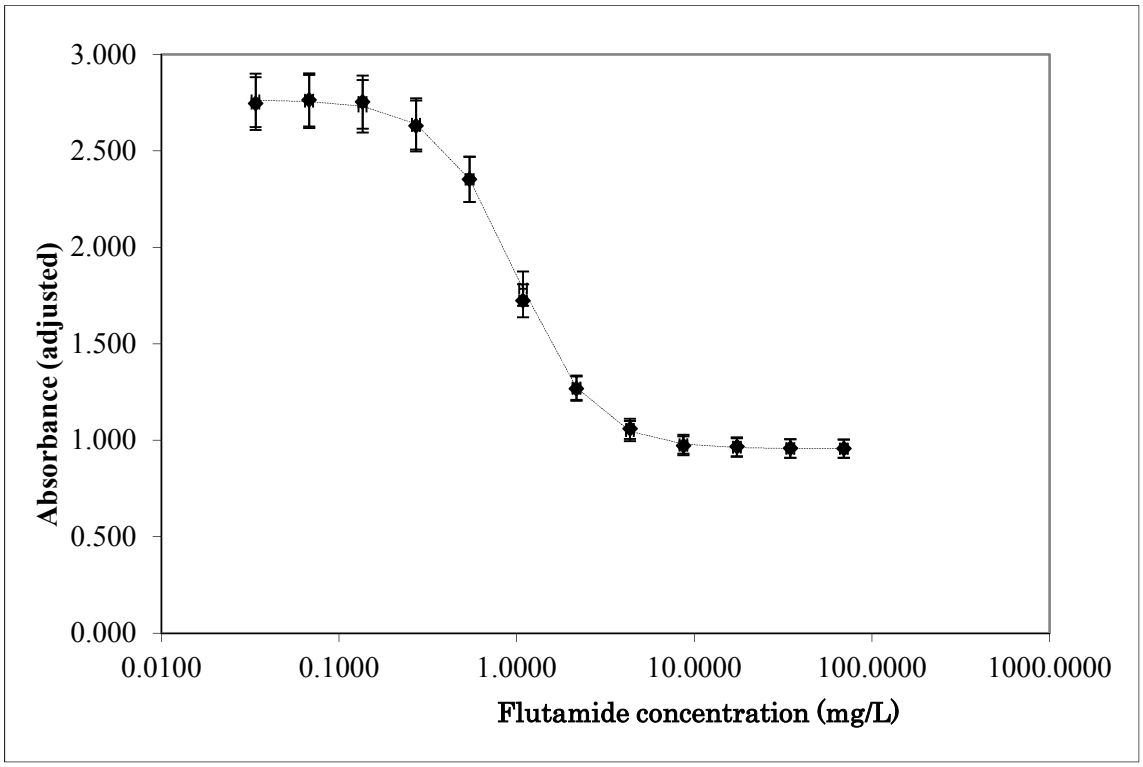


Figure 2.2: Flutamide standard curve showing the plot of corrected absorbance against concentration with the region between the top and bottom plateaux exhibiting strong anti-androgen activity. The EC_{50} of the Standard Curve is measured as 0.95mg/L and is equivalent to $3.44x10^{-6}$M. The dose-response curve is plotted as the mean ± one standard deviation (mean±SD) of the response to three flutamide replicates (n=3).

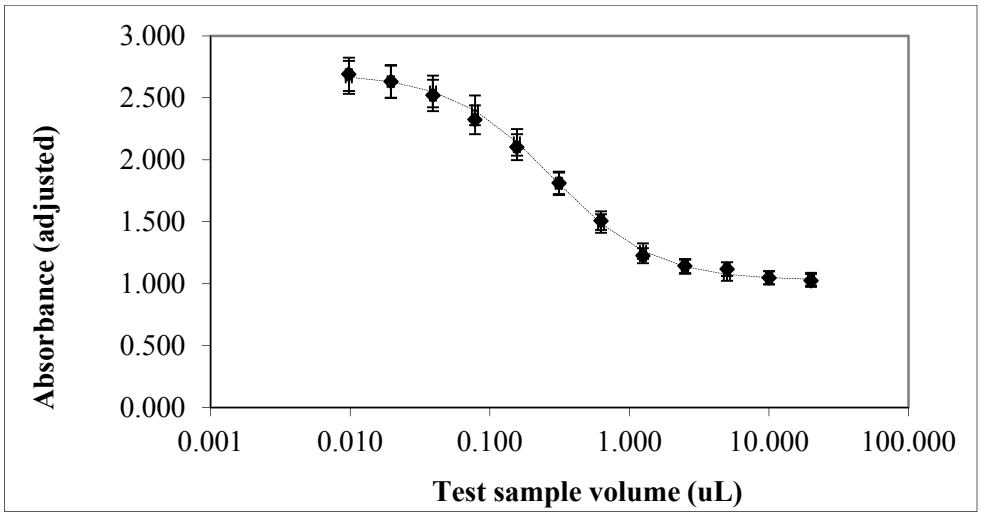


Figure 2.3: The response curve showing the positive response of test samples on AYAS assay. The sample measurement is taken in volume (μL) and the anti-androgenic activity equivalent is determined with respect to the absorbance equivalent of the standard flutamide concentration. The dose-response curve is plotted as the mean ± one standard deviation (mean±SD) of the response to the test sample replicates (n=3).

2.3.5 Evaluation of Recombinant Androgen Receptor Antagonism

The plot of corrected absorbance against concentration for the standard produced reversed sigmoid curves (Figure 2.2) with the top and bottom plateaux corresponding to a maximum and minimum respectively. Similar curves could also be produced when assays of samples containing appropriate dose of anti-androgens are analysed (Figures 2.3 and 2.4) by halving their successive concentrations. The AA is calculated by finding the mid-point between the lowest (minima) and highest (maxima) active points on the curve. The mid-point absorbance from the curve is used to derive a correspondent absorbance on the standard flutamide curve which is extrapolated to produce the EC_{50} and also corresponds to the value of receptor antagonism of the samples. This is related proportionately to the amount of the flutamide contained in the well and that of yeast media added to each well. The overall mathematical evaluation produces a mid-point absorbance and concentration, known as the EC_{50} values, used as the standard measurement to quantify the AA of environmental samples (influent and effluent) under similar laboratory conditions. For the anti-androgenic activity of the samples to be accurately determined, their turbidity readings (taken at 620nm) are comparatively analysed with that of the ethanol blank at the same wavelength. A test sample at any

given concentration is considered active (as indicated by the 540nm values) when its 620nm values are correspondingly roughly equal or greater in magnitude than the mean (-2SD) of the 620nm absorbance readings of ethanol control (see Section 4.1.2.2). However, if the 620nm values of the samples fall significantly below the mean-2SD of the 620nm absorbance values of ethanol control, then it will be regarded as toxic irrespective of the 540nm values recorded. The degree of receptor antagonism was evaluated and expressed graphically using the corrected absorbance values and the corresponding concentrations of the test samples (Figure 2.2).

2.4 Fractionation of Influent and Effluent Replicates in HPLC

The control sample (in Section 2.2) and four aliquot samples comprising two influent and two effluent replicates were fractionated by reversed phase High Performance Liquid Chromatography (HPLC). The HPLC instrument (Waters 600E Millennium 2000 Edition, MA, USA) consisted of a degasser, an automatic sample injector, a pump, a Novak Pak column (4µm particle size 4.6x250mm) and a photodiode array detector coupled together. The combined extracts from each effluent replicate were vortexed and reduced to dryness and re-dissolved in 250µL of methanol-water mixture (1:1 v/v). Similar procedure was repeated for the influent replicates. All the samples were filtered with 0.2µm Whatman Anopore filters to remove the particulates. Aliquot of the control, the effluent and the influent samples were fractionated separately by injecting each in turn into the HPLC at room temperature. The HPLC was operated at a flow-rate of 1mL/min in a linear water-acetonitrile gradient flow starting from 0 min (90:10), 10min (70:30), 65min (0:100) holding for 15min. The acetonitrile mobile phase of the HPLC was prepared by acidifying acetonitrile with 0.16% glacial acetic acid while the water mobile phase was made up by spiking the HPLC graded water with 0.16% and 4% of glacial acetic acid and acetonitrile respectively. The eighty HPLC fractions of each sample were collected and reduced to dryness in a speed vacuum concentrator. Each fraction was then re-suspended separately in 200µL of ethanol and stored in the freezer at -20^0C for further analysis. Also, the residues in the filter sieve of each sample replicate and the control were washed with 200µL each of ethyl acetate, hexane and methanol into separate test tubes to check for any loss of anti-androgenic activity.

2.5 Determination of Anti-androgenic Activity of HPLC Fractions

A 200μL of the yeast growth medium (described in section 2.3.3) was dispensed into the properly dried 96-well microtitre plates to which 30μL each of flutamide, ethanol and the 80 fractions of an effluent replicate above (dried out and reconstituted in ethanol) was previously added (Section 2.4).The fractions were dispensed into the wells across the row such that 30μL aliquot of each fraction was replicated into two adjacent wells. The plates were sealed with tape and carefully shaken on a plate shaker before they were placed into 32^0C incubator for 24hours. The plates were transferred to 24^0C incubator for another 24hours and read afterward on a plate reader at 540nm and 620nm for colour development and turbidity respectively. The two absorbance readings were transferred into an Excel program written to analyse the assay. The program works by averaging each fraction duplicated in the successive wells of the plate and plots the corrected mean absorbance against their retention time (which has automatically becomes the fractions identity throughout this study). The total anti-androgenic activity of the 80 fractions was estimated by determining the inhibition of the YAS assay in response to 5α-dihydrotestosterone (DHT) standard. These values were quantified as correspondent equivalence to flutamide concentration on a flutamide standard curve.

2.6 Results

2.6.1: Determination of Background Noise Level, Limits of Detection and Quantification in AYAS.

Pre-analytical treatments in the laboratory have become a known means by which the accuracy of quantitative and qualitative analytical measurements can be compromised. During experimental preparations in the laboratory, a wide range of analytical signals or interferences are produced at the background of the target sample which can influence the overall experimental results. Such analytical interferences (which exist at the background of the samples) are known as background noise and, in some instance where experimental controls are involved, as control blanks. For the yeast experiment, the background noise and the control blank are constituted by the yeast media in addition to other non-yeast work-ups (e.g. SPE apparatus) and the solvents (e.g. acetic acid, methanol, ethyl acetate and hexane). The total anti-androgenic activity of wastewater samples can be accurately estimated in AYAS when the background noise from the yeast experimental work-up and the blank are known. The analytical signals generated by the blank (or control work-up) of a yeast experiment would record values corresponding to the lowest concentration of anti-androgenic activity of wastewater samples. Therefore, for the assay to produce an analytical signal or response which can be detected or quantified, the anti-androgenic activity of the wastewater samples must produce an analytical signal which exceeds that produced by the background noise. The background noise level of the yeast assay was estimated by measuring the mean (± two times the standard deviation) of the ethanol control. Similarly, the limit of detection (LOD) was determined by addition (or subtraction) of three times the standard deviation of the mean of the ethanol control. For the limit of quantification (LOQ), it was evaluated by adding (or subtracting) nine times the standard deviation of the mean of the ethanol control.

2.6.2 Determination of Anti-androgenic Activity of WwTP Samples by Recombinant Androgen Yeast (AYAS) Assay.

The AYAS assay has been successfully used to determine and estimate the anti-androgenic activity of the wastewater extracts. The reverse sigmoid curve typical of flutamide standard curve was also generated with increasing concentrations of the

wastewater extracts. The EC_{50} for each sample was measured between the sub-minima and sub-maxima at the lower and upper regions of the curves (Figure 2.4).

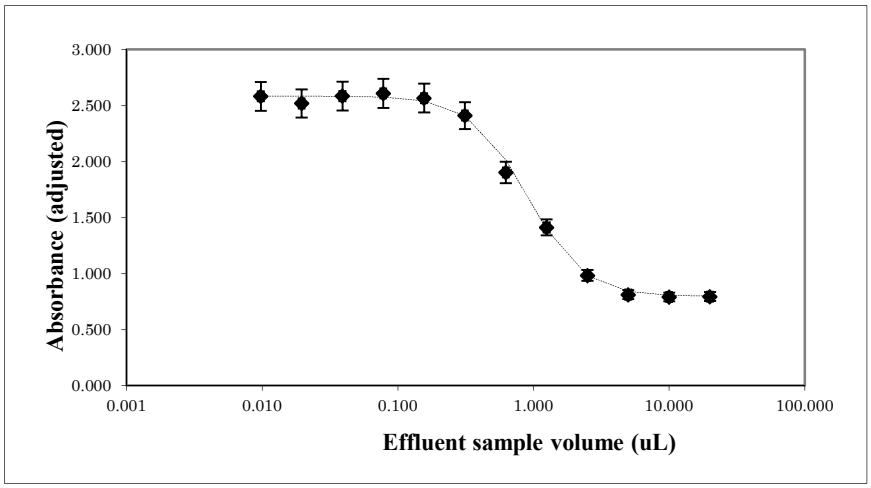


Figure 2.4: The response curve of effluent on the AYAS assay indicating positive response and anti-androgenic activity. The anti-androgenic activity of the effluent is measured in mgFeq/L and it corresponds to the mid-point of the linear response of the curve. The dose-response curve is plotted as the mean ± one standard deviation (mean±SD) of the response to effluent replicates (n=3).

As stated in Section 2.6.1, the anti-androgenic activity of samples cannot be accurately determined until the contributions from the laboratory work ups are known. The background studies to determine the contributions of SPE apparatus and the solvents to the overall anti-androgenic activity were carried out. The three SPE solvent extracts obtained from a set of solely preconditioned cartridge, and another set of cartridges loaded with HPLC-grade water, were assayed after reconstitution in ethanol. The results computed in Table 2.1 below were analysed and evaluated with respect to the limit of detection (LOD).

Table 2.1: Results of background anti-androgenic activity generated from laboratory work-up controls as recorded by the three extraction solvents.

Sample*	Mean AA contribution of Preconditioned cartridges loaded with HPLC-grade water	Mean AA contribution of Preconditioned cartridge only
Methanol (mgFeq/L)	<LOD	$0.007 \pm 5.774 \times 10^{-5}$
Ethyl acetate (mgFeq/L)	<LOD	<LOD
Hexane (mgFeq/L)	<LOD	<LOD

*mgFeq/L is the anti-androgenic activity measurement unit as the anti-androgenic activity contribution of the work-up is quantified in milligram (mg) of flutamide equivalent per litre of sample.

The result (Table 2.1) has clearly shown that each eluting solvent recorded an average anti-androgenic activity below the LOD (0.006mgFeq/L) except for methanol extracts of pre-conditioned cartridges which showed slight background AA. The occurrence of background AA in methanol (Table 2.1), though minimal, reinforces the concern of possible build-up of AA which may arise from work-up during extraction. This explains why cartridges loaded with samples are rinsed with methanolic solution (methanol: water, 5:95, v/v) to remove the work-up anti-androgenic activity. In addition, rinsing with methanolic solution helps to further reduce the AA contributed by the contaminants/impurities in the raw samples.

The plate information about the test samples (effluent and influent) was converted into statistically measureable data on the spectrophotometer. Their anti-androgenic activity as measured on the AYAS is tabulated and evaluated in Table 2.2 and 2.3 respectively. Table 2.2 shows the anti-androgenic activity contributions from polar, moderately polar and non-polar constituents of the influent samples as represented by the methanol, ethyl acetate and hexane extracts respectively.

Table 2.2: The amount of anti-androgenic activity present in all the three SPE extracts of influent sample replicates. A 500mL of sample was extracted and analysed separately as shown in the results of each of the four influent replicates (A, B, C and D).

Sample replicate*	Methanol (mgFeq/L)	Ethyl acetate (mgFeq/L)	Hexane (mgFeq/L)	Total (mgFeq/L)
Influent A	0.190	1.176	1.800	3.166
Influent B	0.416	1.474	0.722	2.612
Influent C	1.200	0.746	0.500	2.446
Influent D	2.182	0.592	0.458	3.232

*mgFeq/L is the total anti-androgenic activity measurement unit which expresses anti-androgenic activity in milligram (mg) of flutamide equivalent per litre of the sample.

The anti-androgenic activity recorded in each of the three solvent extracts (Table 2.2) ranged generally between 0.190 and 2.182mgFeq/L for influent samples. Similarly, the effluent samples recorded anti-androgenic activity values that ranged from below LOD to 0.452mgFeq/L (Table 2.3). It is interesting to note that the three solvent extracts of both the effluent and influent replicates are active on AYAS but the anti-androgenic activities of hexane extracts in effluent occur below the LOD. This would also suggest a possible occurrence of active compounds with a wide range of polarity. The results generally show variability in the total anti-androgenic activity between the four replicate samples. The amount of anti-androgenic activity recorded in each of the three solvents varies from one replicate to another. Due to inconsistency in the variation pattern, it is extremely difficult to predict the spread of active compounds in the samples based on their concentration in the three solvents. Although the variation in the anti-androgenic activity of influent extracts is not unexpected considering that the analytes were grab unfiltered wastewater samples, however, the level of such variability would still be expected to be low. The reason for the variation is not known but the possible explanations for this difference include blockage of the cartridges during loading, incomplete dryness of cartridges due to uneven distribution of pressure around the cartridges and variable solvent retention affinity.

SPE has become a popularly used methodology for purifying and preconcentrating crude samples. It is very effective when the crude samples (especially in aqueous form) are filtered before purification and preconcentration. In cases where crude samples were not filtered, repeatability of such SPE results are sometimes rarely possible as the cartridges could become blocked during extraction. This may be mainly responsible for the variation in the result. It is also possible, due to uneven distribution of pressure around the cartridges, for the period (time-line) required to dry each of the cartridges to vary. However, in this case a uniform drying period was maintained to prevent oxidation and volatilization (that could facilitate losses), and perhaps working under variable laboratory conditions may worsen the losses beside the fact that it will make the results unreliable. Under the existing condition, there is a possible that some cartridges may not be completely dry and the water which was retained in the cartridge' sorbents becomes eluted with the extracts. As a result, the percentage of water in each extract replicate would be different thus making the process of evaporating the samples to dryness longer than would be expected. This could lead to sample losses as extract replicates (especially that having the highest proportion of water) could lose more volatile components due to longer drying duration. Hence the overall anti-androgenic activity will be different in all the replicates. Another reason which is very rare is the solvent retention affinity in the three cartridges. Given that the cartridges are made up of the same material, this reason would not be tenable. However, in the circumstance of variable pressure distribution around the various cartridges (explained as the probable second reason above), it is very possible. It is worthy of note that the pressure applied to the cartridges is proportional to the solvent eluted from the cartridges and, by implication, inversely proportional to the solvent retained by the cartridges. This can lead to variable sample retention by the cartridges.

Effluent samples were processed in quadruplicate akin to the influent samples. Each sample replicate was extracted with the three solvents (methanol, ethyl acetate and hexane) and were assayed. Their AA on the plates was analysed with a spectrophotometer and presented in Table 2.3.

Table 2.3: The amount of anti-androgenic activity present in all the three SPE extracts of wastewater effluent replicates. A 500mL of effluent sample was extracted and analysed separately as shown in the results of each of the four replicates.

Sample replicate*	Methanol (mgFeq/L)	Ethyl acetate (mgFeq/L)	Hexane (mgFeq/L)	Total (mgFeq/L)
Effluent A	0.140	0.074	<LOD	0.214
Effluent B	0.254	0.014	<LOD	0.268
Effluent C	0.452	0.016	<LOD	0.468
Effluent D	0.212	0.084	<LOD	0.296

*mgFeq/L is the total anti-androgenic activity measurement unit which expresses anti-androgenic activity in milligram (mg) of flutamide equivalent per litre of the sample. The AA of the hexane extracts in all the effluent replicates are below the LOD measured as 0.006mgFeq/L.

The anti-androgenic activity values recorded in each solvent extract creates a pattern across all the four replicates. It can be seen that the level of AA in all the three solvent extracts is highest in methanol and much lower in ethyl acetate and hexane extracts respectively. In case of hexane, the anti-androgenic activity was below the LOD. This implies that the percentage anti-androgenic activity contribution from methanol extract alone ranged from 65.1 to 96.4%. Similarly, the anti-androgenic activity of the ethyl acetate extracts account for between 3.4 and 28.3% of the total activity per replicate. In contrast to the results of the influent (Table 2.2), the anti-androgenic activities in these effluent extracts appear to contain more polar bioactive compounds than non-polar judging from the results. Also, the level of anti-androgenic activity in effluent samples were found to be significantly lower in contrast to what was recorded in influent samples (Table 2.4). A drop of 90% in anti-androgenic activity was recorded from influent to effluent samples and this, presumably, may be due to the removal of contaminants after bacterial degradation, chemical biotransformation and sedimentation processes during treatment at the wastewater works. Although there are certain degrees of variability in both samples (Table 2.4), the effluent samples recorded a statistical range that is comparatively better. However, one of the results (replicate C)

appears to be an outlier as it records an AA value that is twice as much as that of replicate A. The level of difference shown affects the mean, the standard deviation as well as the RSD values. Although, the results of the influent samples also show some extreme values which should adversely affect the mean, the standard deviation and the RSD values, but because the values are well spread the effect has been mitigated.

Table 2.4: Summary of TAA present in influent and effluent samples as indicated by the mean, standard deviation and relative standard deviation. The sum total of all the anti-androgenic activity (mgFeq/L) recorded by each of the three solvent extracts is represented in the replicates below.

Sample replicate	A	B	C	D	Mean±Std	RSD
Effluent*	0.214	0.268	0.468	0.296	0.312±0. 110	0.35
Influent*	3.166	2.612	2.446	3.232	2.864±0.394	0.14

*mgFeq/L is the total anti-androgenic activity measurement unit which expresses anti-androgenic activity in milligram (mg) of flutamide equivalent per litre of the sample.

Effluent replicate C (0.468mgFeq/L) is suspected to be an outlier and, as a result, the data was subjected to Grubbs' and Dixon's tests at 5% significance level. The results [(G=1.436; Critical value=1.481; P=0.05; N=4) and (Q=0.677; Critical value= 0.831; P=0.05; N=4)] show that the data cannot be rejected as G and Q are less than the critical values. Similarly, the influent data was tested with the two statistical measurements at significant level of 5% and found that the results (G<critical value=1.481; Q<Critical value=0.831; P=0.05; N=4) of all the suspected data are not outlier which means they cannot be rejected.

2.6.3 HPLC Fractionation of Control, Influent and Effluent Samples

200uL of effluent and 40uL of influent were injected into the High Performance Liquid Chromatography. Below are the 254nm absorbance chromatograms of the control work-up and wastewater samples.

The HPLC chromatogram of the control work-up shows only the solvent peaks occurring between 1 and 2 minutes (Figure 2.5). No other significant peaks are detected on the chromatogram.

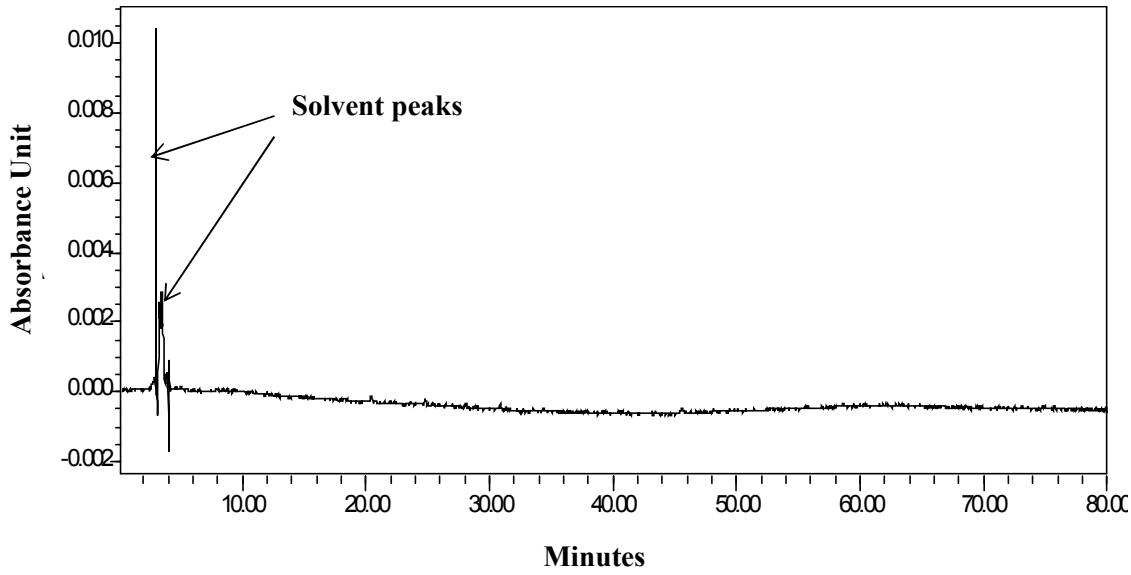


Figure 2.5 HPLC Chromatogram of the control showing the background noise and the solvent peaks monitored at 254nm UV absorbance.

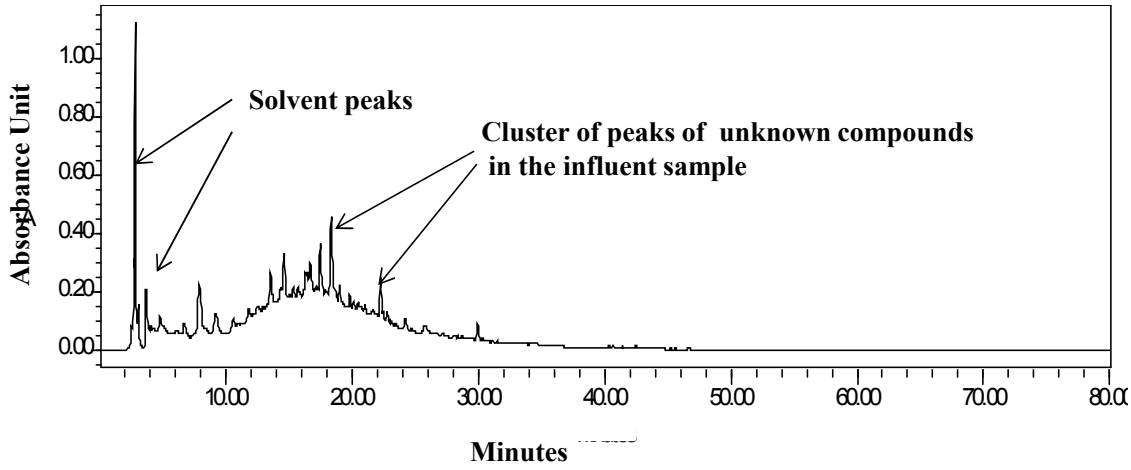


Figure 2.6 HPLC Chromatogram of influent sample showing the solvent peaks and the cluster of peaks of unknown compounds monitored at 254nm UV absorbance.

The HPLC Chromatogram of the influent (Figure 2.6) reflects a different picture to what is seen on the control chromatogram. Whereas the control chromatogram shows only the solvent peaks, the chromatogram of the influent revealed a cluster of peaks of several unknown compounds between the retention time of 5 and 50 minutes in addition to the solvent peaks that occur between the retention time of 1 and 2 minutes. A similar result is observed on the HPLC Chromatogram of the effluent (Figure 2.7) where the solvent peaks emerged between 1 and 2 minutes but an additional cluster of peaks of unknown compounds are observed between the retention time of 5 and 50 minutes.

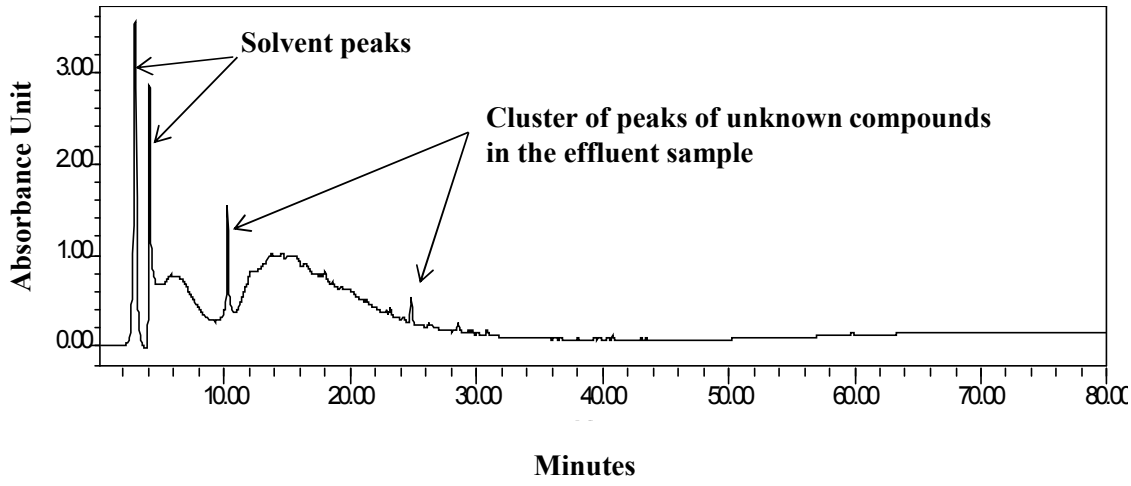


Figure 2.7 HPLC Chromatogram of effluent sample showing the solvent peaks and cluster of peaks of unknown compounds monitored at 254nm UV absorbance.

2.6.4 Anti-androgenic Activity Profile of HPLC fraction by AYAS assay

The AA profiles of the influent, effluent and water (first work-up control) fractions were prepared using the TIE approach. The AA (and the androgenic activity) of the fractions (shown in Figure 2.8) are represented by 80 black vertical lines of absorbance plotted against retention time in the range of 0-80minutes and each line corresponds to the retention time of the fractions. For the fractions of the work-up control, no significant anti-androgenic activity is recorded below the lower background noise level (mean-2SD) indicated with the lower thin reference line (see Figure 2.8). Although no significant trace of anti-androgenic activity is recorded in the control fractions, there are some fractions with retention time of 25 and 30 that show apparently weak androgenic activity (Figure 2.8). These fractions project above the upper background noise level (mean+2SD) marked off at 2.556ABS.

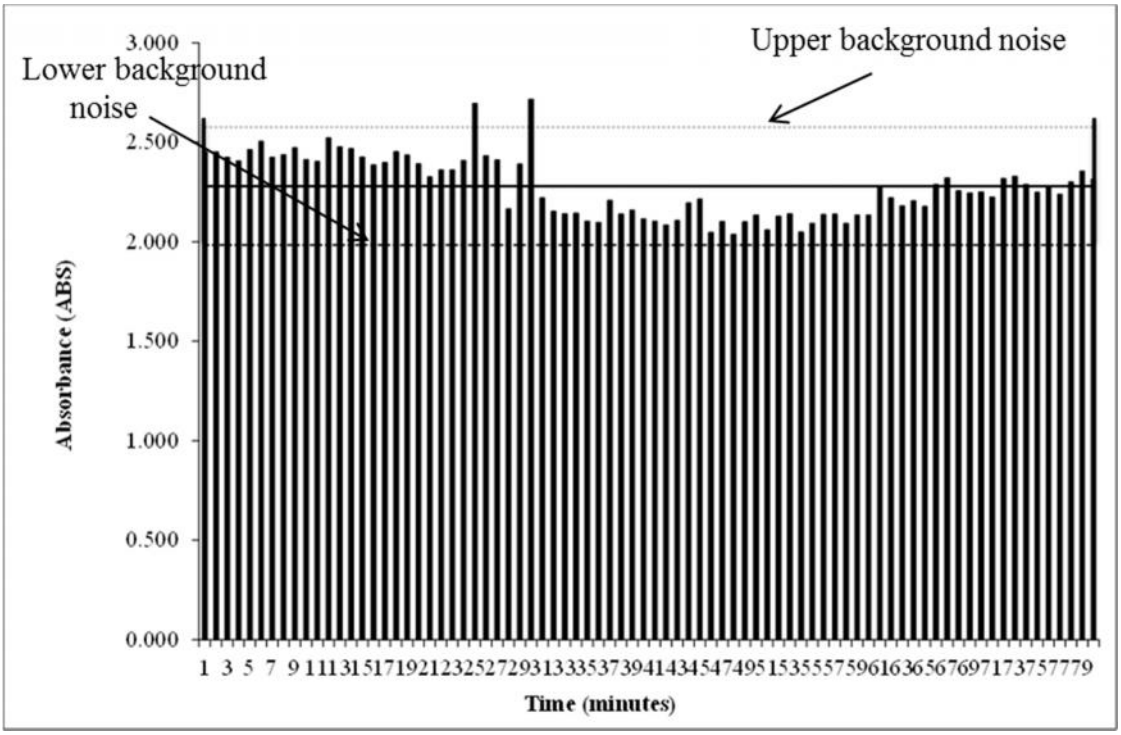


Figure 2.8: Profile of total anti-androgenic activity of the work-up control extracted by Oasis HLB cartridge and eluted with methanol, ethyl acetate and hexane. The mean background level of ethanol media is indicated by the thick horizontal line on the profile (absorbance unit 2.306) while the upper and lower background noise levels, marked off with the faint horizontal lines, are plotted as mean (±2SD) at 2.556 and 2.056ABS respectively.

The profile of the influent A (Figure 2.9) shows the anti-androgenic activity of the eighty HPLC fractions. To establish a significance response, only fractions with values below the background noise level of the assay are recorded to contain anti-androgenic activity. From the profile of influent A sample, sixteen fractions are identified to contain anti-androgenic activity and fractions with 27, 28, 29 and 31 retention times show strong anti-androgenic activity.

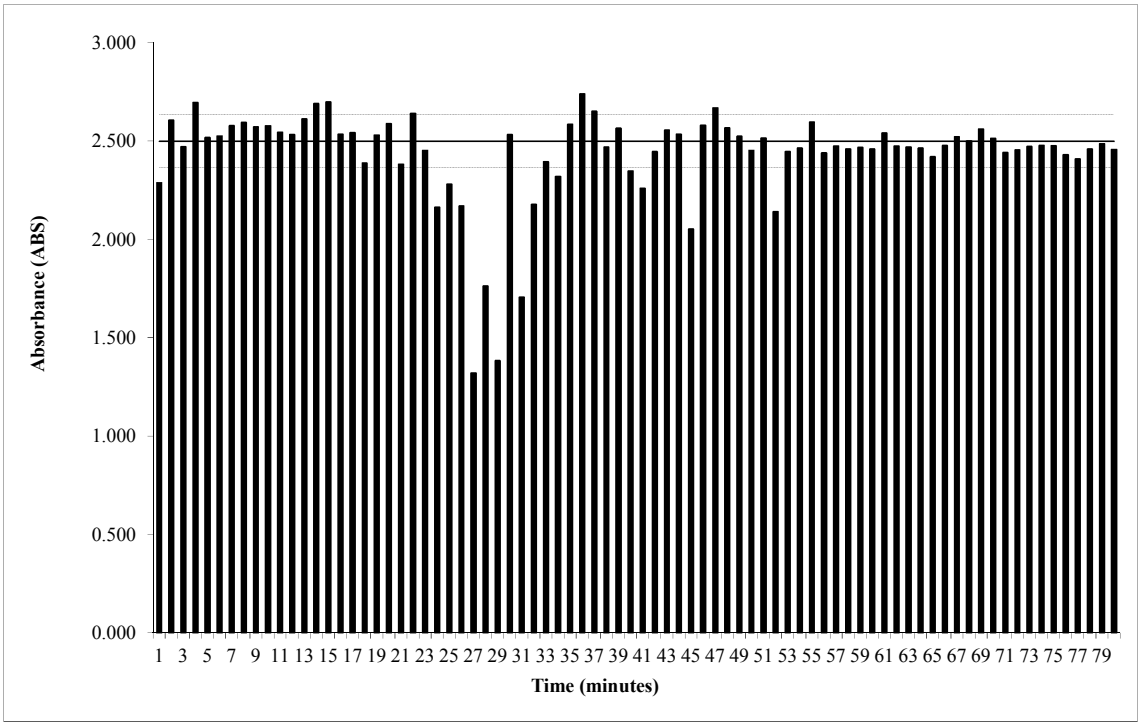


Figure 2.9 Profile of total anti-androgenic activity of influent A sample replicate extracted by Oasis HLB cartridge and eluted with methanol, ethyl acetate and hexane. The mean background level of ethanol media is marked with a broad horizontal line at absorbance unit of 2.50. The upper and the lower background noise levels are plotted as (±2SD) of the mean, marked off respectively at absorbance (ABS) of 2.60 and 2.40 with the thin horizontal lines.

The HPLC profile of anti-androgenic activity in the influent B replicate was analysed under the same laboratory conditions and shows similar results to that of influent A (Figure 2.10). Fractions that are anti-androgen-active in influent B occur at the same retention times as influent A. In addition, fractions 24, 25 and 26 of influent B are anti-androgenic too. In both influent samples, the major fractions identified as potential anti-androgens occur at the moderately polar region of the profiles (i.e.

fractions 25-29, 31). In addition in the influent samples, other less polar fraction (52) also contains anti-androgenic activity.

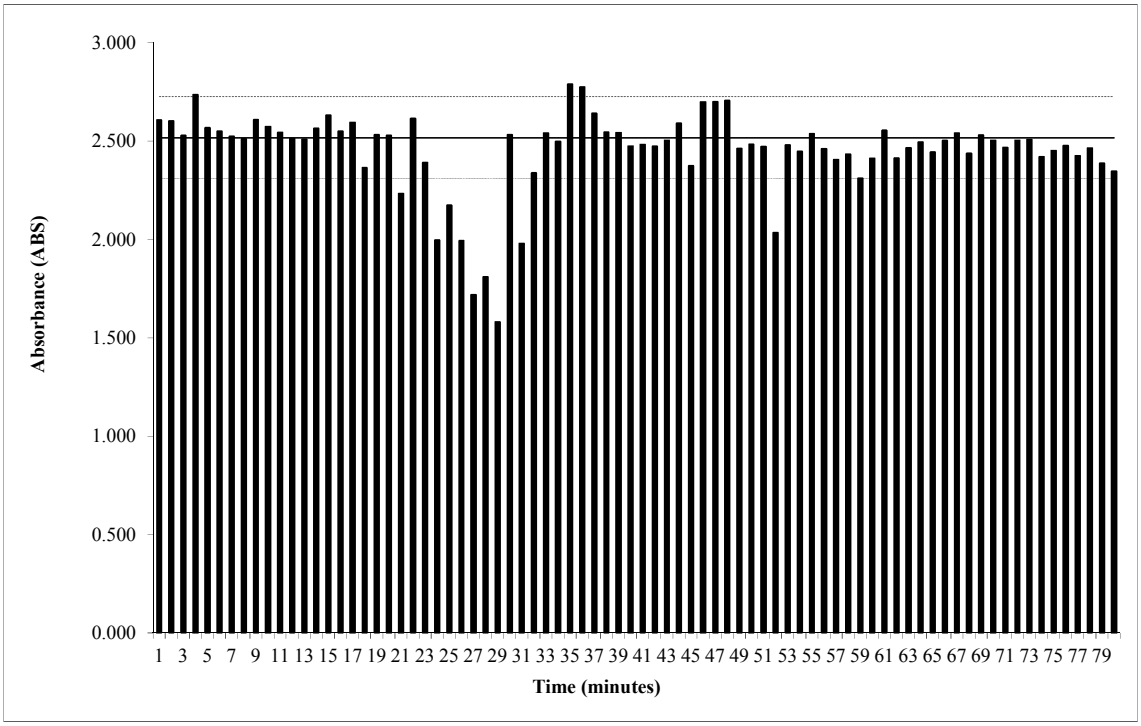


Figure 2.10 Profile of total anti-androgenic activity of influent B sample replicate extracted by Oasis HLB cartridge and eluted with methanol, ethyl acetate and hexane. The mean level of ethanol media is marked with the broad line (absorbance unit 2.40) whilst the upper and the lower levels of the background noise (mean±2SD) are marked off with thin horizontal lines respectively at absorbance (ABS) of 2.55 and 2.25 as shown.

The profiles of effluent A and B (Figures 2.11 and 2.12) show a combination of androgenic and anti-androgenic activity. In the effluent A profile, thirty-eight fractions contain anti-androgens while twenty-five fractions of the effluent B are identified to contain anti-androgenic activity. Precisely, twenty-one fraction pairs from both effluent profiles with evidence of anti-androgenic activity are found to have the same retention time and are common to both effluent replicates.

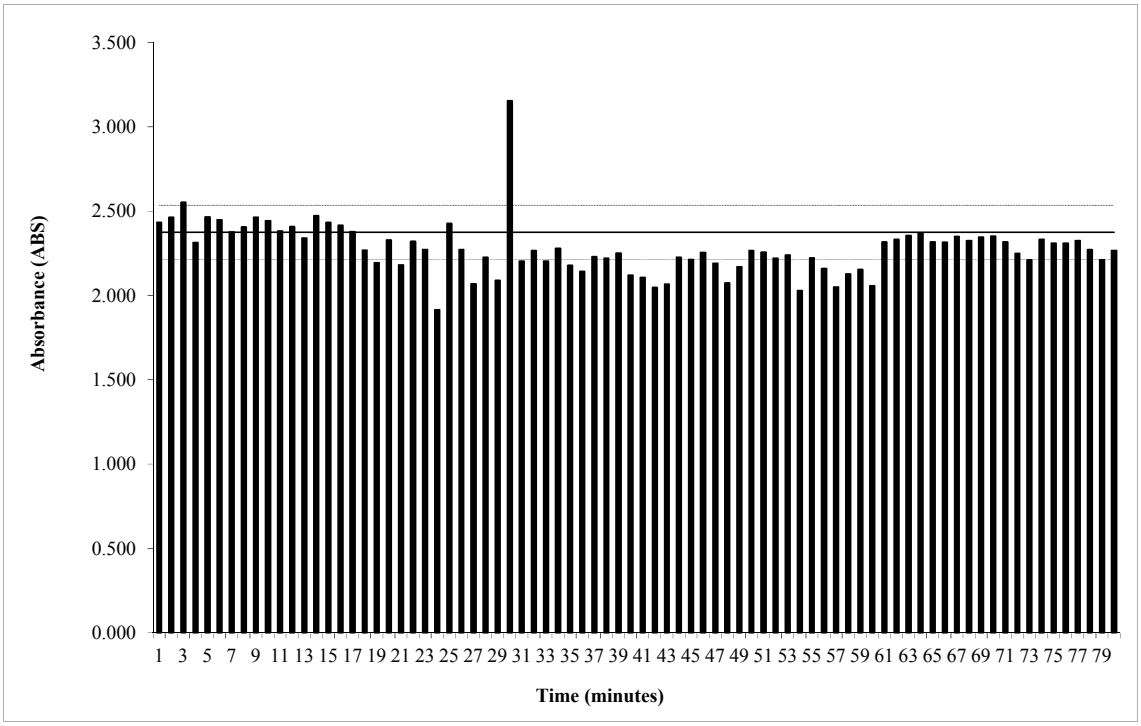


Figure 2.11 Profile of total anti-androgenic activity of effluent A sample replicate extracted by Oasis HLB cartridge and eluted with methanol, ethyl acetate and hexane. The mean background level of ethanol media is marked with the broad horizontal line (absorbance 2.30) and the upper and the lower background noise levels, plotted as (±2SD) of the mean, are marked off respectively at 2.46 and 2.10 absorbance (ABS) as shown with the thin horizontal lines.

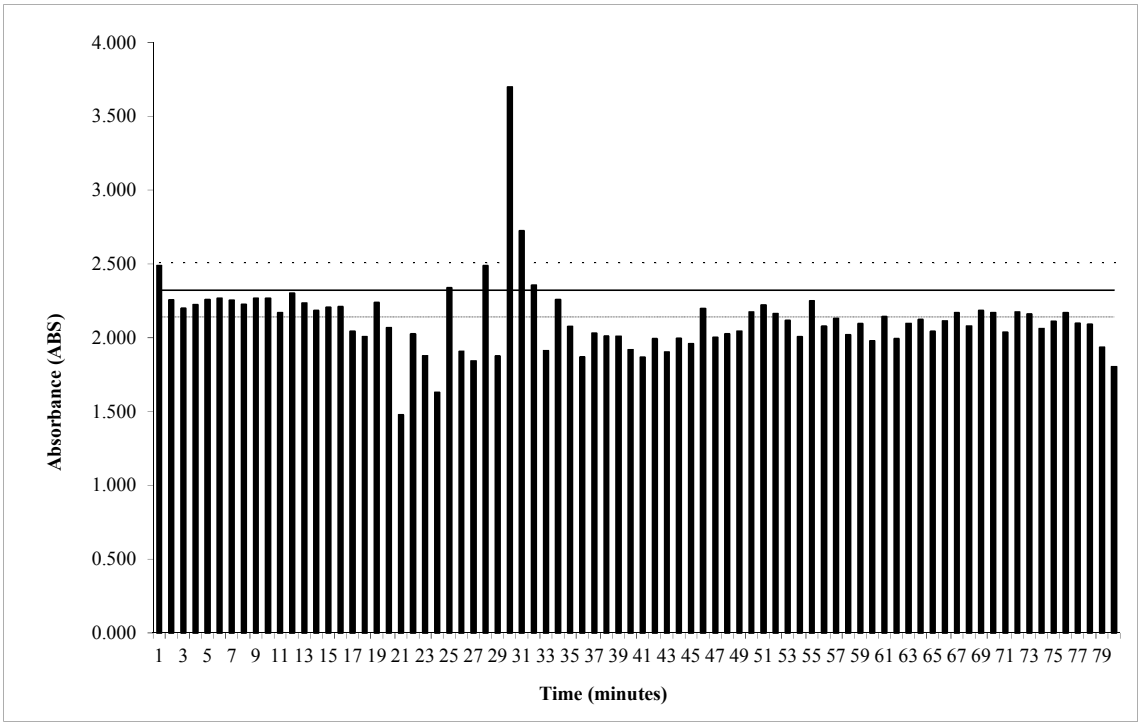


Figure 2.12: Profile of total anti-androgenic activity of effluent B sample replicate extracted by Oasis HLB cartridge and eluted with methanol, ethyl acetate and hexane. The mean background level of ethanol media is marked with the broad horizontal line (absorbance unit 2.155) while the upper and the lower background noise levels, plotted as (±2SD) of the mean are marked off respectively at absorbance (ABS) of 2.40 and 1.91 as shown by the thin horizontal lines on the profile.

The profiles of influent and effluent replicates show some dissimilarity. The fractions containing anti-androgenic activity in the influent are more than those identified in the effluent. This is not unexpected as some of the compounds could have metabolised into non-bioactive form during the wastewater treatment. In addition the effluent samples contain fractions at retention times of 25 and 30 mins which have androgen activity (Figure 2.11 and 2.12). Possibly for three reasons, the potential androgen fractions in the influent extracts are not detected. First, it may be due to the research aim of this work which, in this case, is focussed purely on analysing anti-androgenic activity. Second, there is a possibility that the androgenic activity present in the active fractions in the influent extracts could be masked by the anti-androgenic activity (Weiss et al., 2009; Conroy et al., 2007). For this to occur, the concentration of anti-androgenic activity in the fraction must exceed that of androgens. Third, the concentration of the fractions may be lower than the limit of detection in the influent

extracts. The profiles of the two sample replicates of the influent and effluent extracts reveal four anti-androgenic fractions common to all samples, namely: fractions 24, 26, 27 and 29.

2.6.5: Recovery of Samples after Fractionation

The recovery studies of the total anti-androgenic activity present in active post-fractionated fractions was compared with the TAA of the pre-fractionated extracts. The results are summarised in Table 2.5.

Table 2.5 Recovery of anti-androgenic activity in influent and effluent extracts

Sample	Total Activity (mgFeq/500mL)	TIE Recovery from HPLC (mgFeq/500mL)	Non-TIE Recovery (mgFeq/500mL)	% Total Recovery
Effluent A	1.075×10^{-1}	6.840×10^{-3}	NGG	6.36
Effluent B	1.345×10^{-1}	7.100×10^{-3}	NGG	5.28
Influent A	1.538	1.840×10^{-2}	INC	INC
Influent B	1.331	1.620×10^{-2}	0.848*	64.93

NGG: Negligible recovery value as only the injection vial glassware was extracted in both effluents non-TIE recovery; INC: Inconclusive due to insufficient sample. *Non-TIE recovery involving the additional solvent extraction of test-tubes and injection vial glassware for the influent B.

After fractionation, the recovery of anti-androgenic activity of both the influent and effluent samples was undertaken. In the studies conducted, however, the direct recovery from TIE for both the influent and effluent samples is very poor. Recovery attempts via extraction of the injection vial glassware produced poor results too. While recovery from the effluent glassware is negligible that obtained from the influent replicates are indeed less than 8%. Further recovery attempted when various test-tubes used for sample retention and storage were extracted produced some interesting results. The recovery study for influent B showed that over 57% of the sample anti-androgenic activity was adsorbed to glassware indicating that much of the AA failed to dissolve in the methanol-water solution used for fractionation. As a result, only a fraction of the

influent anti-androgenic activity was available for fractionation in the HPLC. Test-tube recovery results for effluent replicates and a replicate of influent are not available. For that reason, it is inappropriate to conclude that the best recovery effort came from the test-tube wash.

2.7 Discussion

Wastewaters sourced from predominantly domestic environments are expected to contain several chemical contaminants, some of which may act as anti-androgens in the body of wildlife animals and humans. Consumer products for domestic and personal applications such as cosmetics, pharmaceuticals, laundry and cleaning detergents, and those for health and recreational, institutional, agricultural and industrial applications constitute a potential channel by which harmful synthetic chemicals could be unconsciously discarded into the environment. These compounds end up in Wastewater Treatment Plants (WwTPs) either in their original or biologically altered forms. Previously published steroid receptor bio-analytical studies reported the presence of anti-androgenic activity of some chemical compounds in the influent and effluent samples of some WwTPs (Lishman et al., 2006; Ternes et al., 2004). Similar studies extended to wastewater samples from Horsham WwTP, which is primarily constituted by wastewater collections from domestic sources, also revealed the presence of anti-androgenic activity.

Horsham wastewater treatment proceeds in three stages: primary, secondary and tertiary (Section 1.7; Chapter One). The wastewater influents undergo primary treatment as it passes through the fine screening sand to remove particulates within the range of 3-10 mm diameter at a reduced flow to allow the heavy grits to settle down. The emerging water is conducted into a primary sedimentation tank where it remains for between 2-4 hours. In the primary tank, over 70% of the organic matters that are present in the wastewater are removed as sludge. The secondary treatment involves biological filtering and humus sedimentation. The biological filtering beds (trickling filters) contain a wide variety of micro-organisms which include bacteria, protozoa, worms, algae and larvae required for the decomposition of the organic matters. The water leaving these beds is passed into the humus tank where humus particles are separated out. The treatment in the biological filter beds and humus tank spans about 6 hours before it would be ready for tertiary treatments. The tertiary treatment stage involves the interaction of ferric chloride with the wastewater to remove phosphorus, dissolved organic matter and particulates and the coagulants formed are treated as sludge. The final effluent is discharged into River Arun. Removal of chemicals in wastewaters can potentially occur in all the three basic treatment levels in the WwTPs. However, in many instances significant chemical removal takes place at the primary and secondary treatment levels

via biodegradation, volatilisation, sorption to sludge and particulates, biotransformation and photolysis. The efficiency of WwTPs at removing these chemicals has been associated with their technological framework, the nature of chemicals involved (log K_{ow}) and how often the treatment infrastructures are renewed and reviewed. Ternes et al. (1999) showed that due to technology and facility differences at various municipal Sewage Treatment Plants investigated in Germany, Canada and Brazil, the efficiency of chemical removal of any Treatment Plant cannot be the same. Also, Svenson and Allard (2004) reported removal of between 26 and 42% androgen receptor-active chemicals by WwTPs where secondary treatment was by-passed. Similar account of chemical reduction was reported by Kumar et al. (2008) on WwTP effluents in India. This agrees with the findings in this work on Horsham WwTP which detected a substantial decrease in anti-androgen activity during the wastewater treatment process.

In this study, the choice of extraction methods was critically evaluated especially as there has been little information published on the anti-androgens in wastewater samples. Evans (2008) reported that different cartridges with different sorbent composition could retain anti-androgens differently. The choice of Oasis HLB cartridge for this work was informed by comparative deductions from her report (*ibid.*) which considered Oasis HLB favourable for anti-androgen extraction from wastewater samples. Also, based on her recommendations, the samples were not filtered before SPE so as to extract significant amount of targeted analytes from the samples. The three solvents (methanol, ethyl acetate and hexane) considered for elution were chosen as anti-androgens could range from polar to non-polar in nature (Evans, 2008). Another significant step taken was the decision on the suitable choice of assay for this work. Considering the eventual number of fractions that may likely be generated from the analysis, the cost implication and limited time available to carry out the test, this work was based on *in vitro* steroid receptor action using recombinant yeast assay (AYAS).

The preliminary findings of the anti-androgenic activities of Horsham WwTP samples produced some interesting results. The two sets of the work-up controls analysed produce responses on the AYAS. The first work-up control recorded the level of AA in all the three eluting solvents (methanol, ethyl acetate, and hexane) below the LOD (6µgFeq/L). Similarly, for the second, all the three solvents also recorded AA below the LOD except for methanol that recorded AA at the level of LOD. Some inferences can be drawn from these results. The fact that only the second work-up

control recorded an elevated AA suggests that there are some AA contributions made by pre-conditioned cartridges. If the first work-up control was only involved in the analysis of acidified HPLC-grade water, it is possible that the water must have reduced the level of AA interference in the preconditioned cartridges. It should also be remembered that similar function must have been performed by methanol in the second work-up control since it was the immediate solvent introduced into the cartridges after pre-conditioning. It therefore suggests that the AA interference generated by the pre-conditioned cartridges were removed by these (polar) solvents. Given that the suggestion of polar solvent was based on the properties of both solvents involved in this analysis, further investigation would be required to know if non-polar solvents could have performed the same function in absence of polar solvents.

The summation of the anti-androgenic activity contained in each sample replicate of the influent ranged between 2.446 and 3.232 mgFeq/L and their mean ± one SD value was 2.864±0.394mgFeq/L. Their RSD value was 0.14. Similarly, the total anti-androgenic activity of effluent replicates occurred in range of 0.215 and 0.469mgFeq/L while the mean± one SD and RSD were 0.313±0.109mgFeq/L and 0.35 respectively. The mean total anti-androgenic activity in the effluent extracts decreased to 10.9% of that of the influent extracts. The amount of anti-androgenic activity recorded in effluent samples analysed in this study is consistent with the upper range of AA reported on the effluent samples analysed from twenty-five sewage treatment plants across the United Kingdom (Johnson et al., 2007). Their findings showed that wastewater effluents collected between April and May 2003 recorded anti-androgenic activity potency range of 0.021-0.228mgFeq/L (*ibid.*). The July-August samples recorded a much higher anti-androgenic activity potency range of 0.09-1.23mgFeq/L (*ibid*.). In the analytical studies of the influent and effluent samples compared to this work across the United Kingdom, there was a common pattern which stresses the seeming variations in anti-androgenic activity from one treatment plant to the other depending on the time in the year when the works were carried out (Johnson et al., 2000; Kirk et al., 2002; Evans, 2008). These variations may be due to a series of factors such as microbial activities, difference in influent sources, collections and composition, duration of sample storage before analysis, temperature and climatic variations and possibly difference in processing method.

Two fractions containing androgen-like activity were noticed to be common on the profiles of the effluent samples and the control. This seemingly androgenic activity occurred on fractions 25 and 30 in the profiles of the target samples and the control. Although, the androgenic activity was prominent on both fractions of the control, it was absent in the profiles of the effluent and was poor in fraction 30 in the influent profiles. Given that similar androgenic activity was also noticeable in the control, it is much more likely that the contaminants responsible for this would have originated from the laboratory work-up. However, further work is needed to identify the compound(s) responsible for this androgen activity.

The percentage of anti-androgen activity recovered after HPLC fractionation is very poor. The anti-androgenic activity recovered after fractionation for each of the two effluent replicates is estimated to be <7% of the starting AA, and for the influent replicates it is less than 2%. Considering that the TIE recovery is poor for all the samples investigated, it is possible that most of the compounds in the original extracts were insoluble in the injecting solution (methanol-water solution) used for HPLC fractionation. Some compounds may also have adhered to the glass walls of the extraction vessels which would only be removed by washing with additional solvents. All the glass containers used in the extraction of influent wastewater samples were subjected to additional solvent extraction with dichloromethane, and the extracts tested in the AYAS assay. Although, the outcome of test-tube wash produced negligible AA recovery in effluent replicates (as only the injection vial glass was extracted), a significant recovery was achieved in influent B. Given that a single result (analysis) is available for the influent, the success of the post-refractionation recovery technique could not be accurately measured. These results indicate that the entire methodology used for extraction and HPLC profiling should be reviewed in order to reduce losses of anti-androgenic activity during work up.

It appears that most effluent anti-androgenic activities were lost in the course of work-ups in the laboratory. Other possible mechanisms for loses to occur are through repeated reduction of the extracts to dryness before re-dissolving in another solvent; it is expected that for labile (volatile) anti-androgens, the process will not only facilitate their exclusion from test samples it will also create erroneous mass balance. Other probable mechanisms are photo-degradation, solvent incompatibility during re-

suspension, biodegradation (even in storage system) and through fractionation on the HPLC.

2.8 Conclusion

After thorough experimentation on the samples, it is obvious that domestic wastewaters contain some chemicals that are anti-androgenic in nature as several published works have pointed out. However, the nature of these chemicals is largely unknown. The AYAS-based bioassay-directed technique facilitated the capturing of likely unknown anti-androgens (and androgens) in the test samples which may be represented by the active HPLC fractions detected in these samples. However, the methodology of the analytical work needs to be revised so as to improve on the efficiency of the recovery of anti-androgenic compounds.

CHAPTER THREE

Development of Optimised Method for Profiling Xenobiotic Anti-androgens in Effluent and Influent Samples of Horsham Domestic-based Wastewaters.

3.1. Introduction

The preliminary investigation in Chapter Two revealed that the target wastewaters contain over thirty-eight anti-androgenic fractions, many of which were common to both the influent and effluent extracts. The occurrence of several active fractions in the profile is an indication that wastewaters are complex heterogeneous mixture of bioactive environmental chemicals whose concentration and identities are currently unknown. It was discovered that only 5% of the total effluent anti-androgenic activity (i.e. the sum anti-androgenic activity of effluent fractions) was recovered from the HPLC fractionation. There was the possibility that a few of the androgenic fractions observed in the HPLC profiles could have interfered with the estimation of total anti-androgenic activity (TAA) in the wastewater extracts and could have also masked detection of anti-androgenic fractions. However, the recovery studies in Chapter Two has clearly indicated that most of the losses of anti-androgenic activity occurred while undertaking some laboratory work-up steps as additional solvent extraction of the glassware resulted in recovery of significant anti-androgenic activity (AA). Urbatzka and colleagues (2007) also reported that losses of chemicals can occur during fractionation of a sample extract and speculated that such losses may be due to additional work-up treatment to which the fractions had been subjected (*ibid.*). Other likely causes of anti-androgenic activity losses include sample degradation and repeated sample resuspension in solvents. To minimise these losses and optimise recovery, it is necessary that a new methodology for analysing the wastewater samples must be developed.

To develop a new methodology which will serve the general purpose of this study, the various stages of the previous methodology (in Chapter Two) were reviewed and improved. Different cartridges with a wide range of polarity were tested for the ability to clean-up, pre-concentrate and elute standard anti-androgens. The various sorbents contained in these cartridges were made with different materials which possess

divergent ability to retain a wide range of compounds. Those selected for testing were HLB OASIS, Strata-X, SDBL, Phenyl, CN, C18 and C8 and their effectiveness in retaining a range of standard compounds were compared. Compounds that were selected have a wide range of polarities ($\log K_{ow}$: 3.10-7.44) and they include dichlorophene, vinclozolin, p, p'-DDE, PCB-77 and PCB-138. The rationale for selecting compounds with such a broad range of polarity was to produce a mixture replica of environmental samples in the circumstance of which the true recovery efficiency of the cartridges could be determined. These compounds selected are also potential endocrine disruptors which are still being reported in environmental samples today. The AYAS assay procedure was technically modified in order to minimise background noise level and erroneous feedbacks from the samples.

In addition, a broad range of modifications was made to improve on some essential steps in the previous SPE methodology. Ethyl acetate (dielectric constant of 6.0) was replaced with a more polar aprotic dichloromethane (dielectric constant of 9.1). It was suspected that ethyl acetate was less effective at extracting or solubilising some category of polar compounds present in the environmental samples under study. For instance, dichloromethylanthracene (DCMA) is an environmental anti-androgen that is regularly used for recovery studies in our laboratory. It is poorly soluble in ethyl acetate but is soluble in dichloromethane (DCM). Methanol and hexane were regarded as good polar protic and non-polar solvents respectively and were therefore retained in the new method development. Therefore the three eluting solvents selected in the new SPE method are hexane, DCM and methanol.

In this Chapter, the yeast screen assay (AYAS) is used for screening steroid-like anti-androgenic compounds that are present in the wastewater samples and to assess the efficiency of the new methodology. The HPLC methodology is also used to simplify the identification analysis of unknown compounds in the complex extracts while the GC-MS is used to identify the unknown compounds in the fractionated extracts. This approach is often known as bioassay-directed fractionation, or Toxicity identification and Evaluation (TIE).

The aims of this research work include:

1. Developing an improved methodology for optimising recovery of steroid receptor antagonists in wastewater samples (influent and effluent).

2. Profiling the whole mixture of xenobiotic anti-androgens in these wastewater samples using the TIE approach.

3. Identifying environmental anti-androgens in fractions of influent and effluent extracts using GC-MS.

3.2 Materials and Methods

3.2.1 Materials

The HLB OASIS, C18 and C8 cartridges were purchased from Waters Corporation, Milford, MA, USA. Strata-X, SDBL, Phenyl and CN cartridges were purchased from Phenomenex. Glass wool was purchased from Fishers Scientific, United Kingdom. HPLC grade water, acetonitrile, heptane, methanol, ethyl acetate and hexane were purchased from Rathburn, Scotland. Dichlorophene (DCP), vinclozolin (Vz), p,p'-dichloro-2,2-bis(p-chlorophenyl)ethylene (p.p'-DDE), ibuprofen, isopimaric acid, 17β-estradiol, estrone, estriol, 17α-ethynylestradiol, diclofenac and tris(2-chloroethyl)phosphate (TCEP) were purchased from Sigma. 3, 3, 4, 4-tetrachlorobiphenyl (PCB-77), 2, 2', 3, 4, 4', 5-hexachlorobiphenyl (PCB-138) and chloroxylenol were purchased from Sigma-Aldrich, Steinhein, Germany. Acetic acid (≥99% pure) was purchased from Sigma-Aldrich, Steinheim, Germany. Galaxolide was a generous gift from the president of American Research Institute of Fragrance Development (ARIFD). N,N-diethyl-methyl-toluamide (DEET), abietic acid and tris(1-chloro-2-propyl)phosphate (TCPP) were purchased from Fluka. Bisphenol-A was purchased from Aldrich. Triclosan, chlorophene, naproxen, TBEP (KP-140), 3,4-dihydrobenzoic acid, pimaric acid, and 2,4,6-trimethylbenzoic acid were purchased from Tokyo Chemical Industry (TCI), United Kingdom. All the yeast materials and their purchase sources are stated in Chapter Two (Section 2.1).

3.2.2 Method Development and Validation.

Following poor recovery of anti-androgenic activity (AA) in Chapter Two, a revised methodology to screen for, and optimise AA recovery of wastewater samples was attempted. It involved testing recoveries of standards from different SPE cartridges, modifying the AYAS assay and adapting it to evaluate steroid receptor antagonism in polar (e.g. ethanol) and non-polar (e.g. dichloromethane) extracts.

Foremost, different cartridges, ranging from low to high polar affinity, were tested with the purpose of selecting the most suitable one for wastewater extraction. As stated in Section 3.1, the selected cartridges are HLB Oasis (divinyl-benzene-co-*N*-vinylpyrrolidone), C18 (octadecyl-bonded silica), Strata-X (modified styrene divinylbenzene), C8 (octyl-bonded silica), SDBL (styrene divinylbenzene), Ph (phenyl-

bonded silica), and CN (cyanopropyl-bonded silica). For this experiment, each of the cartridges listed above was made up in triplicate with each plugged with glass wool and conditioned with 5mL hexane, 5mL methanol, 5mL dichloromethane and 10mL 1% acetic acid solution(acetic acid: water, 1:99, v/v). A standard mixture was prepared with equal concentration of DCP, p,p'-DDE, PCB-77 and PCB-138 out of which 4µg aliquot (representing 1µg of each contributing compound) was added to 100mL of water for analysis. To develop a work-up solution which will serve as the control for this study, non-spiked HPLC grade water was used. The analysis was carried out in three replicates. The properties of the test compounds selected for this analysis are tabulated in Table 3.1. The sample preparations were loaded into the respective cartridges.

Table 3.1 Summary of standards used for method development with their CAS number, logarithm octanol-water partition co-efficient (log K_{ow}) and solubility in water.

Compound	CAS Number	Log K_{ow}	Solubility in water at 20^0C (mg/L)
Dichlorophene	97-23-4	4.26	30
Vinclozolin	50471-44-8	3.10	3.4
p,p-DDE	72-55-9	6.51	0.12
PCB-77	32598-13-3	6.65	Nil
PCB-138	35065-28-2	7.44	$1.0x10^{-2}$

CAS Number: Chemical Abstracts Service Number.

The cartridges were washed with 5mL 2.5% methanolic solution (methanol: water, 2.5%: 97.5%, v/v) after sample loading and were left to dry on the manifold at full vacuum (5.32 psi pressure) for one hour. Serially, the cartridges were eluted with 2x5mL methanol, 2x5mL dichloromethane (DCM) and 2x5mL hexane. Each load container (beaker) was carefully washed with (every) 5mL of eluting solvent before it was used to elute the standards. Each of the three solvent extracts collected from each cartridge (i.e. eluates) was combined and reduced to dryness and then reconstituted in 100µL of acetonitrile. A 10µL of each replicate was injected to the HPLC-UV at wavelength range of 210-400nm and was run at a gradient flow rate of 1mL/min starting

from 50:50 water/acetonitrile to 100% acetonitrile for 5 minutes. The program was maintained at 100% acetonitrile for 13 minutes. The mobile phase was made up of water (HPLC grade), 5% acetonitrile and 0.2% acetic acid (A) while the acetonitrile mobile phase consisted of acetonitrile and 0.2% acetic acid (B). As precautionary measure taken to account for possible loss of test materials due to poor sample retention on the cartridges and sample adsorption during loading, the load waste (which stands for the discharge that filtered through the cartridges) and the cartridges-wash were collected and were subjected to partition chromatography with DCM. The partition chromatography extracts were analysed on the HPLC and evaluated. The recoveries of test compounds undertaken via the HPLC were estimated by external calibration method, and the calibration curves and their coefficient of determination (R^2) for three of the five test standards are shown in Appendix A. The coefficient of determination (R^2) is a statistical measurement that evaluates the closeness of agreement between the peak area of a sample chromatogram and its concentration and measures how well the regression line represents the plotted values. It also measures the proportion of total variation associated with the data points which is accounted for by the regression line. It can be statistically expressed as the square of correlation coefficient of the data points and the regression model. Its values are expressed such that $0 \leq R^2 \leq 1$. It is sometimes regarded as a relative expression of the goodness of fit of linear plots but its failure to explain the variation of the data points around the regression line is seen as a major limitation (AMC, 1994). It is possible to have regression lines whose R^2 values are close to 1 where no single data point falls on the line. Inability to explain this data geometry makes linear regression approach ineffective and inaccurate to evaluate the goodness of fit of the regression line. Also, the linearity relationship may not exist when the data values are below or above certain data range. For these reasons, the regression model may not be suitable to predict the outcome of future experimental investigations.

3.3 Application of the New Method on Wastewater Effluent and Influent Samples.

Following poor sample recovery recorded in Chapter Two, fresh wastewater samples comprising influents and effluents were collected on the 15th of September, 2008 and the new SPE methodology was applied for purification, pre-concentration, elution and analysis. The results from this application were analysed in Section 3.6.

3.3.1 Collection, Extraction and Purification of Wastewater Samples to Evaluate the New Extraction Method.

The newly developed method was tested on a freshly collected wastewater samples consisting separately of influent and effluent. 3 x 2 litres of each sample was collected into six 2.5 litre-capacity methanol pre-rinsed Winchester bottles as described in Section 2.1.2.2. A 100mL of methanol was added to each Winchester to minimise bacterial degradation while being transported to the laboratory for processing. Each sample (400mL of influent, 400mL of effluent) was prepared in five replicates and loaded on glasswool-plugged HLB cartridges (200mg) fitted to vacuum manifold. After loading, the cartridges were dried under vacuum at 5.32 psi for 1 hour before elution was commenced. The analytes were eluted as described in Section 3.2.2. For each replicate, the eluates were combined and were dried down in a speed vacuum concentrator. The influent was redissolved in ethanol (200µL), vortexed, sonicated and transferred into a new, labelled test-tube. This process was repeated with ethanol (100µL) to fully extract the residual compounds in the sample. The extract was stored away in -20^0C freezer for assay. The extraction procedure above was observed for all other influent and effluent replicates. In all, three replicates of influent and effluent samples (labelled X, Y and Z) were made up in contrast to four replicates (A, B, C and D) which were prepared in Chapter Two as a lot of time is usually required for sample purification. However, due to insufficient sample for further analysis, Effluent-X, Effluent-Y and Effluent-Z were combined to make a single sample named Effluent-W.

3.3.2. Determination of Total Anti-androgenic Activity of Wastewater Samples with Yeast Androgen Receptor Transcription Assay

The procedure employed to determine the total anti-androgenic activity of ethanol extracts of the wastewater samples was similar to that described in Section 2.3.4 except for a modification. It was discovered that polystyrene plates are susceptible to plastic degradation when they are exposed to some non-polar solvents (e.g. DCM). As a result, they were replaced by polypropylene plates. Working with the polypropylene plates, the anti-androgenic activity of non-polar soluble compounds which were not soluble in polar solvents (e.g. ethanol) could be accurately evaluated. The three extracts produced by eluting each cartridge with methanol, DCM and hexane were combined and evaporated to dryness in a speed vacuum concentrator following which they were

resuspended in 100µL of ethanol. The procedure for plating, media preparation and dispensing, incubation, plate reading and the result analysis is as explained in Sections 2.3.4 and 2.3.5.

3.3.3. Profiling of Sample Extracts Using Reversed Phase HPLC.

3.3.3.1 Test of Sample Solubility in HPLC Injection Solvent

The anti-androgenic activity of target analytes can sometimes be lost during sample re-suspension especially when they are not completely soluble in the chosen solvents. Tests to evaluate the level of solubility of test influent and effluent extracts were undertaken using the AYAS techniques. Sample solubilisation was carried out in acetonitrile-water solution in lieu of methanol-water used in Chapter Two. 112µL aliquot of Influent-X was reduced to dryness in a speed vacuum concentrator and reconstituted in 260µL acetonitrile-water solution (80:20, v/v) to make the concentration of the extract non-toxic on AYAS. Similarly, 112µL of Influent-Y was reconstituted in 260µL of acetonitrile-water solution and the 460µL aliquot of Effluent-W was reconstituted in 260µL of acetonitrile-water. The anti-androgenic activity recovery results are recorded in Table 3.4.

3.3.3.2 Fractionation of Sample Extracts on Reversed Phase HPLC

An aliquot of Effluent-W (described in Section 3.3.1), earlier reconstituted in 260 µL of acetonitrile-water solution (80:20, v/v), was dispensed into an Eppendorf tube and was spun in a microcentrifuge at 13000 rotations per minutes for 30 minutes to remove the particulates in the sample extract. The supernatant was transferred into a HPLC vial and placed in an autosampler for injection. A small portion of the remaining sample extract was assayed on AYAS to determine the total anti-androgenic activity of the effluent extract at fractionation. Similar to the approach used in Chapter Two, a reversed-phase HPLC system was connected to a photodiode detector (Waters UK Ltd, Elstree, Hertfordshire, UK) and C18 Nova Pak column (4µL particle size 4.6 x 2500mm) and was set up with the solvent system comprising acidified HPLC grade water (0.2% acetic acid) and acetonitrile hitherto degassed with an on-line degassing system (Alltech Associates Applied Science, Lancashire, UK). The solvent system (water: acetonitrile ratio) was operated on a gradient programme: 0 minute (90:10), 10 minutes (70:30), 65 minutes (0: 100) and 80 minutes (0: 100) with the fractionation

occurring at room temperature in a regular flow of 1 mL/min. The gradient section of this HPLC fractionation program was run for 65 minutes followed by 15 minutes isocratic section (in 100% acetonitrile only). After ensuring a stable pressure, the fractionation was started with the water control and was followed by the standard mixture which comprised β-estriol, 11-ketotestosterone (11-KT), 17β-estradiol (E2), dichlorophene (DCP), technical nonylphenol (t-NP), PCB-77 and p, p'-DDE. 150µL of the standard mixture, made with concentrations between 20 and 50ng of each standard per microliter of acetonitrile-water solution, was injected into the HPLC using the autosampler. After each injection and completion of each run, the autosampler was cleaned five times with methanol (200µL on each occasion) and the system equilibrated between 25-30 minutes. The same procedure was repeated for the analysis of 260µL of effluent W and 150µL each of influent extracts X and Y.

3.4. Toxicity Identification and Evaluation Assay and Recovery Estimation of HPLC Fractions

40µL aliquot replicate of each influent fraction was assayed as described in Section 2.5 and the procedure was repeated on the effluent fractions. However, in this Section, the aliquot replicates taken were assayed directly from the originating solution without reconstitution in another solvent. The anti-androgenic activity of individual fractions in the AYAS was measured using a spectrophotometer, and the data was corrected for differences in turbidity and the anti-androgenic activity present in each fraction was calculated as described in Section 2.5. In addition, the total pre- and post-fractionated anti-androgenic activity of the influent and effluent extracts was also determined using the AYAS (Section 2.3.5).

3.5. Gas Chromatography-Mass Spectroscopy (GC-MS) Analysis of HPLC fractions with Anti-androgenic Activity

3.5.1 Selection of Fractions for GC-MS Analysis

The active fractions identified on the profiles were selected for GC-MS analysis. The fraction selection was necessary in order to maximise the time required to complete the analysis of the highly active fractions and the prospects of identifying the most potent anti-androgens. The criteria for selecting the fractions were based on the level of anti-androgenic activity of the fractions and on the possibility that some compounds

which are present mainly in a fraction could also occur in the neighbouring fractions. In addition, some selections were made based on the target analysis of some known compounds commonly detected in wastewater samples. The fractions were selected because their retention time tally with that of the target compounds using same HPLC program. For such selection pathway, the anti-androgenic activity status of the fractions would not matter much.

3.5.2 Silylation of Standards and Fractions for GC-MS Analyses.

A 400µL aliquot of anti-androgenic fractions confirmed in AYAS assay was measured into a V-shaped derivatisation glass vial after which 50µL (50ng) each of deuterated estrone and p, p'-DDE, both used as internal standards, were added. The internal standards were used to monitor the analytical and instrumentation variability given that their retention time and fragmentation pattern are known. The deuterated estrone was also added to confirm the success of the derivatisation and to determine the concentration of the GC-MS peaks produced (due to its relatively negligible volatility and thermal stability during sample concentration). Similarly, the deuterated p, p'-DDE was introduced to aid in evaluating the concentration of non-polar compounds where derivatisation is either not successful or not required. The mixture in the derivatisation tube was thoroughly vortexed and was evaporated to dryness using the speed vacuum concentrator. Thereafter, 60µL of pyridine/ N, O-bis (trimethylsilyl)-trifluoroacetamide (BSTFA) {1:3, (v/v)} was added and the sample was silylated to ether and/or ester derivatives for 30 minutes in a heat block. Again, the derivatised sample was vortexed and concentrated under nitrogen and transferred into GC-MS injection vials. 1µL of the derivatised sample was injected into the GC-MS. Prior to the analysis of the derivatised samples, pure silylating materials and derivatised internal standards were injected to respectively check the sensitivity of the GC-MS and determine their retention time. The GC-MS chromatograms of the fractions were analysed and the compounds were identified using Xcalibur (Ver. 1.4SR1) and NIST library (Ver. 2.0).

3.5.3 GC-MS Analysis of Effluent and Influent Fractions

The GC-MS used for sample analysis comprised of a Trace Gas Chromatography (Thermoquest, Texas, USA) and a Polaris-Q ion trap mass spectrometer (Thermo, Texas, USA) connected together. Attached to the GC was a fused silica capillary column (30m x 0.25mm x 0.25µm film thickness) where sample

separation was executed. The sample was injected manually into the GC using a microliter syringe (Hamilton Bonaduz AG, Switzerland) and was aided by a carrier gas (Helium) at a constant flow rate of 1.5mL per minute. The oven program was created such that the oven temperature, which was initialised and held at 70^0C for 2min, was raised steadily to 264^0C at the rate of 8^0C/min. After being held at this temperature for 10 min, it increased until it reached a final temperature of 300^0C at the rate of 10^0C/min and was maintained for 5min. The mass spectrometer was operated in full scan to capture mass range from 50 to 650u and analyse a wide range of ions generated by standards, effluent and influent fractions. For the purpose of this analysis, corresponding ions of the internal standards namely deuterated estrone, E1-d_4 (m/z= 346) and p,p-[^{13}C]-DDE (m/z =258, 330) were also monitored.

3.6 Results

3.6.1 Limits of Detection and Quantification in the AYAS and GC-MS Determinations

Limits of detection and quantification are two statistical measurements used in evaluating correctly the anti-androgens identified in wastewater samples. As expressed in Section 2.6.1, LOD is measured as the difference between the mean and three times the standard deviation of the control values whilst LOQ is evaluated as the difference between the mean and nine times the standard deviation of the control values. The LOD and LOQ were calculated for every yeast assay where quantification was necessary. The background noise-to-signal level of each experimental measurement was undertaken in AYAS with replicated ethanol blank, otherwise known as control. The measurement of TAA of wastewater extracts undertaken in the entire yeast assay was evaluated whilst taking into cognisance the response from the control. Similarly, the GC-MS chromatograms of the samples were analysed using the chromatograms of the silylating materials (regarded as the blank) and that of the standards as the foundation. For both the yeast assay and the GC-MS, the blanks were constructed to provide the needed information about the background noise conditions existing in all the experiments. Usually, they form the statistical foundation for defining the limit of detection (LOD) and limit of quantification (LOQ) of analytes. The significance of demarcating actual AA from those generated by experimental work-ups cannot be overemphasised. For instance, during the comparison of chromatograms with that of the blank, the interfering peaks occurring within the range of the analyte's retention time were identified and isolated. During quantification, the interfering peaks were factored into statistical evaluations in order to get accurate results. For the calibration curve, the LOD and LOQ are estimated based on the standard deviation (α) of the y-intercept and the slope (S) of the regression lines of the curves (ICH, 1996). The value of α is estimated from the relation: $\alpha = \sqrt{\left(\frac{\sum(Y_{exp}-Y_{reg})^2}{n-2}\right)}$ where Y_{exp} and Y_{reg} are y-values produced experimentally and those generated on the regression line respectively and n represents the number of points used for generating the curve. The LOD and LOQ are calculated from 3.3α/S and 10α/S respectively (Bartolomeo and Maisano, 2006).

3.6.2 Recovery of Samples on Cartridges

From the previous work in Chapter Two, it appeared that wastewater effluents and influents contain a wide range of anti-androgenic chemicals with differing polarities. To effectively capture these classes of target analytes, high retention cartridges are required. The recovery from different cartridges with variable sorbent properties was investigated using the test standard mixture (Section 3.2.2) on HPLC. High recovery (expressed as a % percentage of the total amount applied) from the HLB SPE cartridge (which was also used in previous extraction in Chapter Two) was demonstrated across all the test standards (Table 3.2). The results suggest that losses of contaminants that may have similar chemical properties in the wastewater extracts are likely to be very low. The calibration curves of recoveries of dichlorophene, vinclozolin and p,p-DDE were plotted with their peak areas (as measured on the HPLC chromatograms) against the corresponding concentrations and the results are shown in Appendix A. The R^2 values for the three test compounds are very high (>0.9998).

Table 3.2: SPE recovery of test standards from seven different cartridges as analysed on the HPLC. The recoveries are recorded in % mean±SD of the peak areas of the test samples.

Cartridge	Dichlorophene	Vinclozolin	p,p-DDE	[a]PCB-77	[a]PCB-138
HLB	99.33±25	111.10±18	100.21±11	94.48±15	101.04±21
C18	63.80±2	79.01±13	53.77±10	-	-
Strata-X	76.94±11	74.36±2	15.39±3	-	-
C8	73.99±1	70.60±15	82.09±15	-	-
SDBL	81.25±5	82.77±3	25.70±7	-	-
Phenyl(Ph)	93.63±2	81.09±12	31.40±2	-	-
Cyanide(CN)	88.37±3	91.90±1	33.28±1	-	-

[a]PCBs were not tested on the cartridges marked with dash sign. Dichloromethane, methanol and hexane were used as the eluting solvents for the test standards.

3.6.3 Total Anti-Androgenic Activity of Influent and Effluent Samples from the New Methodology.

The anti-androgenic activity of the fresh wastewater samples was analysed using the new methodology and the results are shown in Table 3.3.

Table 3.3: Total anti-androgenic activity of influent and effluent sample replicates. The samples were collected from Horsham Wastewater Treatment Plant in September 2008. 400mL each of influent samples (X, Y and Z) and 400mL each of effluent samples (X, Y and Z) were extracted.

Sample[a]	X	Y	Z	Mean±SD	%RSD
Influent	3.225	3.175	3.194	3.198±0.025	0.782
Effluent	0.222	0.210	0.226	0.219±0.008	3.796

[a]The anti-androgenic activity of the sample replicates are measured in mgFeq/L of the volume of wastewater extracted.

Generally, the anti-androgenic activity of the influent extracts range between 3.175 and 3.225mgFeq/L while that of the effluent extracts occur in the range of 0.210-0.226mgFeq/L. The results of TAA (in Table 3.3) significantly demonstrate a high degree of repeatability (or precision) expressed by a range of the following statistical values {range (effluent: influent; 0.016/0.05), standard deviation (effluent: influent; 0.008/0.025) and %RSD (effluent: influent; 3.796%/0.782%)}. The results clearly show that there is a significant improvement over that recorded in Chapter Two (Section 2.6.2). Partition chromatography analysis of loading waste (the solvent that emerged from the cartridges during sample loading) produced a recovery below the LOD which further suggest that HLB cartridges tested possess high sample retention efficiency.

3.6.4 Sample Solubility in Fractionating solvent

The measurement of sample solubility in acetonitrile-water (80:20, v/v) undertaken in Section 3.5 as presented in Table 3.4 indicated that acetonitrile-water solution is suitable as a resuspension solvent mixture for reversed-phase HPLC fractionation. Evaluation of the total anti-androgenic activity of both test samples before and after reconstitution in the injecting medium indicated that there was no loss recorded in between the re-solubilisation process.

Table 3.4: Test on re-constitution of influent ethanol extracts to acetonitrile-water solution.

Influent]	Total activity of re-solubilised ethanol extract in acetonitrile-water*	
	Before (mgFeq/L)	After(mgFeq/L)
X	0.121	0.121
Y	0.130	0.130

*Only two replicates were analysed due to insufficient sample for fractionation.

3.6.5 Activity Chromatograms of blank Control, Effluent and Influent Sample Extracts

The chromatograms of the control and the standards generated during HPLC fractionation (Figures 3.1 and 3.2) provide some useful information for effective identification of potential environmental compounds that are present in the wastewater samples being investigated. The prominent peak seen between 0 and 5 min of the fractionation process represents the HPLC-UV peak of the acetonitrile-water, the sample injection solvent for this experiment. The control chromatogram contained some tiny peaks whose activities are below the LOD. In contrast, the chromatogram of the standards contains several peaks which are distributed relative to the retention time of the constituent compounds (Table 3.5).

Table 3.5 Summary of reference standards injected into the HPLC before sample fractionation to help establish elution pattern of synthetic compounds based on their polarity.

Standard	CAS Number	Retention Time (min)	LogKow
17β-estriol	50-27-1	17	2.81
11-kT	53187-98-7	20	2.00
17α-estradiol (E2)	50-28-2	27	4.01
Estrone (E1)	50-22-0	30	3.75
DCP	97-23-4	37	4.26
NP	104-40-5	52	4.48
p,p-DDE	72-55-9	61	6.51
PCB-77	32598-13-3	61	6.63

The chromatogram (Figure 3.1.) shows the polarity diversity of the standards (Table 3.5) injected into the HPLC during analysis. The injected standard mixture was run using the same HPLC program (Section 3.3.3.2) and the peaks of the standards are identified in the description of Figure 3.1.

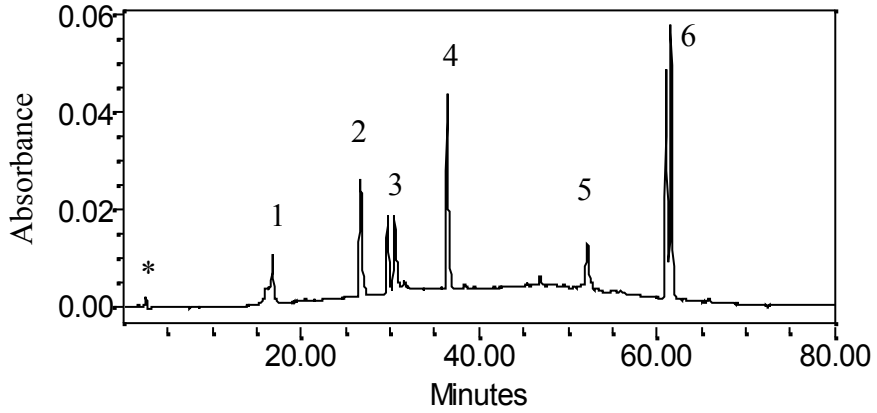


Figure 3.1: HPLC-UV chromatogram of selected standard mixture measured at a wavelength of 254nm: (1) 20ng 17β-estriol (E3), (2) 30ng 17β-estradiol (E2), (3) 30ng Estrone (E1) at 30min and 30ng chloroxylenol at 31min, (4) 40ng dichlorophene (DCP), (5) 20ng nonylphenol (NP), and (6) co-eluting PCB-77 & p, p-DDE (50ng each). The chromatogram asterisked in the diagram represents the solvent peak.

The chromatogram (Figure 3.2) reveals the level of contaminants present in the samples being analysed which may contribute to the background noise interference. The peak eluting between 0-5minutes on the left of the chromatogram represents the refractive index of the injection solvent changing the UV response during analysis. On this chromatogram, the background noise level is very minimal.

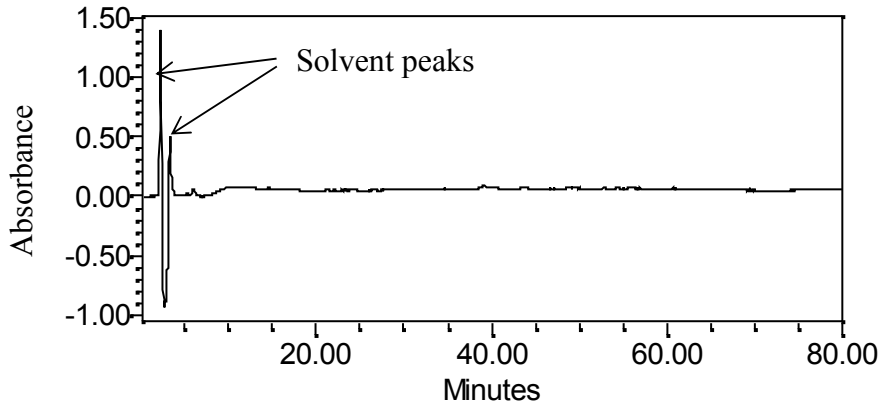


Figure 3.2: HPLC-UV chromatogram of control (methanol-water) at a wavelength 254nm showing the solvent peaks.

The HPLC-UV chromatogram (Figure 3.3) shows the clusters and peaks of unknown compounds occurring in the extract of wastewater effluent. The chromatogram which was monitored at wavelength of 254nm shows some of the

chemical compounds in the wastewater samples that elute from the HPLC column. Most of the chemicals detected at 254nm elute in the moderately polar section of the chromatogram between 0-45minutes.

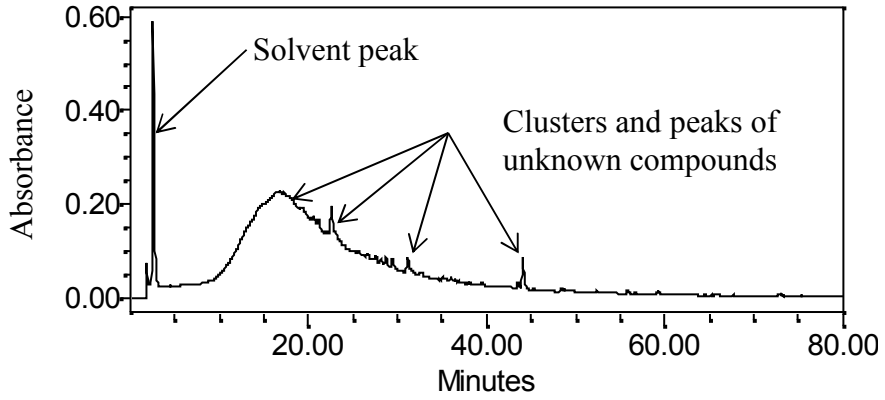


Figure 3.3: HPLC-UV chromatogram of effluent-W extract at a wavelength of 254nm showing the solvent peak and clusters of other unknown compounds.

The influent-X and-Y chromatograms (Figures 3.4 and 3.5) reveal widespread of chemicals across the three polarity divisions in the samples. Although the peaks are distributed across the chromatogram, the density of the chemicals occurs at the polar region. It can also be revealed that the chemical spread on the two chromatograms correlates, indicating that they are replicates.

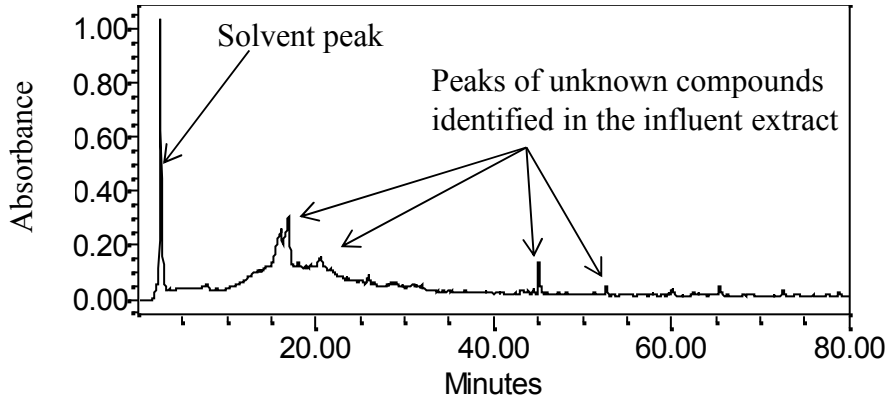


Figure 3.4: HPLC-UV chromatogram of influent-X at a wavelength of 254nm consisting of peaks of unknown compounds.

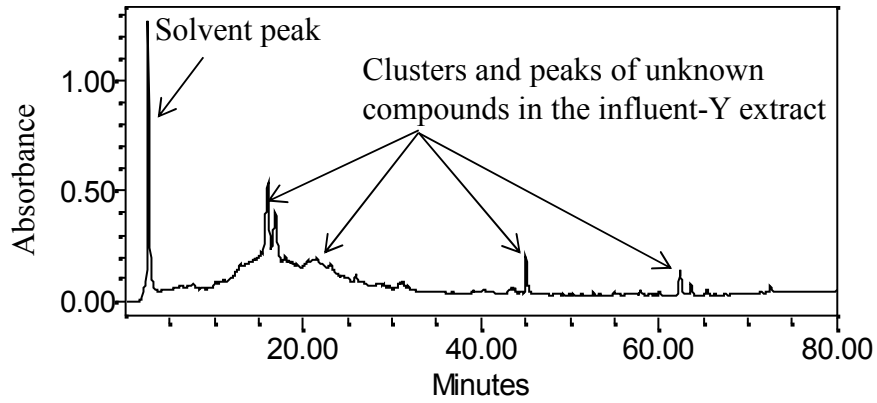


Figure 3.5: HPLC-UV chromatogram of influent-Y at a wavelength of 254nm replete of peaks and clusters of unknown compounds.

3.6.6 Profile of Anti-Androgenic Activity in Influent and Effluent Extracts on AYAS assay

The fractions of the samples generated by the HPLC fractionation process produced a profile of anti-androgenic activity in the AYAS. This AA which are presented in Figure 3.6 were validated and are marked off by the solid line which represents the mean of the ethanol blank while the broken lines correspond to the upper and lower background noise levels (mean±2SD) respectively. Given that a reversed phase HPLC fractionation approach and a water-acetonitrile gradient program were used, the section of the profile ranging from time 0 to 29 corresponds to the strongly polar section while those between the ranges of 30 to 47 and 48 to 80 correlate to the moderately polar and non-polar sections respectively. As explained in Section 2.3.5, the responses of the assay below the lower background noise (mean-2SD) value are considered as fractions with potential anti-androgenic activity and are prioritised for GC-MS analyses. However, it can be observed that some androgenic fractions (a black vertical bar extending above the upper background noise level in the sample profile (Figure 3.6B and C) are detected in both replicates (Y and X) of influent profiles but are not apparent in the profile of the effluent sample or the control (Figures 3.6A and 3.6D). The spread of anti-androgen-active fractions of influent samples cut across all the three divisions except that it mostly occurs in moderately polar and non-polar divisions. No anti-androgenic activity is noticed on the control profile which indicates that the contribution of anti-androgenic activity from the laboratory work-up is minimal (Figure 3.6). Also, a critical assessment of the sample profiles shows that a high proportion of

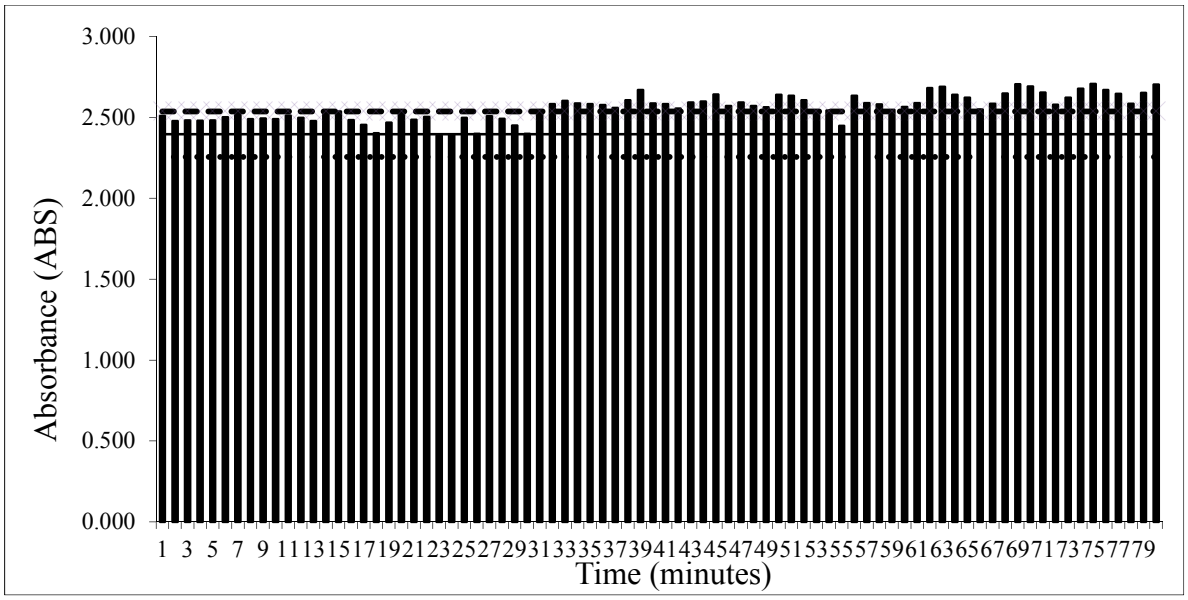
anti-androgenic activity occurs in the strongly polar section of the effluent with patches occurring in the moderately polar and non-polar regions. Initial data collected on influents X and Y, which include their chromatograms (Figures 3.4 and 3.5) and AA (Table 3.3), suggests that both samples are similar and are therefore expected to be analytical replicate. However, the profile analysis of both samples indicates some noticeable differences (Figure 3.6). Profile analysis (in Table 3.6) has shown that twenty-nine anti-androgenic fractions are common to both influent profiles. The same report (Table 3.6) also indicates that more than 40% of the anti-androgenic fractions are different in both influents X and Y which put the ratio of similar and dissimilar anti-androgenic fractions at 0.667. It is statistically evident that the two samples cannot be similar. Although, cases of shift in retention time are sometimes responsible for the variability of profiles especially when they are sample replicates, but such an argument could not explain the structural difference noticed in the two profiles. Therefore, the analysis carried out in Table 3.6 clearly shows that the profiles of both influent replicates express variability which is a classical example of sample replicates that are not analytical replicates. It must also be mentioned that effluent-X, effluent-Y and effluent-Z were combined as effluent-W for fractionation due to insufficient sample (Section 3.3.3.1). As a result, the profile could not be replicated. In general, eleven anti-androgen-active fractions are found to be common to all the samples (Influent X, Influent Y and Effluent W) and they include 26, 33, 34, 35, 37, 38, 41, 42, 48 and 53.

Table 3.6: Profiles of influents X and Y showing active fractions (i) common to each other and (ii) different from each other.

Sample	Active fractions common to both Influent-X and Influent-Y replicates	Active fractions common to both Influent-X and Influent-Y replicates
Influent-X	6, 24, 25, (25)*, 26,33, 34, 35, 36, 37, 38, 40, 41, 42, 45, 48, 49, 50, 53, 58, 59, 60, 61, 62, 64, 65, 66, 67, 68	27, 31, 32, 63
Influent-Y		18, 19, 20, 21, 22, 23, 28, 29, 30, 39, 42, 44, 46, 47, 55, 56, 57

*The circled fraction which is asterisked in the middle lower column occurs in both influent replicates and is presumed to possess androgenic activity.

A



B

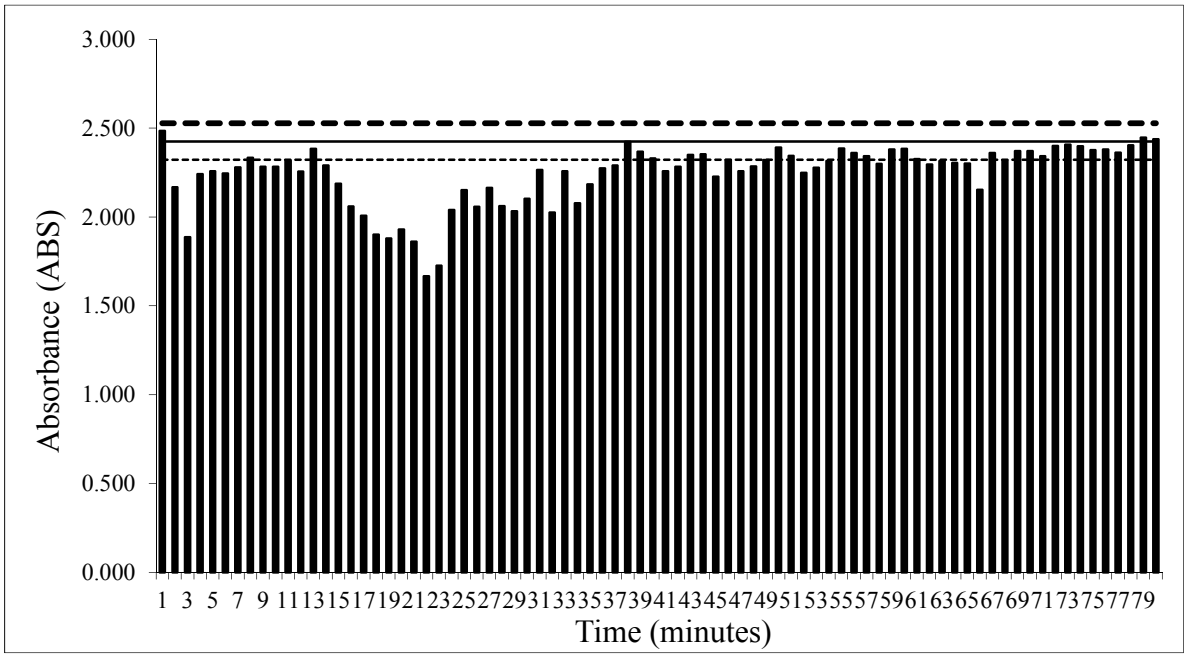


Figure 3.6: Profiles of the total activities of the control and effluent-W samples (marked A and B respectively) extracted by Oasis HLB cartridges and eluted with DCM, methanol and hexane. The mean background level of ethanol control is indicated by the thick horizontal lines on the profile while the upper and lower background noise levels are indicated by the broken horizontal lines defined by mean ethanol control [±2SD].

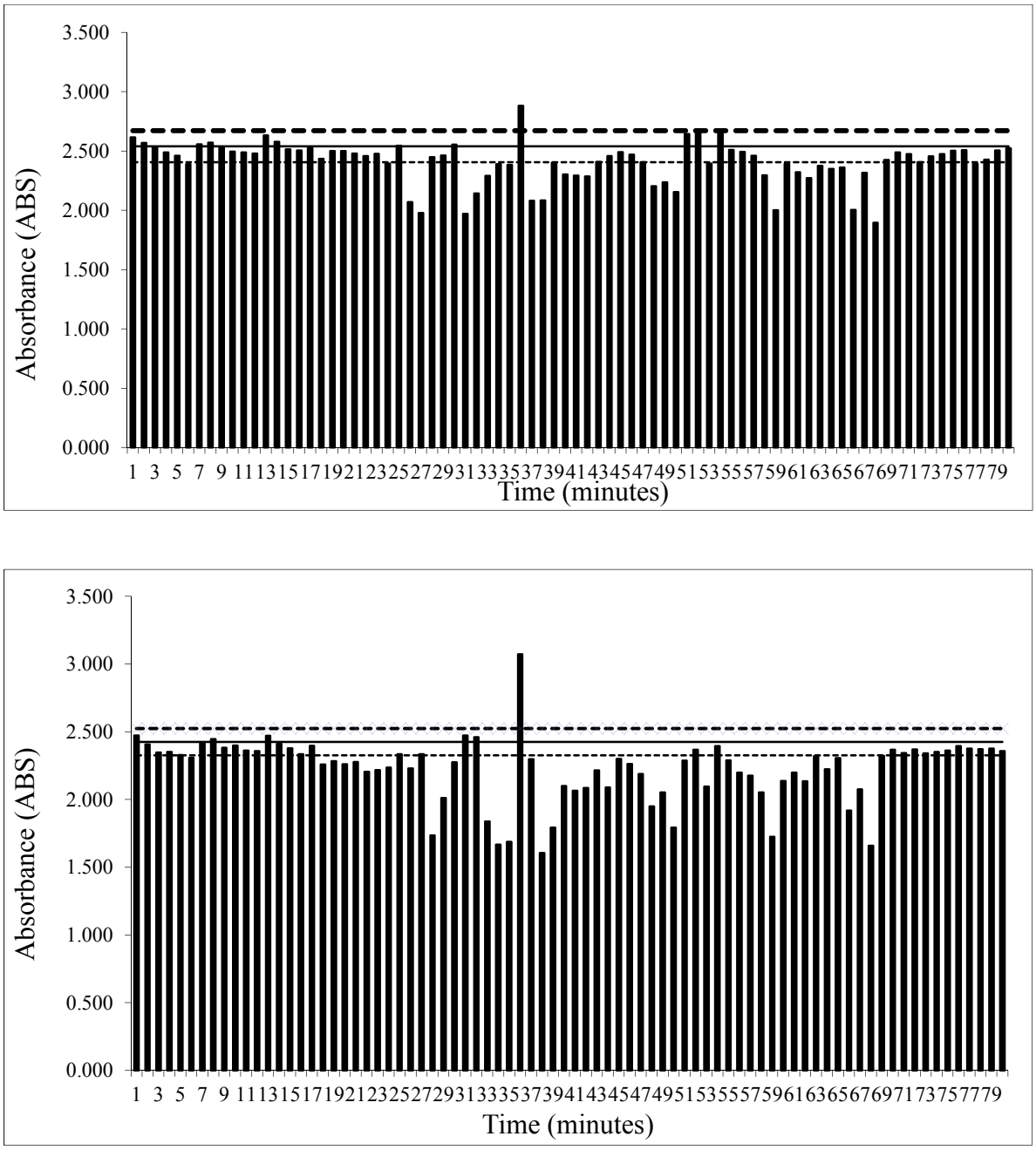


Figure 3.6. Profiles of the total anti-androgenic activity of influent X and influent Y samples (marked C and D respectively) extracted by Oasis HLB cartridges and eluted with DCM, methanol and hexane. The mean background level of ethanol control is indicated by the thick horizontal lines on the profile while the upper and lower background noise levels are indicated by the broken horizontal lines defined by mean ethanol control [±2SD].

3.6.7: Recovery of Anti-Androgenic Activity of Extracts after Fractionation

The recovery studies of the anti-androgenic activity of the extracts were undertaken after fractionation and the results of the studies are reported in Table 3.7. The recovery recorded by the two influent replicate is in the range of 78.63-97.32%. Recovery evaluation of effluent activity, however, cannot be accurately estimated due to availability of a single sample result. There is generally an improved recovery of TAA from the extract results shown in Table 3.7 over that in Table 2.5 (Section 2.6.5 in Chapter Two).

Table 3.7: Recovery of anti-androgenic activity before and after extract fractionation.

Sample	Total anti-androgenic activity (mgFeq/L)		% Recovery
	Before fractionation	After fractionation	
Influent-Y	3.205	3.119	97.32
Influent-X	3.172	2.494	78.63
Effluent-W	0.178	0.147	82.58

3.6.8 Gas Chromatography-Mass Spectroscopy (GC-MS) Analysis

The fractions identified to be anti-androgenic on the TIE assay, together with their neighbouring fractions, were silylated and analysed on GCMS following which the potential compounds responsible for this anti-androgenic activity were identified and analysed respectively using the NIST library and external calibration method. The NIST library fits ≥85% was recorded for all the compounds identified.

3.6.8.1 Internal Standard Fragments Monitored on GC-MS

The internal standards produced fixed fragment(s) whose concentration was used to estimate the quantity of each compound detected in sample fractions. For p, p'-[^{13}C]-DDE with multiple fragment ions, fragment ion 258 was chosen to quantify the compounds. These monitored ions are tabulated in the Table 3.8 below.

Table 3.8: The summary of the fragment ions of the standards monitored on the GCMS

Standard	Retention Time (RT)	Molecular Mass	Monitored ion(s)
E1-d_4	22.12	346	346
p,p-[^{13}C]-DDE	22.38	330	258, 328, 330

It must be stated that during laboratory work-up, a negligible quantity of samples and standards were lost especially by moderately polar and non-polar fractions (Appendix B.5). In these fractions, E1-d_4 standard was thermally stable over the period the analytes were being derivatised and had produced a good recovery in both the influent and effluent fractions. However, the recovery studies of these standards in strongly polar fractions produced poor results (Appendix B). The observed reduction in the concentration of the E1-d_4 and the p, p'-[^{13}C]-DDE in the sample during derivatisation are suspected to be caused by prolonged process of evaporation-to-dryness (liquid removal process) that occurred in the speed vacuum evaporator. The strongly polar fractions contained water in the range of 75-100% which required a longer time to dispose from the fractions. As a consequence, a considerable proportion of the standards and the analytes were lost during evaporation-to-dryness and the relationships between the absorbance, as well as the concentration of the standards and the percentage water-acetonitrile mixture are shown in the linear graphs in Appendix B.

3.6.8.2 GC-MS Chromatogram of HPLC Fractions

A wide range of biologically active and non-active environmental xenobiotics has been identified in the fractions of both the influent and effluent samples analysed. The first compound identified from the huge number of fractions was dichlorophene. Dichlorophene was detected in fractions 36, 37 and 38 of both influent and effluent samples and its occurrence was further confirmed in fractions 34 and 35 of the effluent sample. For a derivatised sample, dichlorophene occurred in the GC-MS chromatogram with a molecular ion, $\mathbf{M}^+$ of 412 which corresponds to the substitution of the hydrogens (in the –OH groups of the compound) as di-trimethylsilated derivative of dichlorophene (Figure 3.7). The $[M-35]^+$ occurred as the most relatively abundant fragment ion (m/z=377) which corresponds to the loss of a chloride radical, $Cl^{\cdot}$. The spectra lines were a good match to the NIST library with the percentage correlation standing at 90%.

Dichlorophene is a chlorinated phenolic compound which has been widely used as bacterioside and in pesticides and fungicides (Anderson, 1982; Takeuchi et al., 1985).

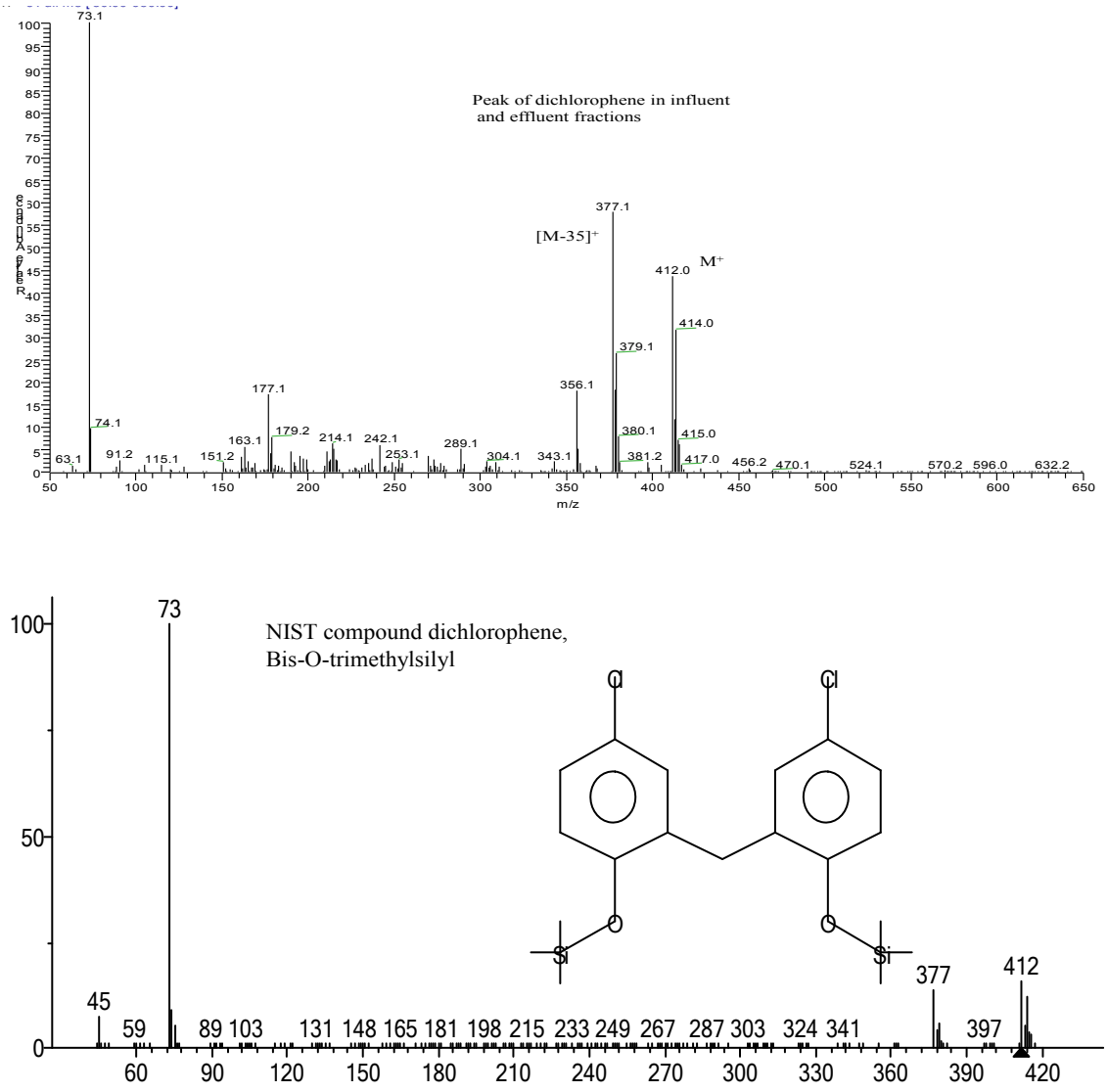


Figure 3.7: Schematic chromatograms comparing silylated dichlorophene compound between the NIST library where it occurred as dichlorophene, bis-O-trimethylsilyl and sample spectra lines.

Galaxolide, which has a molecular mass of 258, produced fragment ion peaks of $[M-15]^+$, $[M-30]^+$ and $[M-45]^+$ respectively. These correspond to fragment ion masses of 243, 228 and 213 generated after consistent loss of a methyl group by the molecular ion. It was identified on the chromatogram at retention time 18.46 min for influent and 18.48 min and 19.23 min for effluent of fraction 58. Emergence of galaxolide at two different retention times in the effluent suggests the possibility of its occurrence in

isomeric form. The mass spectrum of the compound matched well with the NIST library (Figure 3.8).

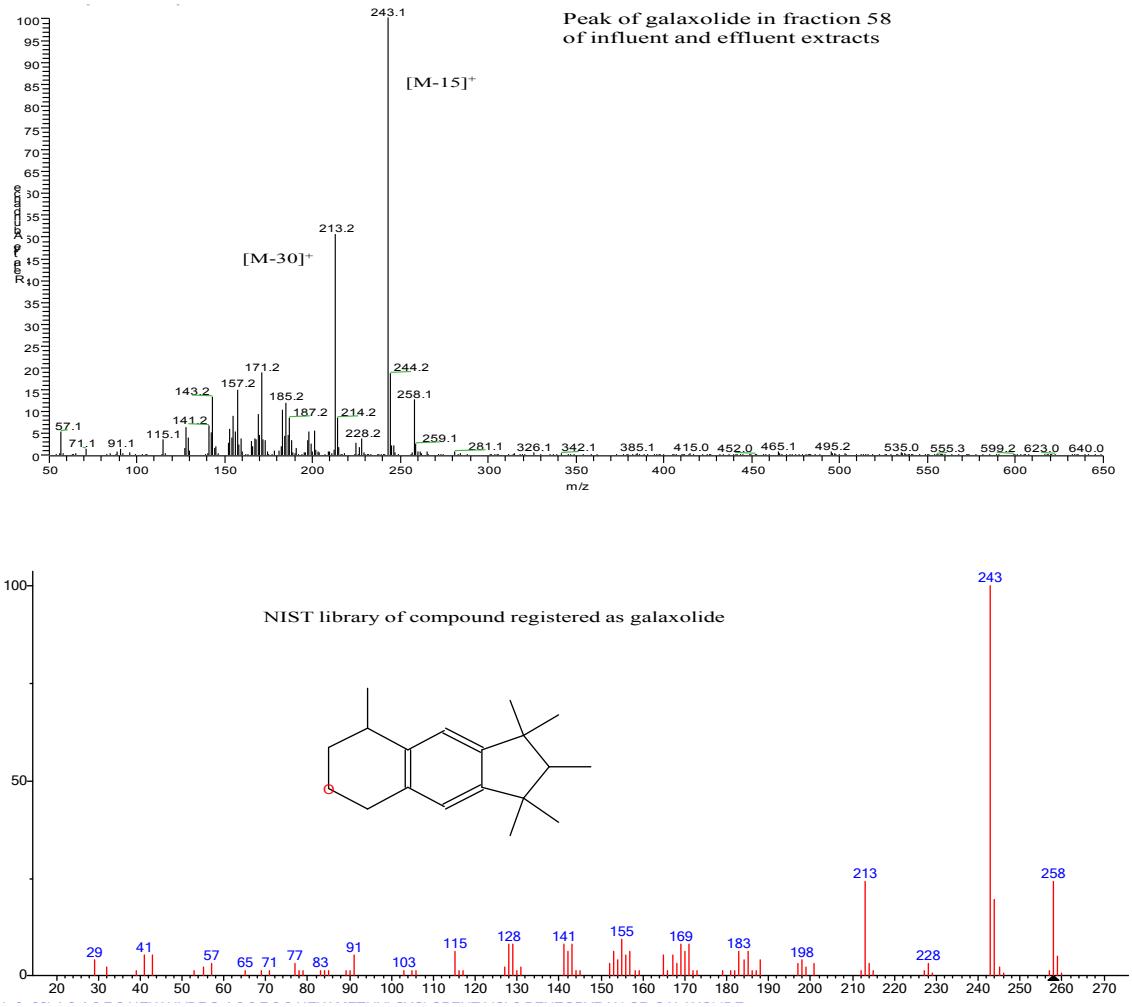


Figure 3.8: GC-MS chromatograms comparing galaxolide detected in sample extract and that in the NIST library.

Galaxolide belongs to the family of polycyclic musks. It is an important components of several personal care products especially cosmetics, detergents, perfumes, cleaning materials, body and hair creams (Muller et al., 1996; Frater et al., 1999).

Triclosan is another environmental compound detected in fractions 45 and 46 of both the influent and effluent samples (Figure 3.9). Triclosan, otherwise known as 5-chloro-2-(2, 4-dichlorophenoxy)phenol, is a broad-based anti-bacterial agent widely used as components of consumer and personal care products (Sabaliunas et al., 2003). Some of these include cosmetic and skin care products (shampoos, toilet soaps,

toothpastes, deoderants, skin creams and lotions). Products such as footwear, plastic wear and carpets are also composed of triclosan. Its regular application domestically could trigger bioaccumulation in the body and compromise human health (Sabaliunas et al., 2003; Lindstorm et al., 2002). Further studies are necessary to clarify its implications with regards to suppression of immune function, carcinogenicity and damage to the liver, kidney and lung.

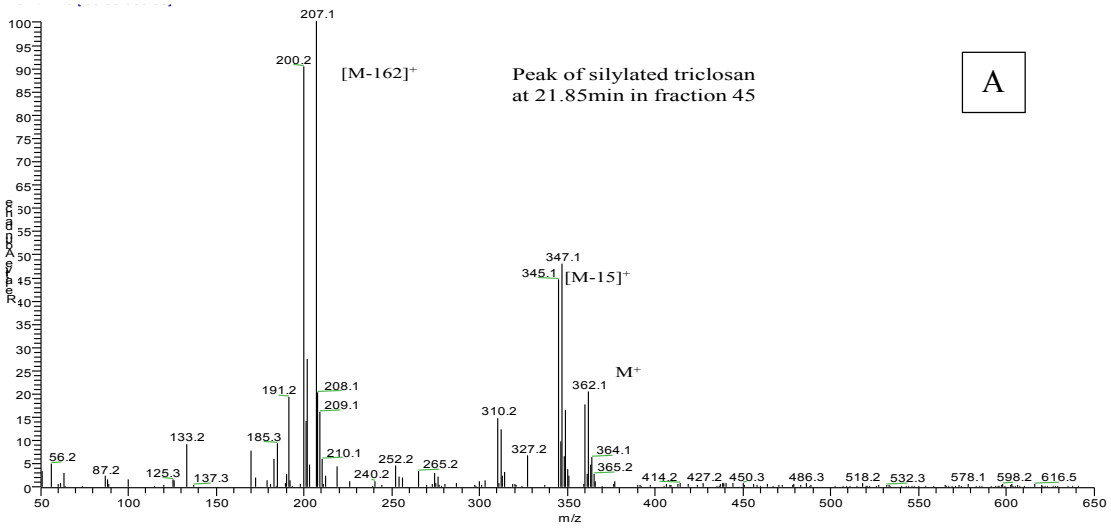


Figure 3.9.A: GC-MS Chromatograms of isotopic triclosan expressing differences on fragment ions 347 and 345 signals shown on A and B respectively.

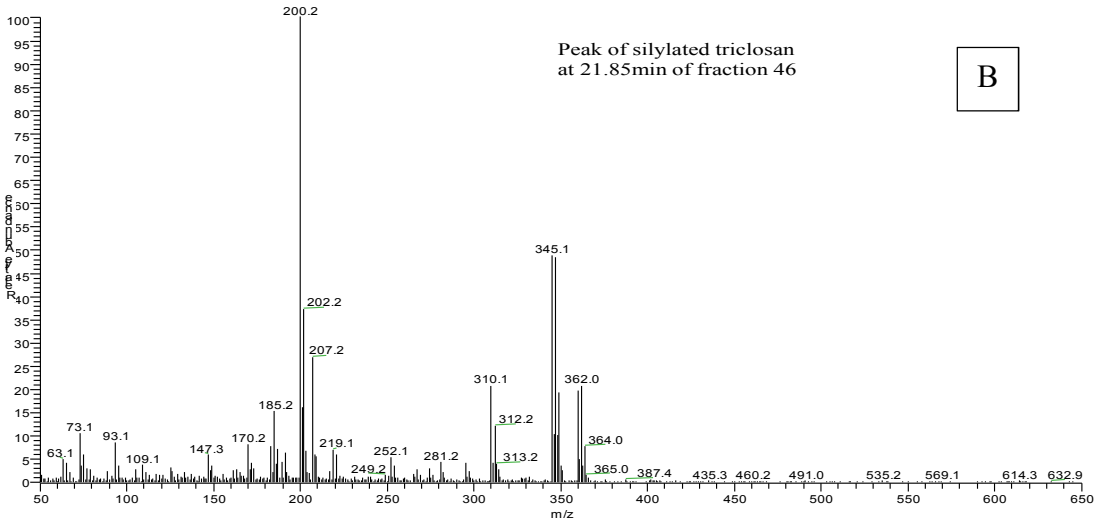


Figure 3.9.B: GC-MS Chromatograms of isotopic triclosan expressing differences on fragment ions 347 and 345 signals shown on A and B respectively.

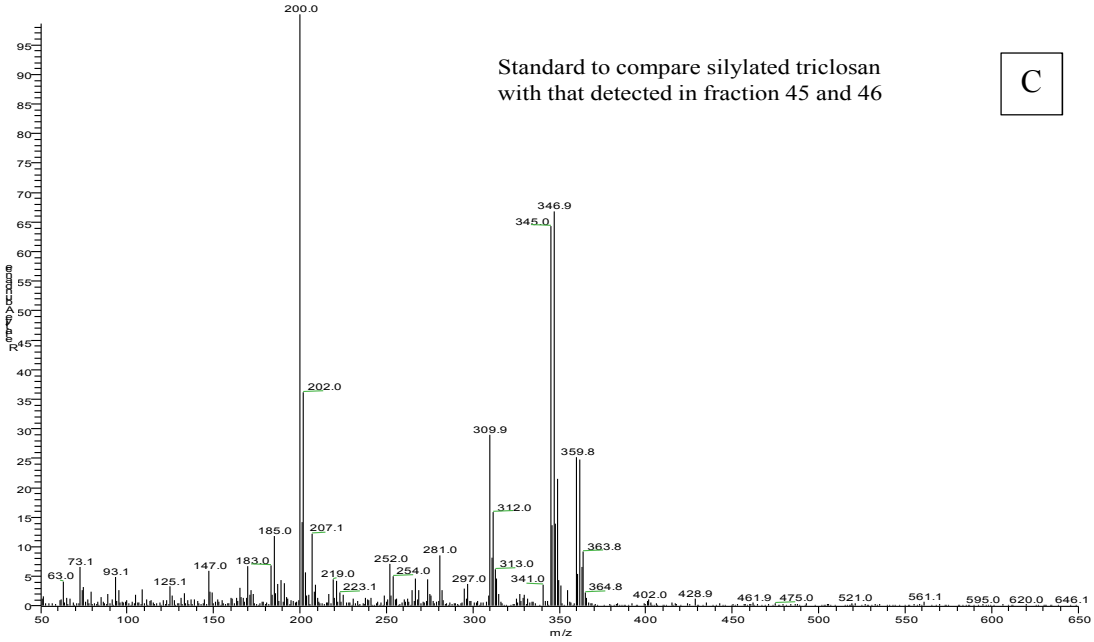


Figure 3.9.C: GC-MS chromatogram of laboratory standard of silylated triclosan used in lieu of triclosan, bis-O-trimethylsilyl (NIST library) to compare sample chromatograms. The silylated triclosan was not available on NIST library.

Triclosan is another chlorinated compound having three chlorine atoms present on the molecular structure. The HPLC confirmatory test on triclosan proved significantly consistent and correlated with its emergence in fraction 45. Triclosan has a molecular ion M+ of 360/362. The fragmentation of m/z = 360/362 resulted in relatively abundant fragment ions with masses 345/347 $[M\text{-}CH_3]^+$, 325/327 $[M\text{-}Cl]^+$ and 200/202 $[M\text{-}C_6H_3Cl_2O]^+$. It is possible for triclosan to exist as isotope in these fractions. On fractions 45 and 46 (Figures 3.9.A and 3.9.B), spectra lines are correspondingly similar except at 345 and 200. Fragments 345 are common to both fractions in approximately equivalent magnitude of intensity or relative abundance. Similarly, fragment 200 and 207 occur in both fractions. While the signal of the former occurs at about the same level of relative abundance in both fractions, that of the latter is more than triple in relative abundance in fraction 45 compare to that in 46. Before a meaningful conclusion could be drawn on its isotopic forms, it is necessary that further investigation be conducted to establish the possibility. Although, NIST library for the silylated compound was not available, the GC-MS chromatogram of the laboratory standard (Figure 3.9.C) was used as a comparative reference.

Another chemical compound found in the samples, which has flame retarding properties, is the tris(1-chloro-2-propyl)phosphate (TCPP) (Figure 3.10). It is an organophosphate ester used as flame retardant and plasticiser (Meyer and Bester, 2004). When TCPP's molecular ion (m/z = 326) fragments, four notable fragment ions are generated in abundant proportion with spectra lines reflecting masses of 99, 125, 157, 175, 201, 215 and 276. The m/z values correspond to the following fragment ions in sequential order of $[H_4PO_4]^+$, $[M\text{-}CH(CH_2)_5O_3Cl_2]^+$, $[Cl_2O(CH_2)_5]^+$, $[CH_3(CH_2)_4O_2Cl_2]^+$, $[CH(CH_2)_5O_3Cl_2]^+$, $[CH(CH_2)_6O_3Cl_2]^+$ and $[M\text{-}CH_2Cl]^+$.

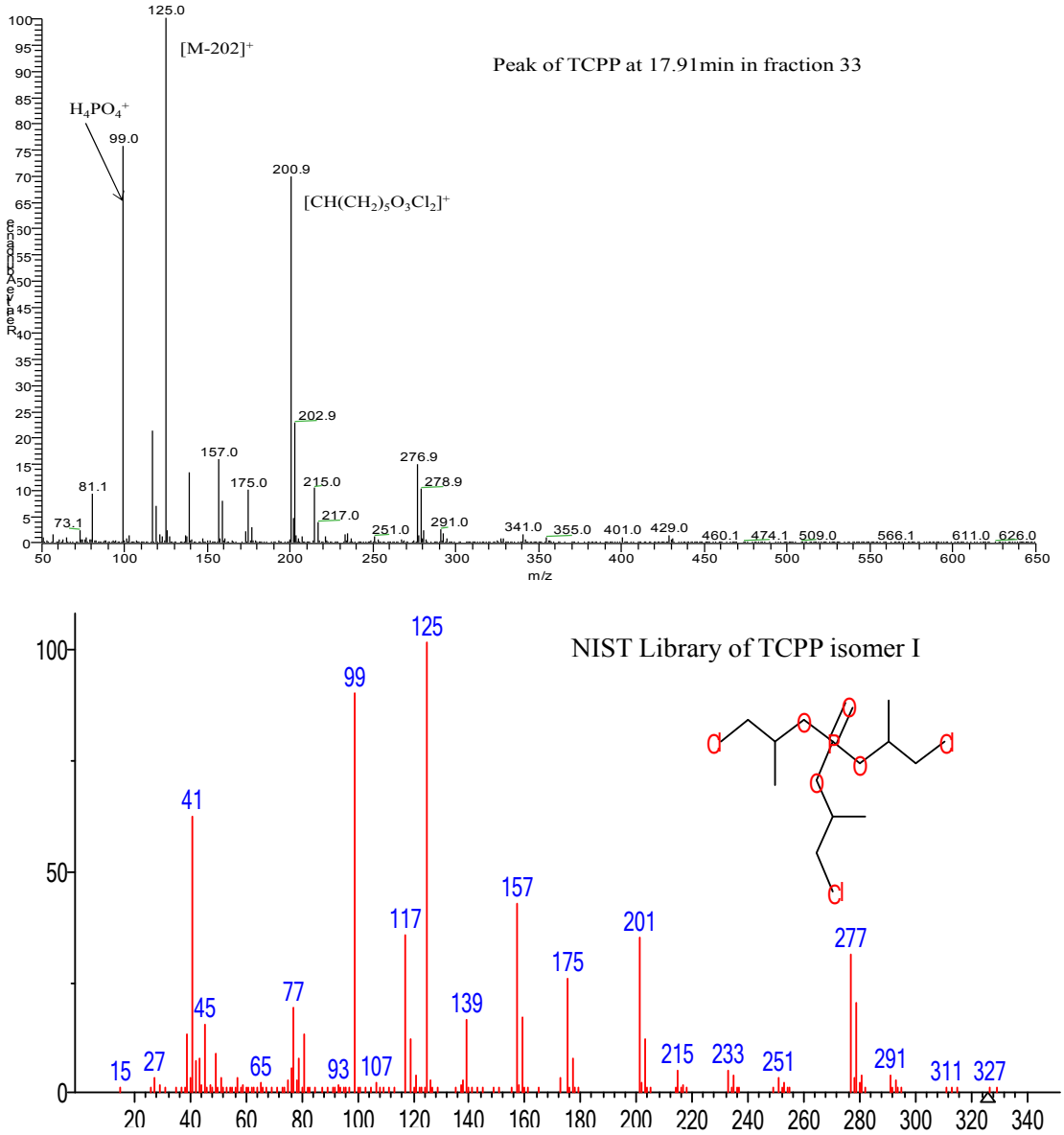


Figure 3.10: GC-MS and sample chromatograms of TCPP isomer I

Two other isomers of TCPP were also detected in wastewater samples. These include bis(1-Chloro-2-propyl)(3-chloro-1-propyl)phosphate (Laniewski et al., 1998) and bis(3-Chloro-1-propyl)(1-chloro-2-propyl)phosphate (Figures 3.11 and 3.12).

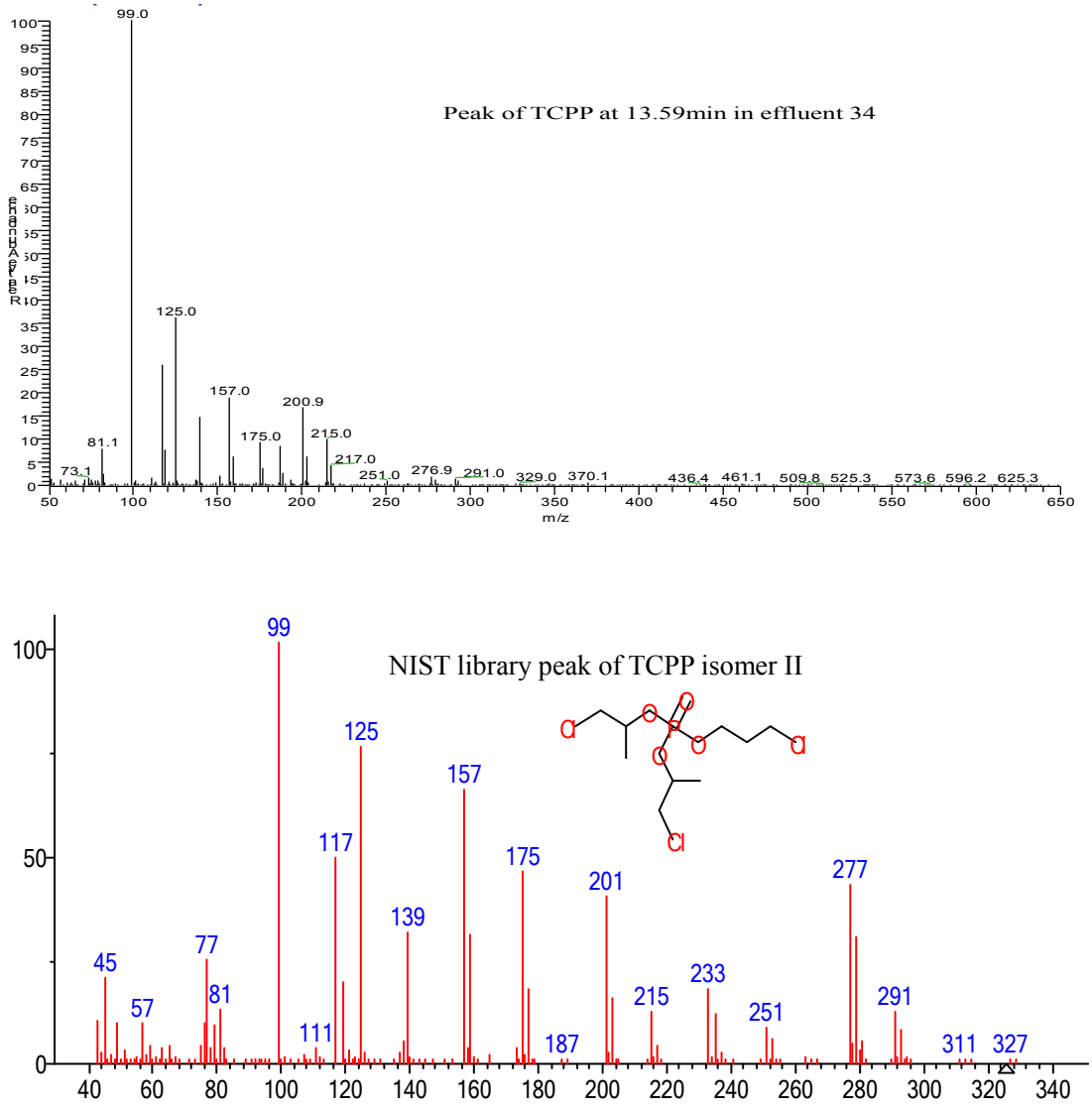


Figure 3.11: Chromatograms of isomer II {bis(1-Chloro-2-propyl)(3-chloro-1-propyl)phosphate}of TCPP detected in wastewater fractions.

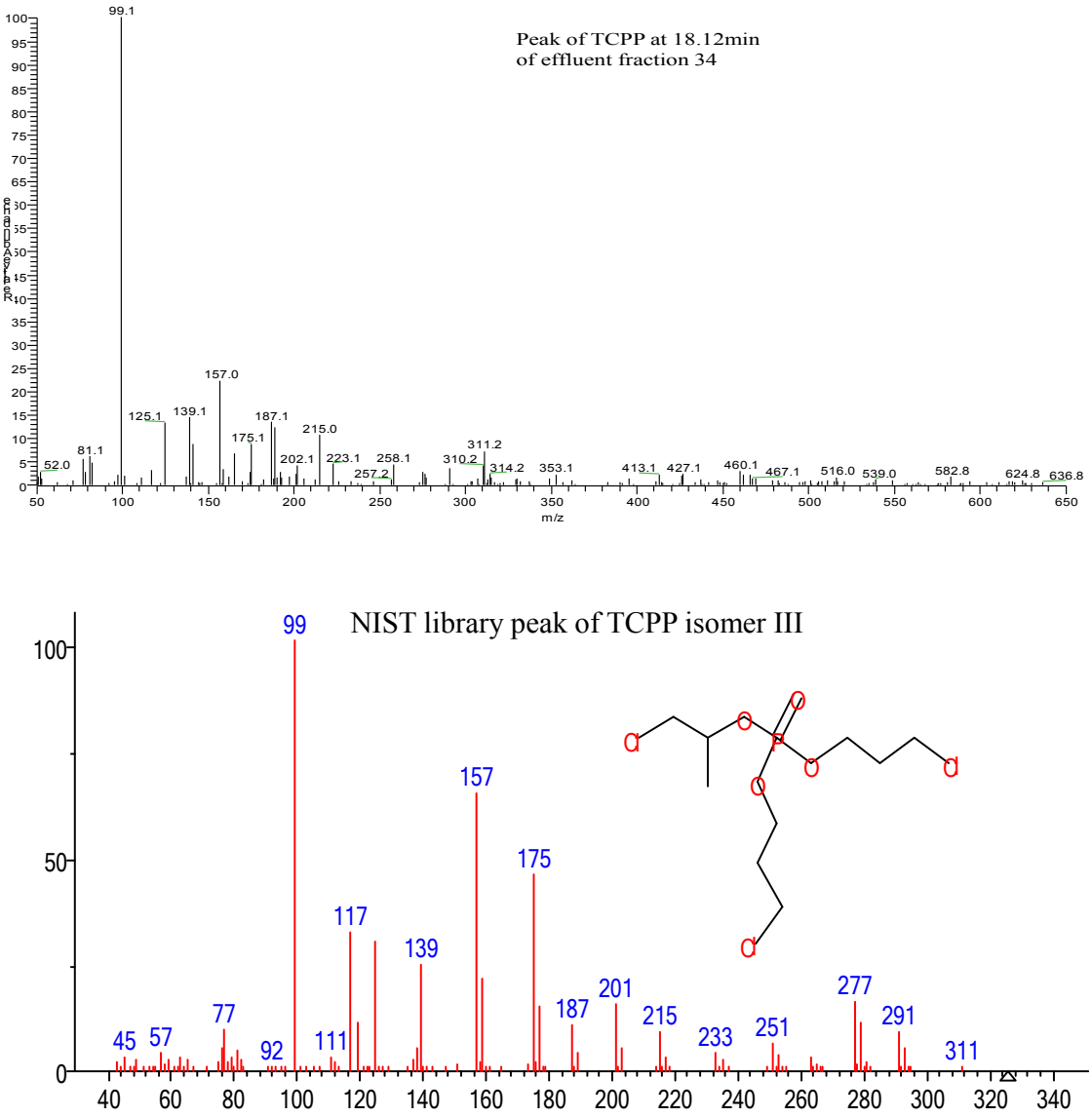


Figure 3.12: NIST library and sample chromatograms of isomer III {bis(3-Chloro-1-propyl)(1-chloro-2-propyl)phosphate}of TCPP found in wastewater fractions analysed.

The three congeneric compounds have more spectra lines that are common to them than those in contrast. The few identified compounds explained are common chemicals that constitute the domestic-based products, which are regularly released into the environment.

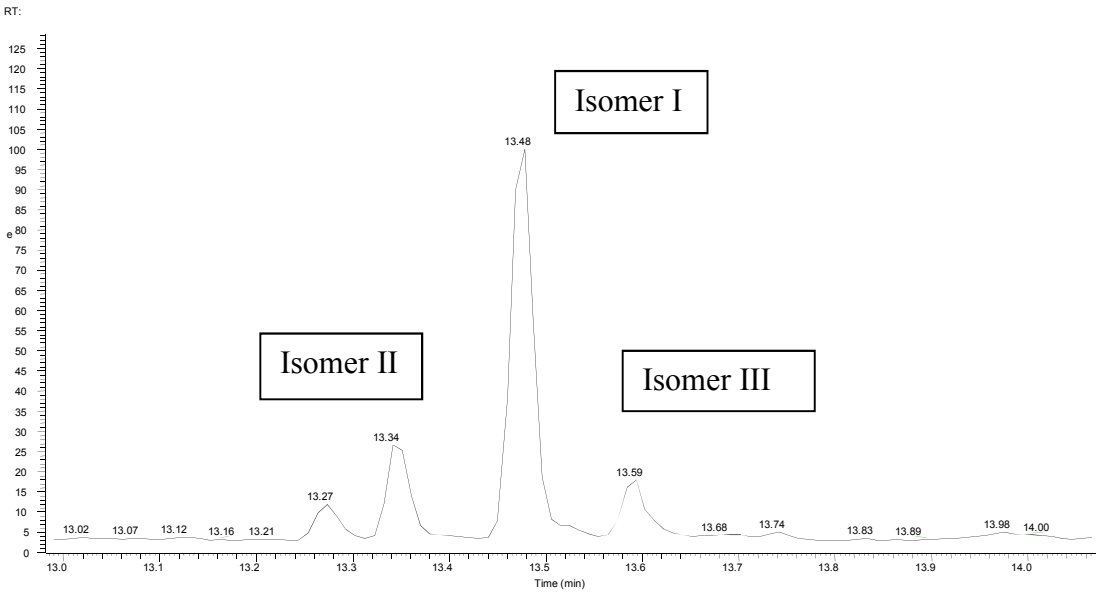


Figure 3.13: The GC-MS Chromatograph showing the three different isomers of TCPP (I, II and III) in an effluent W fraction 34 in retention time 13.48min, 13.34min and 13.59 min respectively.

A comprehensive list of all environmental chemicals detected in the fractions analysed are summarised in Table 3.9. Their corresponding chromatograms are compiled in Appendix C.

Table 3.9: Summary of environmental compounds detected in anti-androgenic fractions of both the effluent and influent samples analysed on GC-MS.

Compound	CAS Number	Fraction	Sample	Retention Time (RT) (min)	Uses	Reference
3-chlorobenzoic acid	114521-53-8	23	Effluent	12.11	Unknown	-
3,4-dihydrobenzoic acid	99-50-3	24	Effluent	18.09	Unknown	-
Tris(2-chloroethyl)phosphate (TCEP)	115-96-8	24	Effluent	17.27	Flame retardant	Regnery et al., 2010
2,4,6-Trimethylbenzoic acid	480-63-7	24	Effluent	13.58	Unknown	-
1-Naphthalenecarboxylic acid	86-55-5	25	Effluent	17.58	Unknown	-
Diethyltoluamide (DEET)	134-62-3	25	Effluent	14.72	Insect repellent	Trenholm et al., 2008
Bisphenol A	80-05-7	27	Influent	22.73	Plastic anti-oxidant	Fromme et al., 2002
Naproxen	22204-53-1	29	Influent	21.25	Anti-inflammatory	Carballa et al., 2004
Chloroxylenol	88-04-0	31	Effluent/Influent	12.32	Anti-bacterial	Kaspryzyk-Hordern et al., 2008

Table 3.9 continued: Summary of environmental compounds detected in anti-androgenic fractions of both the effluent and influent samples analysed on GC-MS.

Compound	CAS Number	Fraction	Sample	Retention Time (RT) (min)	Uses	Reference
Tris(1-chloro-2-propyl)phosphate	13674-84-5	33,34	Effluent/Influent	17.91	Flame retardant	Meyer and Bester,2004
Dichlorophene	97-23-4	34,35,36,37,38	Effluent/Influent	23.83	Pesticide	Hill et al.,2010
Diclofenac	15307-86-5	37	Effluent/Influent	23.67	Anti-inflammatory	Carballa et al., 2004
Ibuprofen	15687-27-1	37,38	Influent	15.35,15.38	Anti-inflammatory	Carballa et al., 2004
Chlorophene	120-32-1	40	Effluent/Influent	19.24	Biocide(germicide)	Boyd et al., 2003
Triclosan	3380-34-5	45,46	Effluent/influent	21.85	Anti-bacterial agent	Sabaliunas et al.,2003
Tris(2-butoxyethyl)phosphate	78-51-3	46	Effluent	24.74	Plasticiser, flame retardant	Bester and Schafer., 2009
Galaxolide	1222-05-5	58	Effluent/Influent	18.48,18.46	Fragrance	Carballa et al., 2004

Table 3.9 continued: Summary of environmental compounds detected in anti-androgenic fractions of both the effluent and influent samples analysed on GC-MS.

Compound	CAS Number	Fraction	Sample	Retention Time (RT) (min)	Uses	Reference
Abietic acid	514-10-3	58,59,60	Influent	24.83	Resin acid	Wang et al., 1995
Isopimaric acid	471-74-9	58.59	Influent	24.04	Resin acid	Lacorte et al.,2003
N-Butylbenzensulfonamide (NBBSA)	3622-84-2	32,33	Effluent/Influent	17.60	Plasticiser	Pederson et al., 2005
Pimaric acid	127-27-5	58,59	Influent	23.91	Resin acid	Lacorte et al., 2003

In addition to the list of compounds tabulated, there are other two categories that would be interesting to report. The first group consists of compounds that have been identified but were not commercially available and, as a result, their mass spectra are not available to compare analytically with that of the corresponding samples (Table 3.10).

Table 3.10: Compounds detected in wastewater samples and could not be tested due to either non-availability commercially or inconclusive on-going analysis.

Compound	Sample	Fraction	Retention Time (min)	Status
1,1-biphenyl-4,4-dicarboxylate	Effluent	19,20	26.52, 26.50	NA
6-hydroxy-α-methylnaphthalene Acetate	Effluent	20	22.13	NA
3,4-dihydrophenylethane	Influent	28	17.88	NA
Carbamazepine ether	Effluent	22	23.39	NT
Carbamazepine	Effluent	22	24.30	NT
1,4-benzenecarboxylic acid	Effluent	24	17.66	NT

NA: Not available commercially for purchase; NT: Identified but not confirmed.

The second group concerns prominent chromatograms of unknown compounds that have been discovered in active fractions of either the effluent or influent but could not be identified. Some chromatograms of these unknown compounds can be found in Appendix D.

3.7 Discussion

3.7.1 Method Development.

The recovery studies undertaken (Table 3.7) have indicated that there is a significant improvement to the overall recovery process. It is difficult to pinpoint whether the improvement to the recovery was brought about by substituting the HPLC injection solvent or by any revised area of the methodology as the data to identify the cause are not available. Also, it is not known if the scheme for this analysis (Section 3.2.2) had significantly helped in reducing the possible channels for losing anti-androgenic activity since stepwise data to justify it are not available. What is known was that the process of sample resuspension occurred about five times throughout the laboratory activities. In Chapter Two, however, sample resuspension exceeded six times during the determination of total anti-androgenic activity alone due to sample toxicity. It is possible for the losses to have occurred during this exercise but there is no statistical data to justify this observation. In the new methodology, ethyl acetate (EtAc) was replaced with dichloromethane (DCM) because it possesses higher dielectric constant (ε_r=9.1) which makes it a better polar aprotic solvent over EtAc. It is not known if the substitution has significantly impacted the process of recovery as their contributions were not measured. For improved recovery of the target analytes, sample recovery test was conducted on seven cartridges using test standards with a range of polarities (LogKow: 3.10-7.44) and produced some remarkable results. Oasis® HLB demonstrated the potential to produce optimal recovery among the cartridges tested as the percentage recovery of test standards was between 94 and 100% (Table 3.2; Appendix A). The results of the test standards justified the choice of Oasis® HLB for this wastewater clean-up and analyte pre-concentration. Evans (2008) suggested the use of Oasis® HLB in her previous work for wastewater clean-up process. Generally, polymeric sorbents (as exemplified by Oasis® HLB) have multiple analyte retention potentials when compared to silica-based extraction sorbents (Benijts et al., 2004). In addition, the microtitre polystyrene plates used for the AYAS assay were substituted with deep well microtitre polypropylene plates which allowed the use of the non-polar hexane solvent as well as DCM for application of compounds which were not soluble in ethanol.

3.7.2 Total Anti-androgenic Activity of Effluent Samples

The effluent extracts recorded total anti-androgenic activities with a mean of 0.219 mgFeq/L and a standard deviation of 0.008 as well as a standard error of mean of 0.005. The results indicated good repeatability of the replicate grab samples. The TAA of the effluent samples occurs within the range reported in other published works. Johnson et al. (2007b) reported anti-androgenic activity in the range of concentration when effluent samples collected and analysed in August 2003 were analysed. Evans (2008) recorded approximately twice the concentration of the Horsham effluent reported in this study for samples extracted in November 2005 (*ibid*.). Five UK WwTPs which were sampled and investigated for anti-androgenic activity in 1999 produced a range of concentrations between 0.055 and 3.770 mgFeq/L (Kirk, 2002). A similar study carried out on forty-three UK WwTP effluent samples reported TAA between zero and 1.231mgFeq/L (Johnson et al., 2007b). The results of the influent samples collected in Chapter Two are comparatively studied with that analysed in this section. While the samples analysed in Chapter Two were collected in February, those analysed in this Chapter were sampled in September. By implication, the equivalent quantity of samples analysed in this section are expected to produce anti-androgenic activity higher in magnitude than those analysed in Chapter Two. This is because samples collected and analysed in summer are expected to contain total anti-androgenic activity more than their equivalent analysed in rainy season. However, the fact that the samples in this Chapter were analysed 72 hours after collection has affected that line of thinking. Given the level of bacterial degradation expected to occur in 72 hours of storage, the amount of anti-androgenic activity recorded in this section is not unexpected as the magnitude would reduce comparatively to that reported in Chapter Two. In addition, the repeatability and precision shown by the results in this section are statistically better than that expressed in Chapter Two.

3.7.3 Total Anti-androgenic Activity of Influent Samples

The influent samples collected from Horsham WwTP were also analysed using AYAS. The average total anti-androgenic activity of influent samples of 3.198mgFeq/L was measured in standard deviation of 0.025 and standard error of mean of 0.014. There are few studies available about anti-androgenic activity of wastewater influent. Moreover,

effluents are mainly the class of wastewaters that are usually released into the receiving rivers after treatment. This may possibly explain why influent samples are less studied compared to effluent samples. Nevertheless, studies conducted on influent samples are used to predict and estimate the level of AA contained in the sewage in-flow and determine to what extent the removal process has been successful. More importantly, the study will be needed to predict the long-term account of bioaccumulation in aquatic environments. As emphasised in Section 3.7.2, the level of anti-androgenic activity of influent samples in this section is higher in magnitude compared to the equivalent analysed in Chapter Two as they were collected in summer. The reason can be attributed to the fact that both samples were collected and analysed at different seasons of the year (indicated in Section 3.7.3). The results of influent samples in this section clearly show a degree of repeatability and precision that are statistically better than those analysed in Chapter Two.

3.7.4 Toxicity Identification and Evaluation (TIE) of fractions

HPLC fractionation and profiling for anti-androgenic activity using the yeast screen is a significant way of determining and characterising the nature of biological activity exhibited by chemical compounds in environmental samples. It can also help in controlling the potential errors which may be associated with the identification of chemical causative agents of some biological effects. The profiles of the control, influent and effluent have offered useful information especially those related to the identification of active compounds present in the analysed wastewater samples. First, the shift in density of anti-androgenic activity from mildly polar and non-polar in influent to polar fractions in effluent samples is remarkable and may be explained in two possible ways: biodegradation and adsorption. Most mildly polar and non-polar compounds are eliminated at the biological stage of wastewater treatment process via biodegradation and chemical adsorption. During the treatment process, less degradable polar compounds are rarely removed and were neither adsorbed into the sediment bed (Li et al., 2000). Most of these compounds are passed into the receiving rivers. In addition, the polar section of the influent may have undergone biodegradation including deconjugation of any glucuronide or sulphate conjugates. These processes, in addition to previously described elimination means, may account for the emergence of anti-androgenic activity in the polar region of the effluent samples.

3.7.5 GC-MS Analysis of Compounds Detected in Wastewater Fractions.

The GC-MS chromatograms of the active influent and effluent fractions analysed generated some interesting results. A variety of chemical compounds which were discovered are largely components of home-use consumer products. These ranged from pharmaceuticals, flame retardants, insect repellent, plastic anti-oxidants, plasticisers, pesticides to wood resin acids. Several studies on wastewater effluents and influents have reported the occurrence of this range of compounds. Pharmaceuticals such as ibuprofen, naproxen and diclofenac have been detected in wastewaters in USA and Europe. These three pharmaceutical compounds are widely used as analgesic and antiphlogistic (anti-inflammatory) drugs in the management of inflammatory and arthritic conditions (Blagbrough et al., 1992). Miao and co-workers (2002) reported the discovery of ibuprofen, naproxen and diclofenac in Canadian wastewater samples taken from two treatment works. In Germany, Ternes (1998) reported the recovery of some other pharmaceuticals in addition to the three (ibuprofen, naproxen and diclofenac) in effluent discharges of Sewage Treatment Plants. Koutsouba and colleagues (2003) discovered diclofenac and ibuprofen in Greece influent and effluent wastewater samples. Similar studies conducted in Spain (Carballa et al., 2004), Austria (Clara et al., 2005), Finland (Vieno et al., 2005), Brazil (Stumpf et al., 1999) and United States of America (Kolpin et al., 2004; Barnes et al., 2008; Stackelberg et al., 2004) have also confirmed the recovery of these three compounds and many other pharmaceutical products in their influent and effluent samples.

In addition to the pharmaceuticals, some pulp and paper mill resin acids comprising of abietic acid, isopimaric acid and pimaric acid were identified in this study. Larcote and co-workers (2003) reported the identification of this class of acids in pulp and paper mill effluents as well as wastewater receiving rivers (see also Kalpin et al., 1997; Leppanen et al., 1998). Significant amount of TCPP, Tris(2-butoxyethyl)phosphate (KP-140) and TCEP, known as flame retardants and plasticisers, have also been detected in wastewater discharges (Fries and Püttmann, 2003; Meyer and Bester, 2004; Martinez-Carballo et al., 2007; Bester and Schafer, 2009). Like other flame retardants, they are composition of foams used for furniture and bedding purposes. Likewise, chemical compositions of cleaning facilities were discovered in wastewater samples and they include chloroxylenol,

triclosan and chlorophene (Sabaliunas et al., 2003; Boyd et al., 2003; Kaspryzyk-Hordern et al., 2008). Other compounds reported in previous literature include dichlorophene, bisphenol A and diethyltoluamide (DEET). The dichlorophene discovered is suspected to emerge from agricultural input given that it is used as pesticide. Evans (2008) likewise reported the occurrence of dichlorophene in the effluent fractions analysed. Bisphenol A is an active component of plastics and plastic materials. Given that it is one of such high production volume compounds, it is expected that the concentration in the environment will be relatively substantial. DEET is a regularly used domestic chemical to reduce the menace of insects. Their occurrence in the environment is highly expected.

The recovery studies also justified the development of the methodology as the results indicated good recovery for both influent and effluent samples.

3.8 Conclusion

Horsham wastewater samples are complex heterogeneous mixture of a wide range of discharges from household, agricultural settlements, hospitals and commercial establishments. The results of this study revealed that Horsham Wastewater Treatment Works are receiving largely from domestic sources. Although the broad spectrum of compounds detected in these samples is a representative of several sources such as household, industrial, agricultural and medicinal consumables, all the compounds identified (except resin acids which originate form paper mill industry) are used in the domestic environment. The results have also provided an evaluation of the WwTP's efficiency at removing these compounds. Contrasting the total anti-androgenic activity of both the influent and effluent in this study, it could be reported that about 90% of the anti-androgenic activity was eliminated after treatment. The TIE studies demonstrate the presence of steroid receptor active chemicals as clearly expressed in the profiles of the influent and effluent fractions. It is impossible for an *in vitro* bioassay to solely account for all internal responses of any intact animal with respect to EDCs. For this reason, it will be necessary for the biological activity of all the compounds detected to be characterised. In addition, the quantity of the active compounds in wastewaters must be evaluated. Above all, their contributions to the total anti-androgenic activity must also be determined in both the fractions and generally in the whole samples.

CHAPTER FOUR

Confirmatory Tests for Receptor-active Xenobiotic Anti-androgens in Wastewater Influent and Effluent Samples

4.0 Introduction

Wastewaters contain numerous xenobiotics that possess androgen receptor antagonist activity. Profile studies of wastewater fractions in Chapter Three showed the occurrence of fractions with anti-androgenic activity from which over thirty xenobiotic compounds have been identified. Wastewaters are known to originate from several sources including hospital, domestic, industrial and agricultural sources. Many compounds identified in Chapter Three have been traced to regular household products such as pharmaceuticals, fragrances, flame retardants, plasticizers, insect repellent, cosmetics and skin care, and other personal care products (Carballa et al., 2004; Eriksson et al., 2002; Pederson et al., 2005) while few others can be linked to industrial sources. The androgen receptor antagonist activity of most compounds identified in the previous chapter is currently unknown; therefore, this study will focus on the determination of their AA.

It is possible for some environmental compounds known to undergo receptor-specific binding to interact with a wide range of other hormone receptors *in vivo* or *in vitro*. Nonylphenol and bisphenol-A are estrogen agonists that could interact with androgen and thyroid receptors to inhibit their binding abilities *in vivo* and *in vitro*. Whereas nonylphenol antagonises the binding of triiodothyronine (T_3) to transthyretin in chicken and bullfrog, bisphenol-A successfully inhibits T_3 binding to thyroid receptor in mammal (Yamauchi et al., 2003; Moriyama et al., 2002). In addition, nonylphenol as well as bisphenol-A could prevent androgen receptor binding and interaction to coactivators (Lee et al., 2003). Gene expression could also be induced when bisphenol-A interacts with progesterone receptors in rat uterus (Krishnan et al., 1993). There is a possibility that exposure to mixtures of receptor-active contaminants could result in health diminishing effects in humans. For instance, the widespread increase of male reproductive disorders in recent decades has been linked to the interactions of environmental anti-androgens (and xenoestrogens) with wildlife and humans (Chen et al., 2007). Laboratory studies have confirmed that exposure to bioactive xenobiotics

such as 4-tert pentylphenol (TPP), dibutylphthalate (DBP) and diethylhexylphthalate (DEHP) can result in disruption of sexual differentiation in rodents (Gimeno et al., 1997; Mylchreest et al., 1998, 1999, 2000; Sharpe, 2006; Sultan et al., 2001a,b). In humans, reproductive health defects that have been linked to chemical contamination include reduced fertility and reduced sperm count, motility and quality (Carlsen et al., 1992; Swan et al., 1997; Andersen et al., 2000), increase in hypospadias, cryptorchidism or undescended testis (Chilvers et al., 1984; Matlai and Beral, 1985; Toppari et al., 1996 and Paulozzi et al., 1997), increases in abnormal genitalia, testicular cancer (Adami et al., 1994; Forman and Moller, 1994), intersex (Reeder et al., 1998) and impaired hormone production (Roy and Pereira, 2005).

A wide variety of bioactive chemicals identified as endocrine disruptors occur in the environment as mixtures. While it is possible for bioactive chemicals to act mutually exclusively of another, it is also possible for some to exhibit biological activity together that could be additive, inhibitive or synergistic. The collective activity of bioactive compounds in environmental samples is determined largely by the potency of the contributing compounds and their concentrations as well as their overall chemical interactions at the receptor level which will influence the final activity of the sample mixture. When a compound demonstrated high level of activity at minimum concentration in an isolated state or sample mixture it is considered more potent than that which produced the same result at high concentration. To estimate the contribution of each anti-androgenic compound in a complex environmental mixture, it is necessary that its potency relative to a standard must be known. For that reason, this study will not only identify potentially androgen receptor antagonists from the list of compounds identified in Chapter Three but it will also estimate their potency relative to flutamide. Screening such a wide array of compounds for anti-androgenic activity can effectively be achieved with the *in vitro* yeast androgen screen assay (AYAS) which has been proven successful and reliable in previous uses in this study. Much as AYAS has demonstrated to be suitable for this screening exercise, care needs to be taken in ensuring that a depression in the response of the assay is due to receptor antagonism and not due to toxicity to the yeast or the galactosidase reporter gene product.

The aims of this study are to:

1. Determine the receptor antagonist activity and potency of the xenobiotics identified in the wastewater extracts in Chapter Three.

2. Determine the degree of their anti-androgenic activity (potency) relative to the standard anti-androgen flutamide.

3. Quantify the concentration of anti-androgenic compounds both in the fractions and in the total wastewater samples.

4.1 Materials and Methods

4.1.1 Materials

Galaxolide was a generous gift from the president, American Research Institute of Fragrance Development (ARIFD). Naproxen, dichlorophene, chlorophene, bisphenol-A, diclofenac, ibuprofen, abietic acid, isopimaric acid, pimaric acid, triclosan, diethyltoluamide (DEET), 2,4,6-trimethylbenzoic acid, 1-naphthalene carboxylic acid, 3-chlorobenzoic acid, tris(2-butoxyethyl)phosphate (TBEP), tris(1-chloro-2-propyl)phosphate (TCPP), tris(2-ethylchloro)phosphate (TCEP), N-butylbenzenesulfonamide (NBBSA), chloroxylenol, deuterated estrone and deuterated p,p-DDE were purchased from Tokyo Chemical Industry (TCI), UK, Sigma-Aldrich, Fluka, Aldrich, Sigma and micronOrganics (see Section 3.2.1). All the materials used for the yeast assay are outlined in Chapter Two, section 2.1.1.

4.1.2 Methods

4.1.2.1 Confirmation of Anti-androgenic Activity of Compounds Using Yeast Screen Assay.

Yeast androgen screen (AYAS) assay for this experimental work (described in Section 2.2.3.4, Chapter Two) was prepared and set-up such that the first three rows of the plates were assayed with flutamide standard curve, the media blank and the ethanol blank respectively. The other five rows were used to assay test samples. This plate arrangement was replicated in two other separate plates in every test exercise undertaken. The test compounds were prepared in ethanol in a range of concentrations ($1x10^{-10}$M to $1x10^{-5}$M) and were assayed in replicate to establish the degree of repeatability of the screen. Each test concentration was replicated on different plates to prevent interference through intra-well creeping. The absorbance values of the test samples were taken with a spectrophotometer at 540nm to determine and measure the anti-androgenic activity, and at 620nm to determine the turbidity of the yeast cells. The results were analysed and are expressed as mean±SD and %RSD as described in Section 3.6.3.

4.1.2.2 Identification of Turbidity of Endocrine Disrupting Chemical Compounds on Yeast Androgen Receptor Transcription Screen

Xenobiotics demonstrate androgen receptor antagonism by inhibiting the true response on yeast androgen transcription screen (AYAS). The true response on AYAS corresponds to the linear expression of the androgen standard (DHT) on absorbance 2.45 (Figure 4.1). At the cellular level, interactions of the yeast cells in the AYAS with anti-androgenic compounds could either bind the androgen receptor to inhibit the expression of the androgen standard (DHT) or interfere with the usual cellular functions. At concentrations below the lethal dose (LD), anti-androgens bind with the androgen receptors which are incorporated on the yeast cytoplasm to produce a response that is directly proportional to the concentration of the compounds applied. The responses generated by the yeast cells over a wide range of concentrations on the plate are expressed as a reverse-sigmoid curve replica of the flutamide standard curve (Figure 4.1). The AA of these chemical compounds can be accurately estimated when the turbidity measurement at 620nm (which expresses variation in the growth of the yeast cells) is mathematically incorporated into the AA measurement undertaken at 540nm. The turbidity removes the overestimation that is likely to occur during 540nm measurement thus adjusting it to the appropriate value of AA (see Section 2.3.5). However, when the yeast environment becomes unfavourably toxic, the anti-androgenic activity cannot be accurately determined as the cellular configuration may be ruptured thereby impeding the growth of the yeast cells in addition to disrupting the regular biological activity carried out by the cells. Such unfavourable environment could be created when the concentrations of the anti-androgens reach or exceed the LD. Sample toxicity is estimated from the 620nm readings of the test compounds in relation to the ethanol controls. If the 620 values of the sample fall below the mean±2SD of the ethanol controls, then the sample is deemed to be toxic in the AYAS and an estimate of anti-androgenic activity cannot be accurately made at that particular sample concentration. An example of an absorbance-concentration curve of a sample showing no toxicity and one showing clear toxicity in the AYAS are given in Figures 4.2A and 4.2B respectively. Under toxic condition, the difference between the 620nm readings of both the test compound and the ethanol control can become relatively large (Figure 4.2B). In this study, it must be stated that any sample absorbance which falls within the mean-2SD of the ethanol reading at 620nm is considered non-toxic.

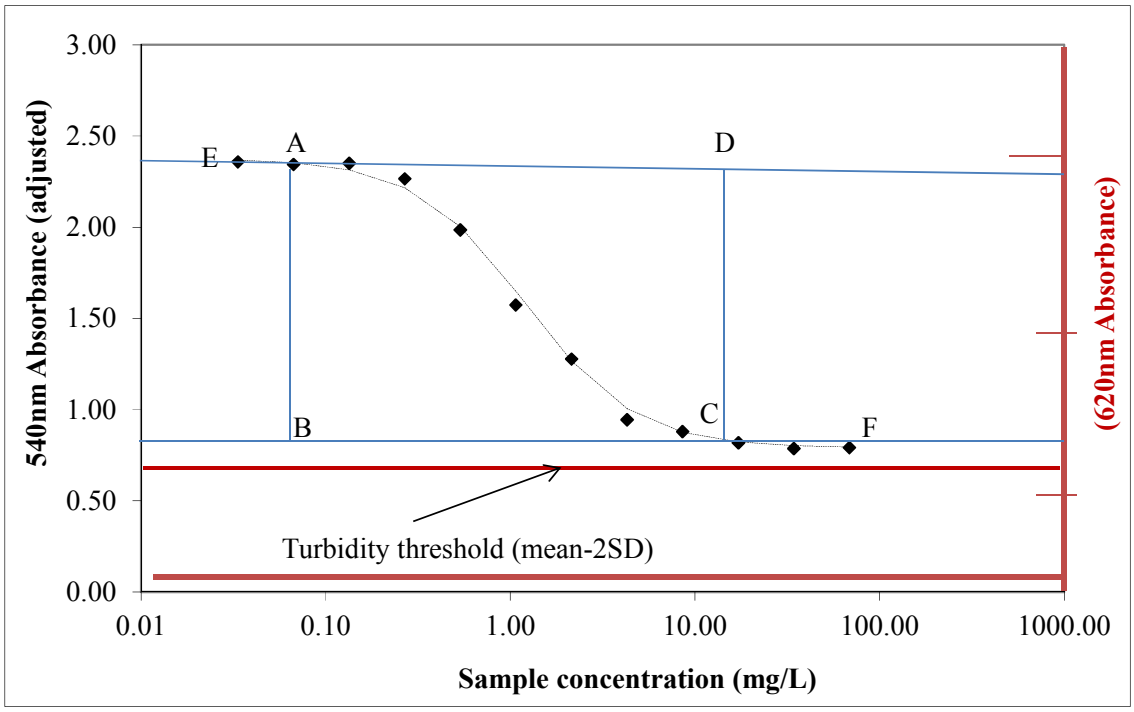
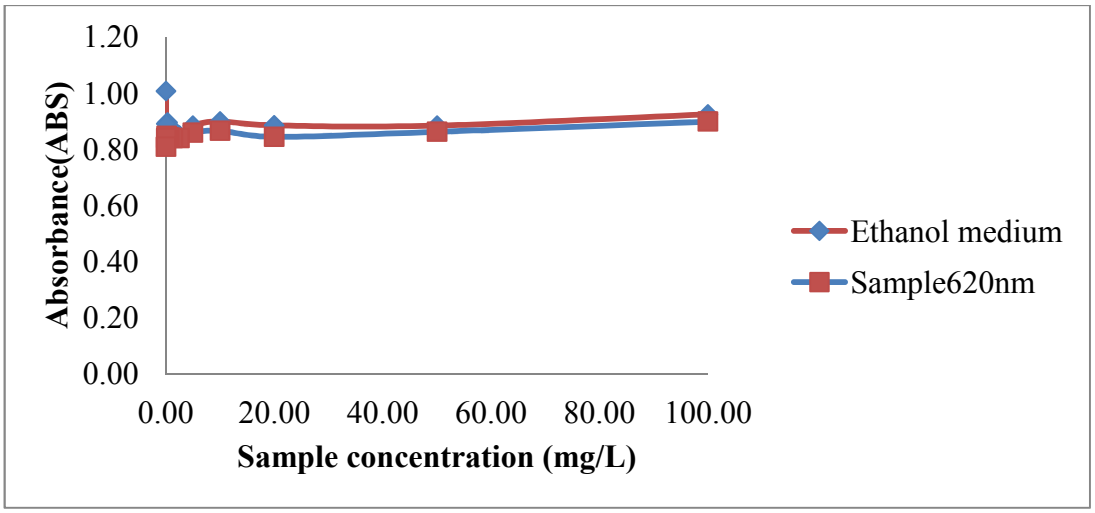


Figure 4.1: Schematic illustration of anti-androgenic activity-concentration relationship of endocrine disrupting chemicals expressed on the yeast assay. The plots are expressed in 540nm and 620nm absorbance. The curve section AC measures the effective response (anti-androgenic activity) of the compounds on yeast. It represents 10-90% of the dynamic signal range. Lines ED and BF correspond to the lowest and highest concentration of the test compounds on the assay plate. The turbidity threshold, measured in 620nm, refers to the background noise (mean-2SD) line and it demarcates the region of activity (above the turbidity threshold line) and toxicity (below the turbidity threshold line)

A



B

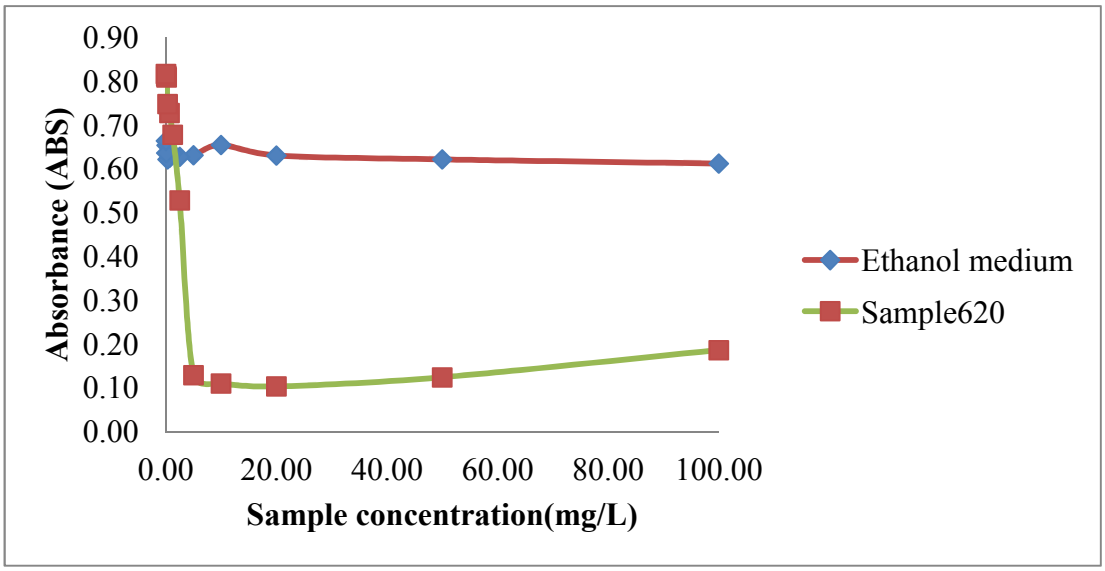


Figure 4.2: Diagrams showing the two possibilities at turbidity threshold at absorbance of 620nm. Figure 4.2A shows very close absorbance values of both the ethanol medium and the sample (sample620nm). Figure 4.2B shows a wide deviation in absorbance values between the ethanol medium and the sample values. Figure 4.2A indicates turbidity of the sample is similar to that of the ethanol medium, whereas Figure 4.2B indicates that the test compound is showing toxicity.

4.1.2.3 Determination of *In Vitro* Biological Potency of Identified Anti-androgenic Compounds.

AYAS was adapted for determining the potency of anti-androgenic xenobiotics identified in Section 4.1.2.1. The plate was prepared similar to Section 4.1.2.3 such that the flutamide standard curve, the medium blank and the ethanol blank occupied rows one, two and three respectively. The test compounds obtained as pure standards were prepared over a range of concentrations and were replicated into different plates to eliminate interference which may be caused by inter-well creeping. The plates were incubated and read using a spectrophotometer at 540 nm and 620 nm. The absorbances of the standard and the test samples were incorporated on the Excel Program designed to analyse the results. The EC_{50} concentration (mg/L) of the test compounds and the standards was determined within the range of common mid-value (EC_{50}) absorbance. The EC_{50} values were calculated in terms of the standard equivalence using standard non-linear regression analysis methods. The potency was evaluated based on the EC_{50} concentration (mg/L) of the test compounds and that of the standard. Potency (P) of any given anti-androgenic compound is calculated as

$$P = \frac{EC_{50}(\text{Standard})}{EC_{50}(\text{Anti} - \text{androgenic Compound})}$$

The EC_{50} corresponds to the concentration of the standard or test compounds that will produce 50% maximal effects on the sigmoid curve.

4.2 Calibration and Quantification of Anti-androgenic Concentration in the Target Fractions of the Wastewater Samples.

The concentration of the identified anti-androgens in the fractions of wastewater samples was determined using calibration curve method. A range of stock solutions containing the mixture of all the confirmed anti-androgenic compounds was made up. The concentration of each contributing compound which ranged from 300pg to 1000ng, was spiked with 300ng each of deuterated estrone (E1-d_4) and p,p-DDE (internal standards). After reconstitution in 60µL BSTFA/pyridine solution (3:1, v/v) as described in Section 3.6.2, they were heated for 30 minutes in a heat block. 1µL of the derivatised calibration samples was injected automatically into the GC-MS using the program described in Section 3.6.3. The calibration curve was constructed by measuring

the peak areas of each compound and the internal standards in the fractions using the most abundant fragment ions (for compounds) and molecular ion (for E1-d_4). The ratio of the peak areas of the compound and the molecular ion of E1-d_4 (m/z= 346) was evaluated and used to produce the calibration curve with respect to the concentration of the compound and the results are shown in Table 4.4.

4.3 Results

4.3.1: AYAS Assay of Anti-androgenic Compounds Tested.

The AA of the compounds detected in sample fractions in Chapter Three was determined using AYAS (described in Section 2.3.4) and the readings were analysed using the Excel program designed for it. The positive response on the yeast assay resulted in a reverse-sigmoid curve (Figure 4.1) and 620nm absorbance values within the (mean-2SD) turbidity allowance. The cross-section of TCPP plates indicated a transition from orange colour (anti-androgenic points on the curve) to red (non-anti-androgenic points) at flutamide concentration explained in Section 4.1.2.2. However, the plot of TCPP response is somewhat short of full sigmoid (Figure 4.3). At concentration of greater than 400mg/L, TCPP is generally toxic.

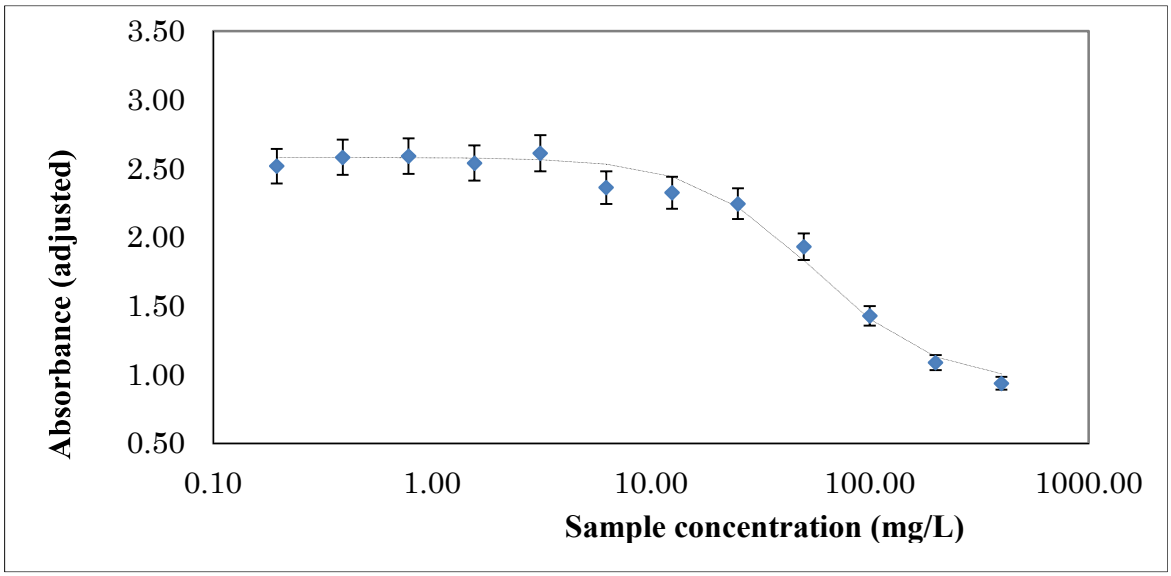


Figure 4.3: The plot of absorbance against concentration (0.20-400mg/L) expressing anti-androgenic response of TCPP on yeast anti-androgen screen (AYAS) assay. The dose-response curve is plotted as the mean response of the TCPP replicates (n=3) and the error bar is expressed as one standard deviation (±SD) of the response.

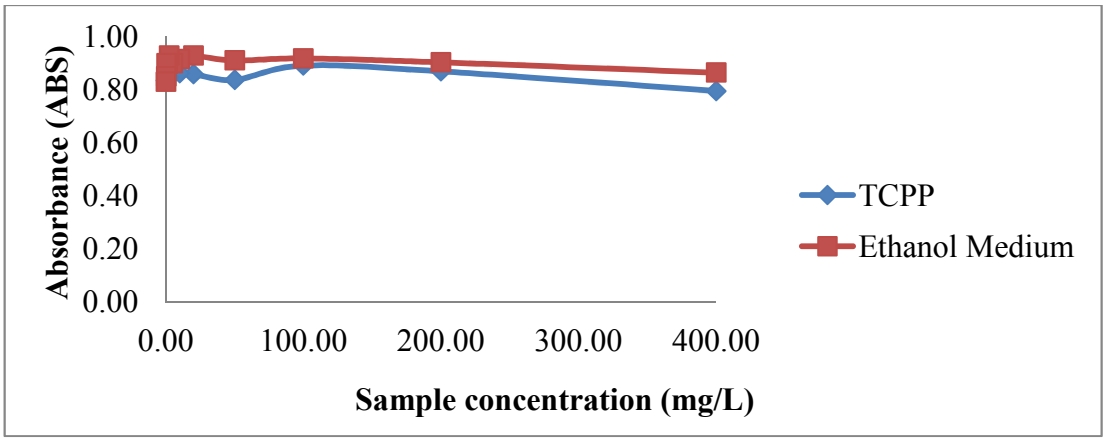


Figure 4.4: Comparison between the 620nm absorbance values of the ethanol medium and that of TCPP sample.

Although three isomers of TCPP were identified in the wastewater samples only isomer I was commercially available for testing. The response curves (Figures 4.3 and 4.4) generated on the AYAS indicated that TCPP (isomer I) is anti-androgenic at the level of the test concentration used. At a corresponding absorbance that produced a flutamide concentration of 2.04mg/L at EC_{50}, TCPP produced an EC_{50} concentration of 41.78 mg/L that results in a relative potency of 0.04.

Conversely, chloroxylenol produced a good response on the AYAS (Figure 4.5) confirming that it possesses anti-androgenic activity at the test concentration. At flutamide EC_{50} absorbance, flutamide and chloroxylenol produced EC_{50} of 2.12mg/L and 10.62 mg/L respectively resulting in a relative potency of 0.20. Chloroxylenol showed no indication of toxicity at a concentration range of 0.02-100mg/L. The 620nm absorbance readings, presented graphically in Figure 4.6, show deviation that falls within the (mean-2SD) absorbance of ethanol medium which is the turbidity allowance.

Triclosan is another compound whose response on the yeast assay produces a good curve (Figure 4.7) and has been identified to have anti-androgenic activity at concentration range of 0.02-20mg/L. The comparison between the 620nm absorbance values of triclosan and ethanol medium (Figure 4.8) indicates that triclosan is turbid at the test concentration. It produces a relative potency of 5.20 from concentrations of 0.41mg/L (flutamide) and 2.12mg/L (triclosan) at EC_{50} absorbance of flutamide.

Similarly, the assay of abietic acid produced a positive anti-androgenic response at a concentration range of $1.25x10^{-3}$-5mg/L (Figure 4.9). The expression of toxicity was

clearly demonstrated when the concentration of abietic acid was greater than 5mg/L (Figure 4.10) and the yeast assay also showed some traces of cell lysis indicating the extent of toxicity at such concentration.

Other compounds tested and found to express anti-androgenic activity on the AYAS are reported in Figures 4.11 as well as Tables 4.1 and 4.2.

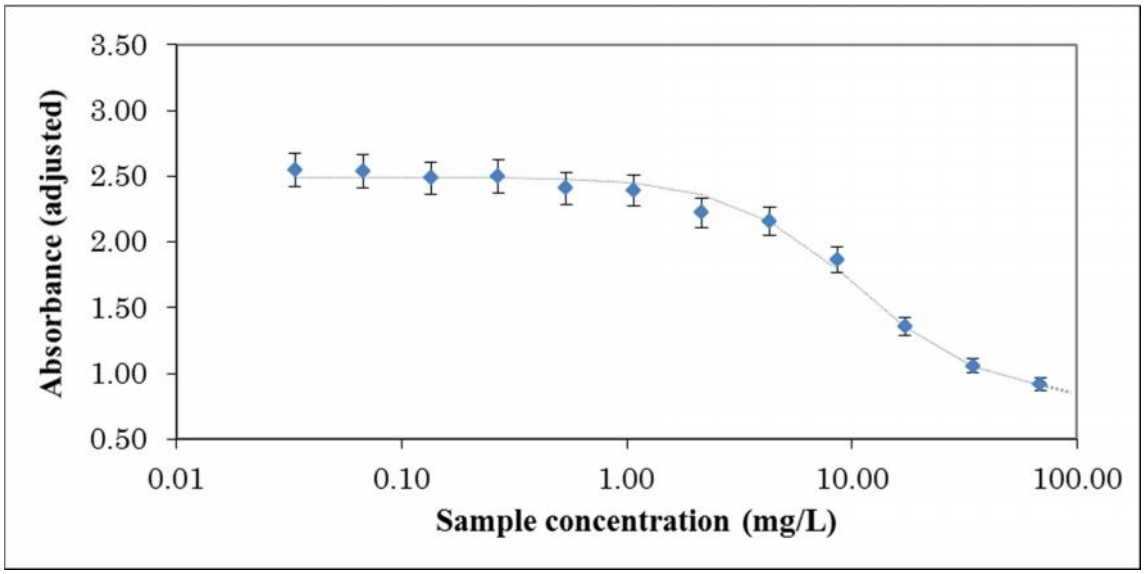


Figure 4.5: The plot of absorbance against concentration (0.05-100mg/L) expressing anti-androgenic response of chloroxylenol on yeast anti-androgen screen (AYAS) assay. The dose-response curve is plotted as the mean response of the chloroxylenol replicates (n=3) and the error bar is expressed as one standard deviation (±SD) of the response.

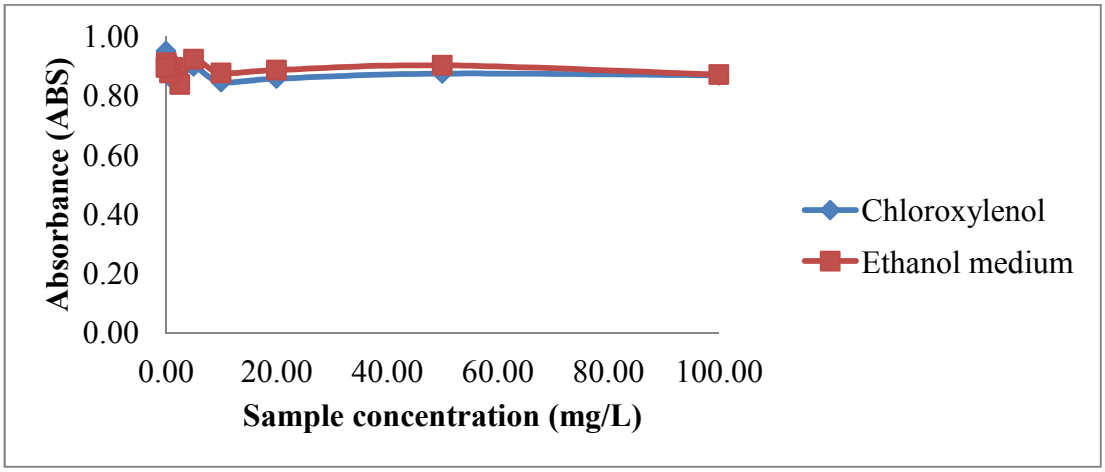


Figure 4.6: Diagram of 620nm absorbance of chloroxylenol and ethanol medium.

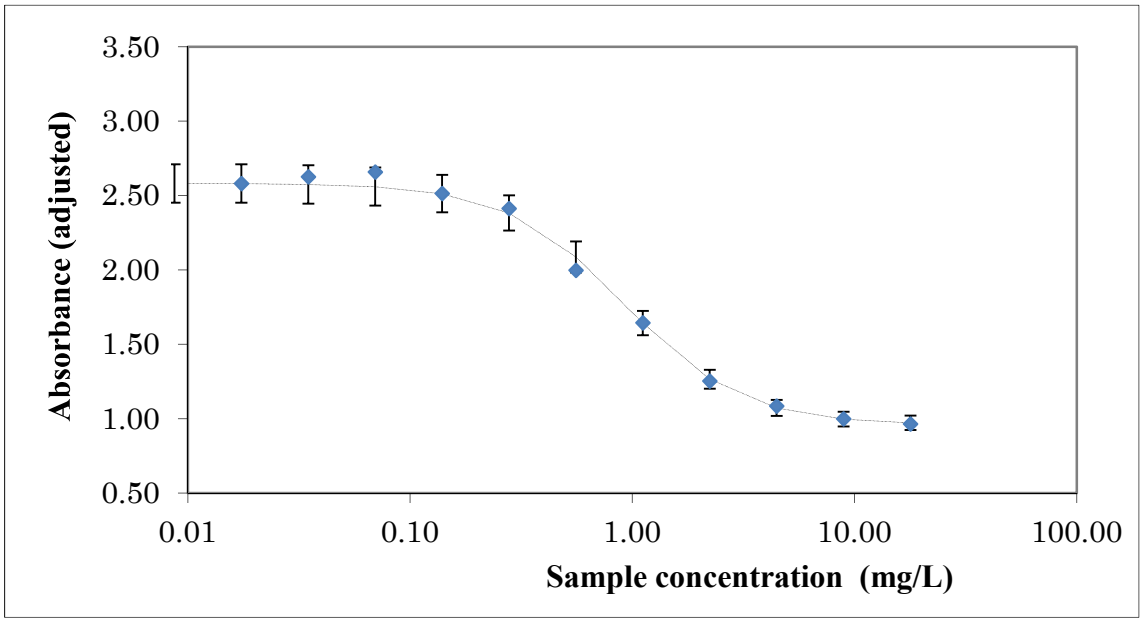


Figure 4.7: Graphical relationship between absorbance and concentration (0.02-20mg/L) expressing anti-androgenic response of triclosan on yeast anti-androgen screen (AYAS) assay. The dose-response curve is plotted as the mean response of the triclosan replicates (n=3) and the error bar is expressed as one standard deviation (±SD) of the response.

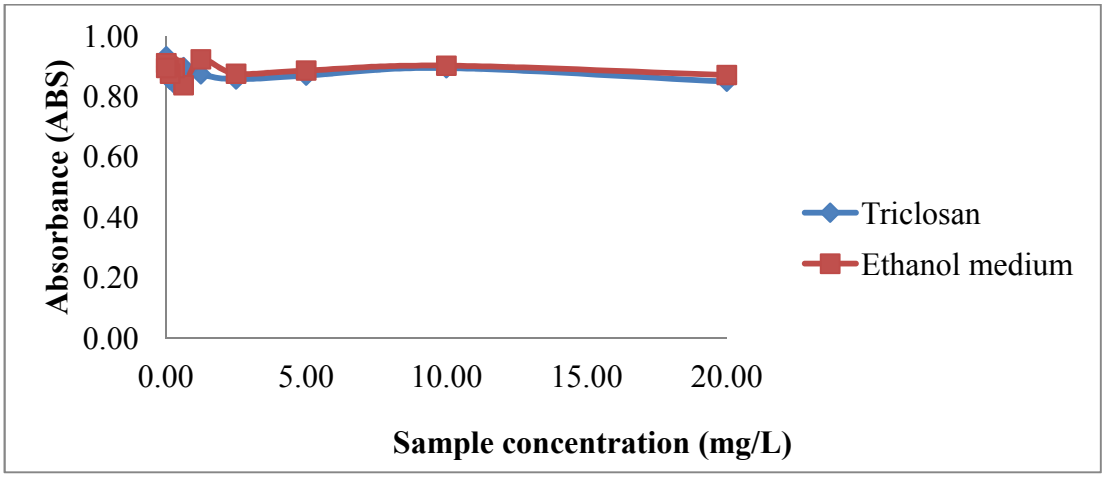


Figure 4.8: Response of triclosan and ethanol medium measured relative to sample concentration on the AYAS at 620nm absorbance to evaluate the possible occurrence of toxicity during the AA investigation.

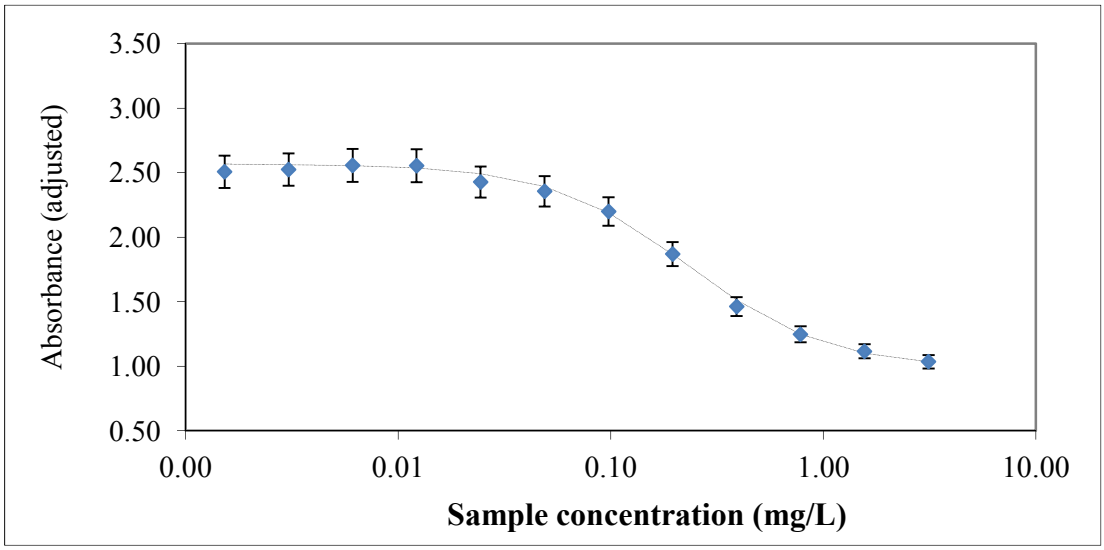


Figure 4.9: The plot of absorbance against concentration expressing anti-androgenic response of abietic acid on yeast anti-androgen screen (AYAS) assay. The dose-response curve is plotted as the mean response of the abietic acid replicates (n=3) and the error bar is expressed as one standard deviation (±SD) of the response.

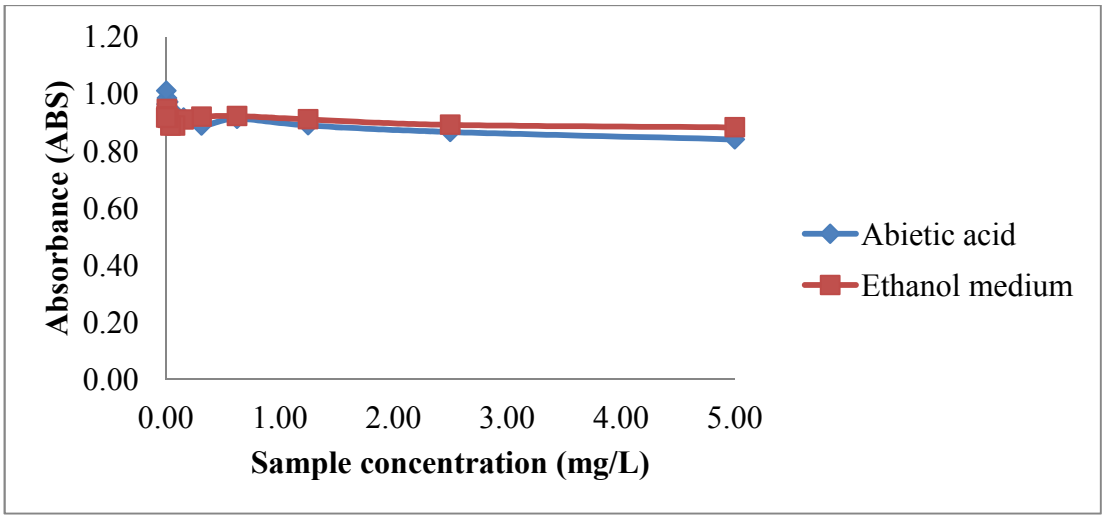


Figure 4.10: Comparison between the 620nm absorbance readings of abietic acid and ethanol medium to determine possible occurrence of toxicity during the AA evaluation.

4.3.2: Potency of the Anti-androgenic Compounds Detected with Respect to Flutamide

The potency of anti-androgenic compounds is determined by measuring the concentration that correlates with the EC_{50} absorbance of flutamide on the concentration-absorbance scale. The repeatability of the assay is evaluated by taking the mean of the results generated from the replicated assay. The summary of the relative potency of all the anti-androgens as well as their chemical structures are provided in Table 4.1 and Figure 4.11 respectively.

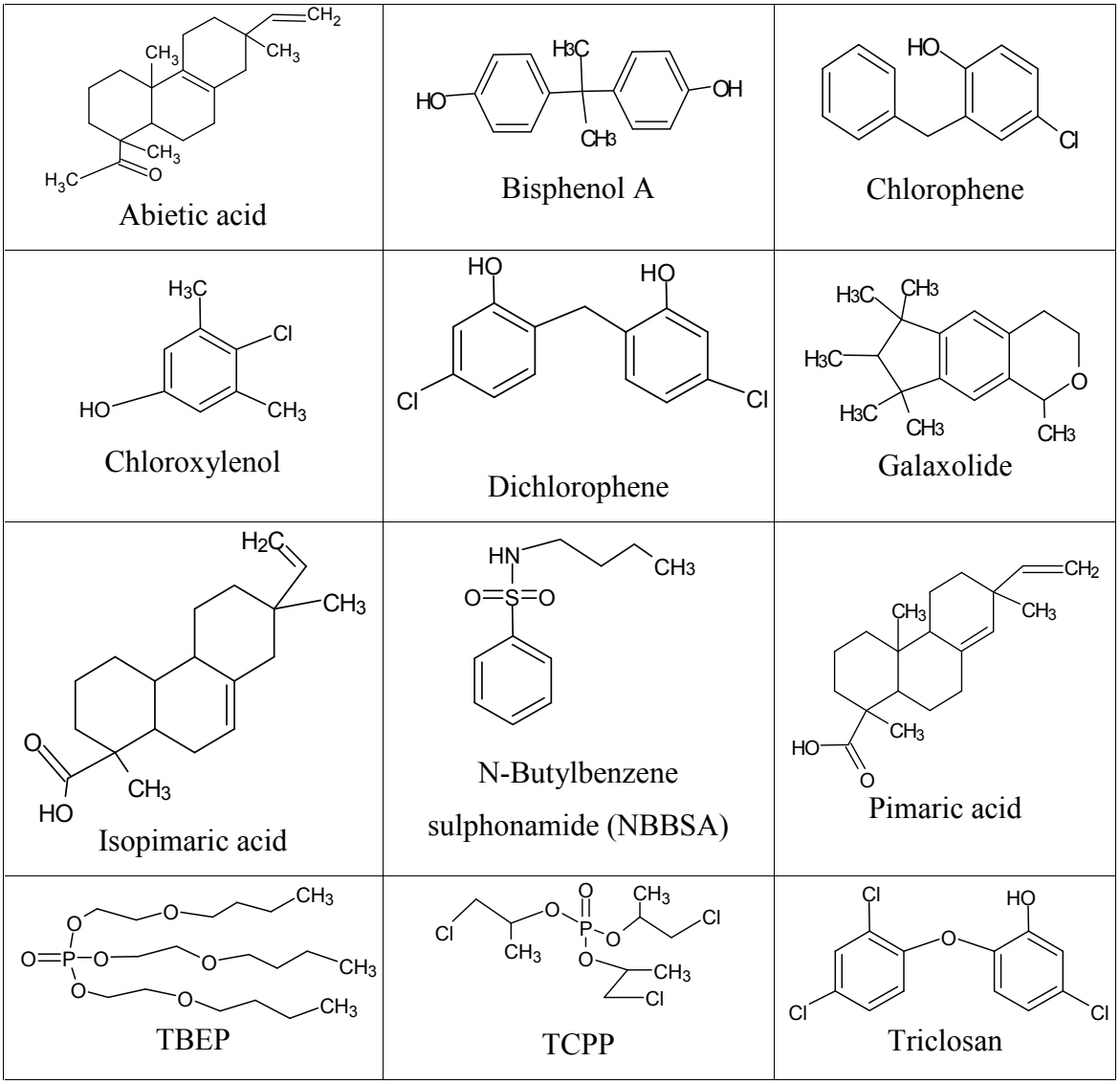


Figure 4.11: Structures of compounds tested and found to exhibit androgen receptor antagonistic activity *in vitro*.

Table 4.1: Table of the relative potency and anti-androgenic activity of compounds identified in WwTP samples.

Compound	Mean EC_{50} of Compound (mgFeq/L)*	Mean EC_{50} of Flutamide (mgFeq/L)	Potency relative to flutamide	Mean±Std EC_{50} of test compound (mgFeq/L)	%RSD EC_{50} of measurement in AYAS
Abietic acid	0.49	2.10	4.29	0.49±0.01	2.04
Bisphenol A	3.73	2.09	0.56	3.73±0.03	0.80
Chlorophene	0.16	2.12	13.3	0.16±0.02	12.5
Chloroxylenol	10.62	2.12	0.20	10.62±0.06	0.56
Dichlorophene	0.43	2.26	5.26	0.43±0.01	2.33
Galaxolide	32.29	2.26	0.07	32.29±0.01	0.03
Isopimaric acid	0.39	2.09	5.36	0.39±0.02	5.13
NBBSA	13.51	2.43	0.18	13.51±0.01	0.07
Pimaric acid	0.57	2.09	3.67	0.57±0.01	1.75
TBEP	34.89	2.10	0.06	34.89±0.03	0.09
TCPP	41.78	2.04	0.05	41.78±0.02	0.05
Triclosan	0.41	2.12	5.20	0.41±0.08	19.60

*The mean EC_{50} of the test compounds was measured with a minimum of three experimental runs.

The potency of the compounds relative to flutamide ranged from 0.05 to 13.4. TCPP possesses the least relative potency of 0.05. In all the compounds identified, chlorophene is the most potent anti-androgen amongst the environmental compounds analysed. The %RSD values range fairly between 0.03 and 12.50% except for that of triclosan which was 19.60%. The following compounds (Figure 4.12) were tested in addition to those in Figure 4.11 but were discovered to be inactive.

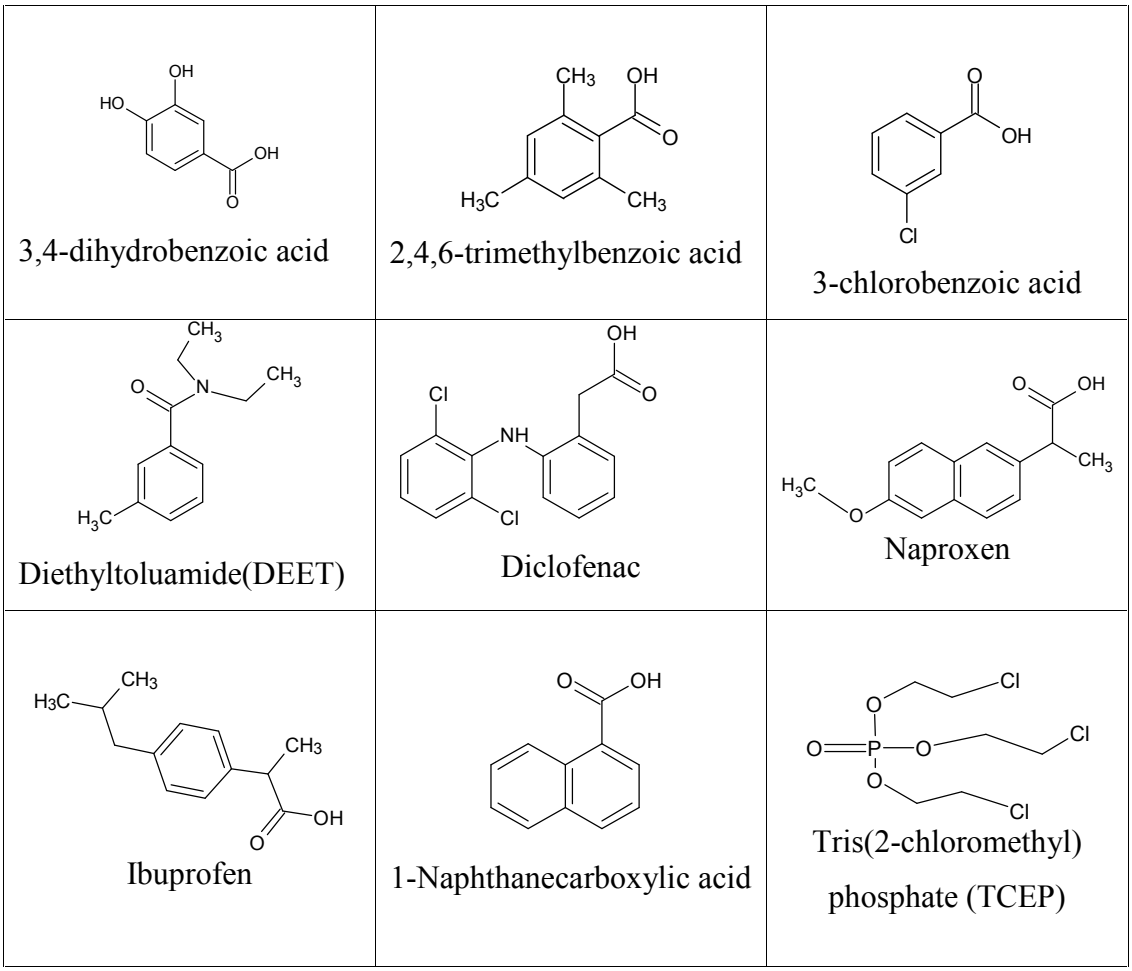


Figure 4.12: The structures of chemical compounds identified in wastewater samples which are inactive on AYAS.

4.3.3: Calibration and Quantification of Anti-androgenic Compounds in GC-MS

The calibration was carried out using the internal standards (deuterated estrone, E1-d_4 and p, p-DDE-d_4). The various identified compounds were injected as a mixture at a wide range of concentrations spiked with the internal standards (30ng). For the purpose of calibration and quantification, a fragment ion identified to possess the highest intensity in the mass spectra was selected for each of the test compounds (Table 4.2).

Table 4.2: Fragment ions used for the quantification of silylated derivatives of anti-androgens and the labelled isotope of the internal standards on the GC-MS chromatograms.

Compound	Mass of fragment ion used for quantification	Molecular Mass of non-silylated compound	Molecular Mass of silylated compound
Abietic acid	256*	302.45	374
Bisphenol A	357	228.29	372
Chlorophene	290	218.68	290
Chloroxylenol	213	156.61	228
Dichlorophene	377	269.12	412
Galaxolide	243	258.14	243
Isopimaric acid	256*	302.45	374
NBBSA	77	213.30	285.30

*Although the fragment ion 256 is common to abietic acid and isopimaric acid in addition to having the same molecular mass, which makes the task of distinguishing one from another difficult, they can be distinguished by the presence of other fragment ions and their different retention times on the GC-MS.

Table 4.2 continued: Fragment ions used for the quantification of silylated derivatives of anti-androgens and the labelled isotope of the internal standards on the GC-MS chromatograms.

Compound	Mass of fragment ion used for quantification	Molecular Mass of non-silylated compound	Molecular Mass of silylated compound
Pimaric acid	121	302.45	374
TBEP	57	398.47	398.47
TCPP	99	327.57	327
Triclosan	200	289.54	362
Estrone-d_4	346	274.40	346

4.3.3.1: Quantification Parameters and Least-Square Linear Regression Analysis.

The quantity of each anti-androgenic compound was estimated using internal calibration. The peak area corresponding to the selected fragment ion of the required compound was isolated on the chromatogram and related to the peak area of the internal standard (estrone-d_4) measured with the selected fragment ion. This ratio of the required compounds to the internal standard was evaluated at various concentration measurements. The relationship drawn from these parameters was used to estimate the quantity of each anti-androgen identified in this study (Section 4.3.3.2, Table 4.3). Examples of such a plot are illustrated with chloroxylenol in Figures 4.13 and 4.14.

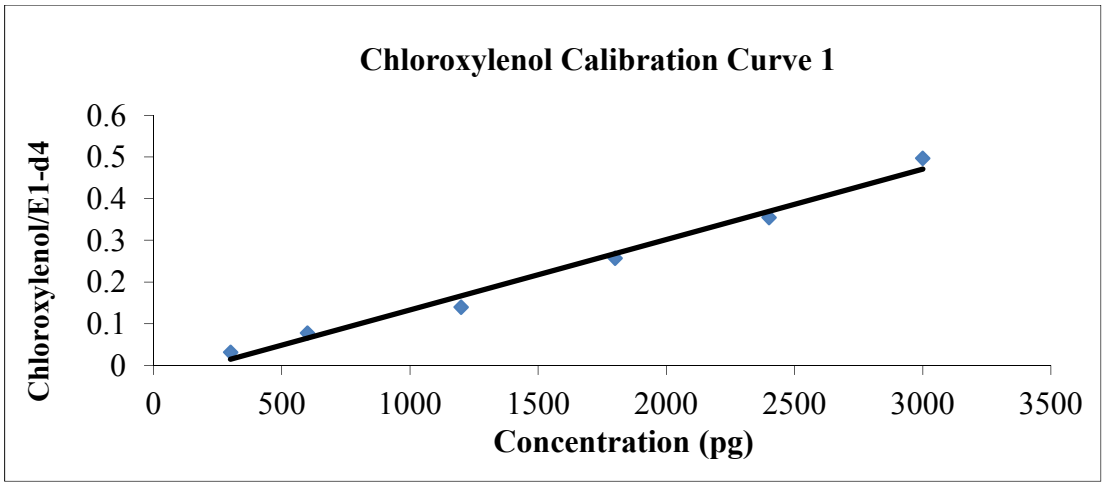


Figure 4.13: The calibration curve of chloroxylenol plotted with linear concentration range of 300-3000pg.

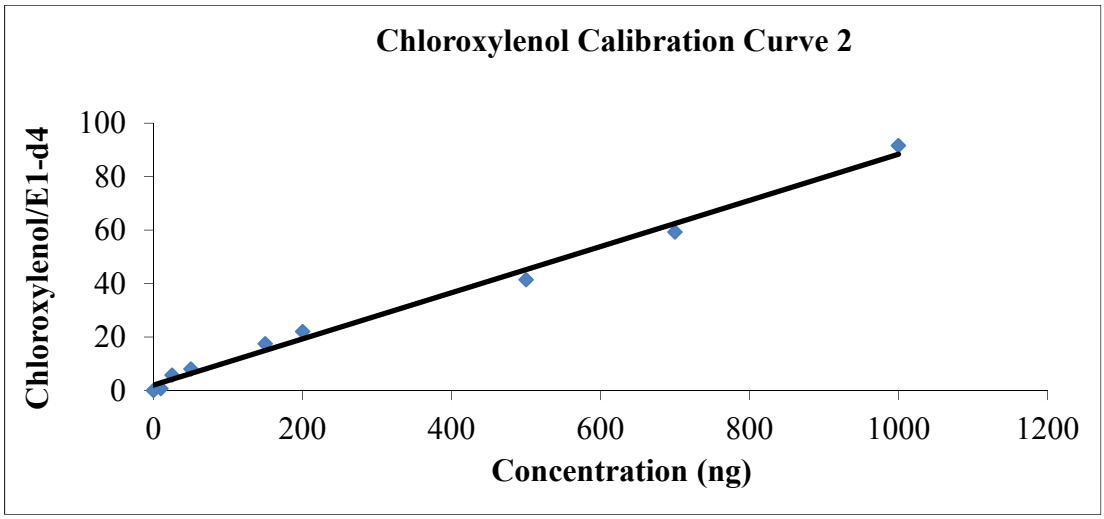


Figure 4.14: The calibration curve expressing linear relationship between chloroxylenol/E1-d_4 and corresponding chloroxylenol concentration range of 10-1000ng.

4.3.3.2: Linearity of the Calibration Curves Used for Quantification of Anti-androgens at Various Concentration Ranges.

The linearity of the calibration curves was estimated by taking into account the various concentration points that form the linear dynamics of all the curves. The calibration plots used for the quantification of the compounds were generated with no fewer than six concentration points using the least square linear regression approach. For the regression plot to be useful, it is necessary to establish whether there is a relationship between the two variables employed to generate the curve. Otherwise, the regression approach will not be appropriate for the analysis. The two statistical parameters commonly used for estimating the suitability of any calibration plots are coefficient of determination and p-values. They are determined using linear square regression analysis and chi-square respectively. For example, the chloroxylenol calibration plots (Figures 4.13 and 4.14) illustrate typical least-square linear regression used for quantification. At 5% level of confidence, the linear plot points for the first calibration curve indicate a goodness of fit as they fall within the normal distribution ($P>0.05$; $n=6$). However, the first two points on the second calibration curve show a slight variation from the range of the normal distribution.

Considering that several linear calibration graphs were prepared to quantify the wide range of chromatograms associated with each compounds, it is therefore necessary to select correctly. Generally, selection of calibration plots was made such that each estimated peak area/E1-d_4 in the fraction lies within the range of the calibration plot. For the purpose of identifying the various calibration plots, a combination of alphabets and a number codes (EL Reference codes) are assigned to each of the calibration plots (see Tables 4.3 and 4.4). The low range calibration graphs were generated within the range of picogram-microgram concentrations whilst the high range calibration graphs were produced within the range of microgram-milligram concentrations of the corresponding compounds.

Table 4.3: Linearity of the calibration plots for quantifying anti-androgens analysed on the GC-MS chromatograms.

Compound	Linear range	Equation of Linearity(EL)	EL Reference Code	Coefficient of Determination (R^2)
Abietic acid	300-2400pg	Y=5E-05x-0.0013	AB1	0.986
	1.2-25ng	Y=0.116x-0.19	AB2	0.945
Bisphenol A	NC	NA		NA
Chlorophene	1.2-25ng	Y=0.125x-0.1878	CP1	0.951
Chloroxylenol	300-3000pg	Y= 0.0002x-0.036	CX1	0.986
	10-1000ng	Y=0.0856x-2.5524	CX2	0.992
Dichlorophene	300-2500pg	Y=2E-05x+0.0007	DC1	0.979
	1.2-50ng	Y=0.0464x-0.0743	DC2	0.992
Galaxolide	300-2500pg	Y=1E-04x+0.008	GA1	0.997
	1.2-50ng	Y=0.1006x-0.015	GA2	0.997
	1.2-150ng	Y=0.1081x-0.0841	GA3	0.999
Isopimaric acid	300-2500pg	Y=2E-05x+0.0029	SP1	0.993
	1.2-50ng	Y=0.0546x-0.078	SP2	0.984

NA: Not available as at the time of preparing this report; NC: Not computed due to non-availability of calibration plot. The EL Reference Code quoted for each calibration plot in the table indicates the calibration plot selected to correctly estimate the amount of the compound present in that fraction.

Table 4.3 continued: Linearity of the calibration plots for quantifying anti-androgens analysed on the GC-MS chromatograms.

Compound	Linear range	Equation of Linearity(EL)	EL Reference Code	Coefficient of Determination (R^2)
NBBSA	5.0-15ng	Y=0.014x+0.0135	NB1	0.997
	5-20pg	Y=2E-04x-0.0177	NB2	0.989
Pimaric acid	2.4-25ng	Y=0.0103x-0.0195	PM	0.965
TBEP	10-700pg	Y=6E-05x+0.0014	TB1	0.998
	0.3-2.0ng	Y=4E-03x+1.762	TB2	0.906
TCPP	3-200ng	Y=0.039x+0.0458	TC1	0.900
	25-1000ng	Y=0.004x+0.1269	TC2	0.981
Triclosan	300-2500pg	Y=0.0001x-0.0049	TR1	0.988
	25-1000ng	Y=0.1525x+2.7794	TR2	0.985
	1.2-25ng	Y=0.2003x+0.2235	TR3	0.958

The EL Reference Code quoted for each calibration plot in the table indicates the calibration plot selected to correctly estimate the amount of the compound present in that fraction.

4.3.3.3: Estimation of AA in Each Fraction Using the Calibration Plots

The amount of the compound (W) present in each proportion of the fraction analysed was determined by substituting the peak area/E1-d_4 (M) as the Y-value of the appropriate linear graph (EL) expressed in the form of Y=mx+C. The X-value resulting from the mathematical equation is equal to W where m and C are constants representing the gradient and the intercept on the Y axis respectively. The detailed summary of anti-androgenic compounds present in each proportion of influent and effluent fractions analysed is prepared in Tables 4.4 and 4.5.

Table 4.4: The concentration of each anti-androgenic compound (measured in flutamide equivalent) present in a certain proportion of the influent fraction analysed. They were estimated using their corresponding peak area/E1-d_4.

Compound	Fraction	Peak area /E1-d_4 (M)	EL Reference Code	Amount of Compound derived from EL (ngFeq/L) (W)
Abietic acid	58	2.509	AB2	23.267
	59	3.004	AB2	27.535
	60	0.244	AB1	4.906*
Bisphenol -A	27	10.773	-	-
Chlorophene	40	0.920	CP1	8.862
Chloroxylenol	31	14.626	CX2	200.682

W is the amount of each compound present in the proportion of influent fraction analysed. This is derived from the related linear calibration equation.*Indicates compounds estimated using low range calibration plots.

Table 4.4 continued: The concentration of each anti-androgenic compound (measured in flutamide equivalent) present a proportion of the influent fraction analysed. They were estimated using their corresponding peak area/E1-d_4.

Compound	Fraction	Peak area /E1-d_4 (M)	EL Reference Code	Amount of compound derived from EL (ngFeq/L) (W)
Dichlorophene	34	-	-	-
	35	-	-	-
	36	0.032	DC1	1.565*
	37	0.897	DC2	20.933
	38	8.260×10^{-3}	DC1	0.378*
Galaxolide	58	3.786	GA2	37.783
Isopimaric acid	58	1.682	SP2	32.234
	59	0.420	SP2	9.121
NBBSA	32	13.406	NB1	956.607
	33	3.873×10^{-1}	NB2	2.025*
Pimaric acid	58	0.307	PM	31.893
	59	0.031	PM	4.903
TBEP	46	2.566	TB2	201.041
TCPP	33	10.319	TC2	2548.025
	34	0.198	TC2	17.775
Triclosan	45	0.192	TR1	1.969*
	46	31.440	TR2	187.938

W is the amount of each compound present in the proportion of influent fraction analysed. This is derived from the related linear calibration equation.*Indicates compounds estimated using low range calibration plots.

Table 4.5: The concentration of each anti-androgenic compound (measured in flutamide equivalent) present in a proportion of the effluent fraction analysed. They were estimated using their corresponding peak area/E1-d_4.

Compound	Fraction	Peak area /E1-d_4 (M)	EL Reference Code	Amount of compound derived from EL (ngFeq/L)(W)
Abietic acid	58	-	-	-
	59	-	-	-
	60	-	-	-
Bisphenol -A	27	0.016	-	-
Chlorophene	40	2.305	CP1	19.942
Chloroxylenol	31	5.303	CX1	26.695*
Dichlorophene	34	4.29×10^{-3}	DC1	0.180*
	35	2.85×10^{-3}	DC1	0.108*
	36	9.16×10^{-3}	DC1	0.423*
	37	10.942	DC2	234.420
	38	8.67×10^{-3}	DC1	0.398*
Galaxolide	58	6.228	GA3	58.391
Isopimaric acid	58	-	-	-
	59	-	-	-
NBBSA	32	10.784	NB1	769.321
	33	7.39	NB1	526.893

W is the amount of each compound present in the proportion of effluent fraction analysed. This is derived from the related linear calibration equation.*Indicates compounds estimated using low range calibration plots.

Table 4.5 continued: The concentration of each anti-androgenic compound (measured in flutamide equivalent) present in a proportion of the effluent fraction analysed. They were estimated using their corresponding peak area/E1-d_4.

Compound	Fraction	Peak area /E1-d_4 (M)	EL Reference Code	Amount of compound derived from EL (ngFeq/L) (W)
Pimaric acid	58	-	PM	-
	59	-	PM	-
TBEP	46	8.416	TB2	1663.50
TCPP	33	11.816	TC2	2922.275
	34	0.734	TC2	151.775
Triclosan	45	0.211	TR1	2.159*
	46	3.235	TR3	15.035

W is the amount of each compound present in the proportion of influent fraction analysed. This is derived from the related linear calibration equation.*Indicates compounds estimated using low range calibration plots.

4.3.3.4: Quantity and Contributions of Identified Compounds to Total Anti-androgenic Activity of Wastewater Fractions

The amount of each compound estimated using the calibration graphs (Section 4.3.3.3) corresponds to the peaks of the chromatograms as well as the anti-androgenic activity of the compounds present in the proportion of the fractions analysed. Given that a representative proportion of the samples was analysed and that such measurements were estimated in flutamide equivalent, it will be appropriate to estimate the AA of the compounds contained in the whole sample as well as express the outcome in a universal measurement independent of flutamide. The process of estimating the amount of AA can be achieved in two steps. First, the amount of AA present in 1mL of the fraction in flutamide equivalent must be determined. Second, the amount of each compound present in the whole sample in ng/L must also be estimated (Tables 4.6 and 4.7).

Chloroxylenol has been chosen to explain how the concentrations of compounds which were originally estimated in flutamide equivalent are estimated in ng/L measurement independent of flutamide. It can be recalled that the amount of chloroxylenol contained in the fractions of wastewater fractions was estimated with the low and high calibration graphs (CX1 and CX2) relative to flutamide (Figures 4.13 and 4.14). The calibration graphs were generated with the best points of fitness which produced coefficient of determination above 0.95. Chloroxylenol was detected in influent and effluent fractions 31 with peak area/E1-d_4 of 14.626 and 5.303 respectively. To estimate the amount of anti-androgenic activity (C) in 1mL of each fraction in flutamide equivalent, the following equation is used,

$$C = \frac{1000W}{V}$$

where W= the concentration of compound generated from the appropriate calibration plot using the peak area/E1-d_4; V= the volume (μL) taken for analysis from 1mL of fraction produced during sample fractionation on the HPLC and 1000= the conversion factor (from μL to mL). Therefore, for chloroxylenol having W of 200.682ngFeq/L in influent (Table 4.4) and 26.695ngFeq/L in effluent (Table 4.5) and V of 500μL (for both influent and effluent fractions), their C values will be 401.364ngFeq/L (Table 4.6) and 53.39ngFeq/L (Table 4.7) respectively. It is important to note that 77.75mL of influent sample (as well as 1700.2mL of effluent sample) fractionated on the HPLC resulted in

1mL of each fraction. Therefore, it can be inferred that each anti-androgenic compound estimated in these fractions is equivalent to the TAA of the compound in 77.75mL of influent sample (or 1700.2mL of effluent sample) analysed.

The next process is to express the amount of each compound present in each fractions of the whole sample in ng/L given that they currently exist in flutamide equivalent. By applying the equation of potency in Section 4.1.2.3 and the relative potencies of the compounds, the values of the compounds (C), estimated in flutamide equivalent, can be converted to ng/L. The amount of AA (ng/L) is expressed mathematically as

$$AA = \frac{1000PC}{H}$$

where P is the potency of the compound, C is the amount of the compound present in 1mL of the fraction and H is the volume-equivalent of either influent or effluent samples fractionated. The value of H for influent and effluent is 77.75mL and 1700.2mL respectively. The quantification step (with the ratio $\frac{1000}{H}$) has simultaneously factored the amount of wastewater samples analysed into the results. Where a compound is present in more than a fraction, the TAA of the compound is calculated by adding the AA present in all the fractions. For chloroxylenol with relative potency of 0.20, the amount detected in fraction 31 was estimated as 40.14ng in 77.75mL of influent and 5.34ng in 1700.20mL of effluent which correspond to 1032.45ng/L and 6.28ng/L of the samples. In addition, their calibration plots are also provided in Appendix E. The percentage contribution of each compound to the TAA of each fraction was also calculated by finding the percentage of AA in the fraction (C) relative to the TAA of the fraction (see Appendix F). This can be related mathematically thus:

$$\% \text{ Contribution of the compound to TAA of the fraction} = \frac{C}{TAA} x100$$

As explained above, C is the amount of AA in 1mL of the fraction and it is expressed in ngFeq/L. For chloroxylenol, its percentage contribution to the TAA of the influent and effluent fractions is 80.27% and 1.72% respectively. The comprehensive summary of the various masses of each anti-androgenic compound detected in wastewater samples is presented in Tables 4.6 and 4.7.

Table 4.6: The summary of estimated masses of each anti-androgenic compound detected in wastewater influent samples evaluated relative to its potency and percentage contribution to the total anti-androgenic activity.

Compound	Fraction	Amount of compound derived from EL (ngFeq/L) (W)	Volume analysed (VμL)	Concentration in 1mL of fraction(C) (ngFeq/L)	Potency (P)	Mass of compound (1000PC /77.75) (ng/L)	% Contribution of anti-androgenic activity in fraction
Abietic acid	58	23.267	400	58.168	4.29	3209.53	1.35
	59	27.535	400	68.836	4.29	3798.16	0.96
	60	4.906	400	12.265	4.29	676.74	0.33
Bisphenol A	27	NC	400	NC	0.56	NC	NA
Chlorophene	40	8.862	400	22.156	13.30	3790.03	0.57
Chloroxylenol	31	200.682	500	401.364	0.20	1032.45	80.27
Dichlorophene	34	-	400	-	5.26	-	-
	35	-	400	-	5.26	-	-

NC: Not calculated due to non-availability of the calibration plot; NA: Not available because NC was not known.

Table 4.6 continued: The summary of estimated masses of each anti-androgenic compound detected in wastewater influent samples evaluated relative to its potency and percentage contribution to the total anti-androgenic activity.

Compound	Fraction	Amount of compound derived from EL (ngFeq/L) (W)	Volume analysed (VµL)	Concentration in 1mL of fraction(C) (ngFeq/L)	Potency (P)	Mass of compound (1000PC /77.75) (ng/L)	% Contribution of anti-androgenic activity in fraction
Dichlorophene	36	1.565	400	3.913	5.26	264.69	0.00
	37	20.933	400	52.333	5.26	3540.47	2.09
	38	0.378	400	0.945	5.26	63.93	0.01
Galaxolide	58	37.783	400	94.458	0.07	85.04	2.20
Isopimaric acid	58	32.234	400	80.586	5.36	555.52	1.87
	59	9.121	400	22.802	5.36	1571.96	0.32
NBBSA	32	956.607	500	1913.214	0.18	4429.31	95.66
	33	2.025	400	5.063	0.18	11.720	0.07

Table 4.6 continued: The summary of estimated masses of each anti-androgenic compound detected in wastewater influent samples evaluated relative to its potency and percentage contribution to the total anti-androgenic activity.

Compound	Fraction	Amount of compound derived from EL (ngFeq/L) (W)	Volume analysed (VµL)	Concentration in 1mL of fraction(C) (ngFeq/L)	Potency (P)	Mass of compound (1000PC /77.75) (ng/L)	% Contribution of anti-androgenic activity in fraction
Pimaric acid	58	31.893	400	79.733	3.67	3763.60	6.77
	59	4.932	400	12.330	3.67	582.03	1.85
TBEP	46	201.041	500	402.083	0.06	310.290	9.81
TCPP	33	2548.025	400	6370.063	0.05	4096.50	82.73
	34	17.775	400	44.438	0.05	28.58	0.57
Triclosan	45	1.969	500	3.938	5.20	263.48	0.16
	46	187.938	500	375.877	5.20	25139.02	9.17

Table 4.7: The summary of estimated masses of each anti-androgenic compound detected in wastewater effluent samples evaluated relative to flutamide concentration with peak area/E1-d_4, relative potency and percentage contribution to the total anti-androgenic activity.

Compound	Fraction	Amount of compound derived from EL (ngFeq/L) (W)	Volume analysed (VµL)	Concentration in 1mL of fraction(C) (ngFeq/L)	Potency (P)	Mass of compound (1000PC /1700.2) (ng/L)	% Contribution of anti-androgenic activity in fraction
Abietic acid	58	-	400	-	4.29	-	-
	59	-	400	-	4.29	-	-
	60	-	400	-	4.29	-	-
Bisphenol A	27	NC	400	NC	0.56	NC	NA
Chlorophene	40	19.942	500	39.885	13.30	312.00	1.73
Chloroxylenol	31	26.695	500	53.386	0.20	6.28	1.72
Dichlorophene	34	0.179	400	0.449	5.26	1.39	0.01
	35	0.107	400	0.268	5.26	0.83	0.01

NC: Not calculated due to non-availability of the calibration plot; NA: Not available because NC is not known.

Table 4.7 continued: The summary of estimated masses of each anti-androgenic compound detected in wastewater effluent samples evaluated relative to flutamide concentration with peak area/E1-d_4, relative potency and percentage contribution to the total anti-androgenic activity.

Compound	Fraction	Amount of compound derived from EL (ngFeq/L) (W)	Volume analysed (VμL)	Concentration in 1mL of fraction(C) (ngFeq/L)	Potency (P)	Mass of compound (1000PC /1700.2) (ng/L)	% Contribution of anti-androgenic activity in fraction
Dichlorophene	36	0.423	400	1.057	5.26	3.27	0.04
	37	237.420	500	474.841	5.26	1469.04	16.96
	38	0.398	400	0.873	5.26	2.70	0.03
Galaxolide*	58	58.391	400	145.978	0.07	6.01	5.41
Isopimaric acid	58	-	400	-	5.36	-	-
	59	-	400	-	5.36	-	-
NBBSA	32	769.321	500	1538.643	0.18	162.90	28.49
	33	526.893	500	1053.786	0.18	111.56	12.55

*The other isomer detected in fraction 58 (retention time 19.20min) was not quantified for lack of information about its anti-androgenic activity and potency. NC: Not calculated for non-availability of the calibration plot; NA: Not available because NC is not known.

Table 4.7 continued: The summary of estimated masses of each anti-androgenic compound detected in wastewater effluent samples evaluated relative to flutamide concentration with peak area/E1-d_4, relative potency and percentage contribution to the total anti-androgenic activity.

Compound	Fraction	Amount of compound derived from EL (ngFeq/L) (W)	Volume analysed (VµL)	Concentration in 1mL of fraction(C) (ngFeq/L)	Potency (P)	Mass of compound (1000PC /1700.2) (ng/L)	% Contribution of anti-androgenic activity in fraction
Pimaric acid	58	-	400	-	3.67	-	-
	59	-	400	-	3.67	-	-
TBEP	46	1663.500	400	4158.750	0.06	146.76	86.64
TCPP	33	2922.275	500	5844.554	0.05	171.88	69.58
	34	151.775	400	379.438	0.05	11.16	7.74
Triclosan	45	2.159	400	5.398	5.20	16.50	0.08
	46	15.035	400	37.587	5.20	114.96	0.78

4.3.3.5: Estimate of Anti-androgenic Activity Removed in Influent at the WwTP.

In the course of influent treatment at the WwTP, certain proportions of some chemicals are being removed. The variation in this proportion (from one chemical compound to another) is due largely to the differences in their physicochemical properties. The following figures (Table 4.8) summarise the estimated anti-androgenic activity of each compound in the fractions that were removed at the Treatment Works. The analysis should be treated with caution as it is based on a single sample of an influent profile and a single sample of an effluent profile. Nevertheless, it does give an indication of the propensity for chemical removal during the treatment processes.

Table 4.8: Relative mass of compounds removed in Horsham Wastewater Works

Compound	Amount in Influent (ng/L)	Amount in effluent (ng/L)	Estimated AA* removed (ng/L)/(%)
Abietic acid	7684.44	-	7684.44(100)
Bisphenol A	NC	NC	NA
Chlorophene	3790.03	312.00	3478.88(91.77)
Chloroxylenol	1032.45	6.28	1026.17(99.39)
Dichlorophene	3869.09	1477.61	2291.48(61.81)
Galaxolide	85.04	6.01	79.03(92.93)
Isopimaric acid	7127.47	-	7127.47(100)
NBBSA	4441.03	274.46	4166.57(93.82)
Pimaric acid	4342.18	-	4342.18(100)
TBEP	310.23	146.76	163.46(52.69)
TCPP	4125.08	183.04	3942.04(95.56)
Triclosan	25402.39	131.47	25270.93(99.48)
Total	**62209.44**	**2537.63**	

NC: Not calibrated due to limited time; * The AA removal is estimated in ng/L while the percentage removal is enclosed in the bracket; total anti-androgenic activity of the whole influent and effluent fractions analysed is 3.11mgFeq/L and 0.15mgFeq/L respectively.

4.3.3.6 Contribution of the Identified Compounds to the Total Anti-androgenic Activity of the Effluent and Influent Wastewater Samples.

Following the evaluation of the total anti-androgenic activity (TAA) in the samples, the contributions of the various identified anti-androgens in the fractions to the TAA of whole samples were estimated. The mass of each compound (C) in each fraction was measured (see Tables 4.6 and 4.7) and where the compound occurs in more than one fraction, the AAs were added up (∑C) (Table 4.9). The percentage contribution made by each identified compound to the overall anti-androgenic activity was estimated relative to the TAA of the wastewater effluent (150000ngFeq/L). That is

% Contribution of a compound to the TAA of the sample$=\frac{\sum C}{M} x100$, where M is the TAA of influent sample (i.e. 3110000ngFeq/L) or effluent sample (which is 150000ngFeq/L) under consideration.

In this study, chlorophene and dichlorophene contributed a majority of the TAA (representing 0.21% and 0.99% respectively) of the wastewater effluent. Analysis further shows that NBBSA ranks third contributing 0.18% to the TAA of the wastewater effluent. Each of TCPP, TBEP and triclosan contributes AA below 0.13% of TAA while that of chloroxylenol occurs below the LOD of the overall effluent's anti-androgenic activity (see Table 4.9). It is significant to note that there is no record of AA contribution from the resin acids (abietic acid, isopimaric and pimaric acid). Collectively, the AA contributed by the identified compounds in Table 4.9 to the TAA of wastewater effluent is 1.69%. This implies that 98.31% of the TAA in effluent wastewater are yet to be accounted for.

Similarly, analysis to evaluate the various contributions of compounds to the TAA of wastewater influent produced some interesting results (Table 4.10). Contrary to the wastewater effluent results, the highest AA contribution to the overall AA of the wastewater influent is produced by triclosan with 0.82%. The resin acids which include abietic acid, pimaric acid and isopimaric acid produced a combined AA of 0.62%. Dichlorophene and chlorophene contribute 0.12% each to the TAA of the wastewater influent while NBBSA added about 0.14% to the TAA. Each of the remaining compounds except bisphenol-A was quantified and reported to express AA

less than 0.13%. The total AA contributions made by all the compounds to the TAA of the wastewater influent is 2.00% indicating that over 98.00% are yet to be identified.

Table 4.9: Contribution of each identified compound to the total anti-androgenic activity in wastewater effluent.

Compound	Fraction(s) where compound was detected	TAA of compound in effluent fractions (∑C) (ngFeq/L)	Compound contribution to TAA of effluent wastewater (%).
Abietic acid	-	-	-
Bisphenol A	27	NA	NA
Chlorophene	40	312.00	0.21
Chloroxylenol	31	6.28	0.00
Dichlorophene	34, 35, 36, 37, 38	1477.61	0.99
Galaxolide	58	6.01	0.00
Isopimaric acid	-	-	-
NBBSA	33	274.46	0.18
Pimaric acid	-	-	-
TBEP	46	146.76	0.10
TCPP	33,34	183.04	0.12
Triclosan	45,46	131.47	0.09
		Total=	**1.69**

NQ: Not quantified; NA: The data is not available due to NC that was not known; The TAA of all the wastewater effluent fractions is 150000ngFeq/L

Table 4.10: Contribution of each identified compound to the total anti-androgenic activity of the wastewater influent.

Compound	Fraction(s) where compound was detected	TAA of compound in influent fractions (∑C)(ngFeq/L)	Compound contribution to TAA of effluent wastewater (%).
Abietic acid	58,59,60	32967.77	0.25
Bisphenol-A	27	NA	NA
Chlorophene	40	50418.17	0.12
Chloroxylenol	31	206.49	0.03
Dichlorophene	34,35,36, 37,38	19522.70	0.12
Galaxolide	58	5.95	0.00
Isopimaric acid	58, 59	38289.99	0.23
NBBSA	32,33	2906.91	0.14
Pimaric acid	58, 59	15867.39	0.14
TBEP	46	186.17	0.01
TCPP	33,34	206.26	0.13
Triclosan	45,46	132092.38	0.82
		Total=	**2.00**

NQ: Not quantified; NA: The data is not available due to NC that was not known; The TAA of all the wastewater influent fractions is 3110000ngFeq/L.

4.4 Discussion

Xenobiotic anti-androgens are a recognised class of endocrine chemicals with the potential to exert adverse effects on the reproductive biology of wildlife and humans. Taking into account that different environmental chemicals have been identified in different compositions of wastewaters and that comprehensive knowledge of their anti-androgenic characteristics are still unavailable, determining the biological status of the environmental compounds discovered in the analysed wastewater samples is one way of resolving some of the many questions surrounding their endocrine disrupting potentials. Urbatzka and co-workers (2007) identified nonylphenol, tert-octylphenol, iprodione, p,p'-DDE, prochloraz, cyproterone acetate, fenthion, pentachlorophenol, ketoconazole, fenitrothion, lindane, dieldrin, mirex, flutamide, vinclozolin and metabolites (M1 and M2) and bisphenol-A in anti-androgenic fractions of sediments and water samples taken from the river Lambro, a watercourse receiving wastewaters from domestic, industrial and agricultural sources (see also Viagano et al., 1999). These compounds are components of some unknown compounds responsible for the total anti-androgenic activity of these samples. Exposure of experimental animals to these chemicals has led to discovery of some abnormal reproductive effects. Nonylphenol, which was detected by Urbatzka and colleagues (2007), has the potential to inhibit sperm production, impair testicular development and the formation of ovo-testis (intersex) in some male fish species (Jobling et al., 1996; Gray and Metcalfe, 1997; Panter et al., 1998).

Horsham wastewater samples analysed have been shown to contain over 95% of domestic waste contributions and have shown to be repositories of a wide range of environmental chemicals (Chapter 3). In previous studies on Horsham wastewaters within and without our laboratory, a wide range of androgen receptor antagonist activity was reported in the range of 0.01-1.69mgFeq/L (Passmore, 2007; unpublished) and 66.7-76.2mgFeq/L (Johnson et al., 2007). The present investigation has identified the musk fragrance galaxolide, organophosphates TCPP and TBEP, antibacterial dichlorophene, triclosan and chloroxylenol, resin acids abietic acid, isopimaric acid and pimaric acid, chlorophene, plasticizer n-butylbenzenesulfonamide (NBBSA) and bisphenol A that is used in plastic industry for epoxy resin and polycarbonate production. It is important to say that galaxolide and bisphenol A had been hitherto reported in some studies to possess anti-androgenic activity *in vitro* (Schreurs et al.,

2005; Sohoni and Sumpter, 1998). Bisphenol-A was reported to possess same anti-androgenic potency as flutamide *in vitro* (Sohoni and Sumpter, 1998). Galaxolide was also reported to possess androgen receptor antagonism with potency five times less than flutamide *in vitro* (Schreurs et al., 2005).

Galaxolide has recently been widely reported to occur at influent, primary, secondary and effluent levels of wastewater treatment plants (Carballa et al., 2004). Fragrance industries have experienced increased expansion following widespread use of polycyclic musks and other nitro musks as fragrances in a spectrum of consumer products globally. It is reported that over 5600 tons of polycyclic musks are produced globally annually (Zhou et al., 2009). As a consequence, some representatives of this polycyclic family have been found in the environment at relatively high concentrations. The polycyclic musk galaxolide has been shown to bioaccumulate in the body of fish and the human breast milk (Gatermann et al., 1999; Schreurs et al., 2005). Zhou and co-workers (2009) reported a range of galaxolide concentration in three Beijing Sewage Treatment Works with the influent samples recording 1649ng/L, 1251.40ng/L and 3003.80ng/L respectively. Their effluent concentrations also were measured in sequence as 492.80ng/L, 729.90ng/L and 1258.3ng/L. Similar studies conducted on wastewater treatment plants' samples in Guandong, China reported influent and effluent concentration of galaxolide in ranges of 11500-146000ng/L and 950-2050ng/L respectively (Zeng et al., 2007). Lee and colleagues (2000) recorded a concentration range of 830-1570ng/L when secondary clarifier effluent was analysed. The analysis of five wastewater treatment plant samples in Canada put the average concentration of galaxolide in the effluent as 2980ng/L (Lishman et al., 2006). The concentrations of both influent and effluent samples in my study (expressed as 85.05ng/L in influent and 6.01ng/L in effluent) occur below the range of concentrations reported in all the studies above. The reason for this is not known but may be due to the loss of anti-androgenic activity during the work-up in the laboratory or due to 72-hour biodegradation during storage. The reduction in the concentration of galaxolide compared to what are recorded in literatures could also be due to high efficiency of removal in the WwTPs in United Kingdom. For instance, the rough estimate in my analysis indicates that 92.93% of galaxolide was removed at the WwTPs. Although there are no available data to suggest so, it is possible that the level of galaxolide used in United Kingdom is very minimal as compared to other classes of fragrances.

Non-steroidal organophosphates are another category of environmental xenobiotics detected in the analysed wastewater samples. They are used as chlorinated organophosphate esters and in non-derivatised forms for a wide range of chemical applications. Organophosphates are predominantly used as flame retardants (in plastics, textiles and timber), plasticisers and lubricants. Some, especially the non-derivatised forms, are used to control the sizes of pores occurring on building structures and materials (Meyer and Bester, 2004). The quantity of organophosphates produced annually in Western European countries runs into ten thousands of tons which makes them rank among the high production volume (HPV) chemicals (*ibid*.). It is on the basis of their widespread use that some members of this family are widely detected in environmental samples across the world. Their presence in wastewater samples is not unconnected with humans' domestic usage from where they get to the wastewater treatment plants via the central sewers. Although, organophosphates identified in wastewaters in Europe and America include tris(2-chloroethyl)phosphate (TCEP), tris(1-chloro-2-propyl)phosphate (TCPP), tris(butoxyethyl)phosphate (TBEP), tris-(1,3-dichloro*iso*propyl)-phosphate (TDCP), *iso*-tributylphosphates (T*i*BP) and *n*-tributylphosphates (T*n*BP) only the first three were detected in my wastewater samples analysed (Meyer and Bester, 2004). The reason for non-detection of other organophosphates is possibly because their concentrations in the analyte samples occur below the LOD. The experimental findings (Tables 4.6 and 4.7) in the study clearly showed a progression of the concentration of TCPP and TBEP from influent. In the assay analysis, TCEP was discovered not to have anti-androgenic properties as it could not elicit about 65% sub-maximal response in the assay but TCPP and TBEP were both active as androgen-receptor antagonists. TCPP was quantified and reported to have concentration of 4128.08ng/L in influent and 183.04ng/L in effluent. TBEP was present in 310.23ng/L of influent and 146.76ng/L of effluent. The quantity of TBEP was measured in two samples sourced from different wastewater treatment plants in ranges of 2300-6100ng/L and 2400-6100ng/L in influent and 290-790ng/L and 250-750ng/L in effluent (Meyer and Bester, 2004). Of the two organophosphates identified as anti-androgens in this study, only TCPP has been identified to have carcinogenic effect in animals (*ibid*).

Another environmental chemical of interest which was detected in both samples of wastewater analysed is dichlorophene. It functions as one of the commonly used

antimicrobial agents in personal care products and cosmetics (Yamarik, 2004). Because of its effectiveness in inhibiting aerobic metabolism, dichlorophene is used to treat some cestode infection and fasciolopsiasis (Idris et al., 1980; Standen, 1963). It is also used to control mould and algae growth to protect textile and horticulture materials (Brown et al., 1986). It is applied to control moss in turf (*ibid*.). In humans and animals, it is used to eradicate/treat tapeworm infection and fungal infection in athlete's foot (Kintz et al., 1997). The few recognised effects of dichlorophene on humans include wheezing (Watt, 1991) and dermatitis (Meynardier et al., 1992). In the influent fractions 37 and 38, dichlorophene contributed anti-androgenic activity of 11.02% and 0.01% to the AA of their respective fractions. Dichlorophene in effluent fractions 34, 35, 36, 37 and 38 equally contributed AA of 0.01%, 0.01%, 0.04%, 16.74% and 0.03% respectively to the AA present in their fractions. On the whole, dichlorophene contributed 3869.09ng/L in influent and 1477.61ng/L in effluent samples analysed. In similar work undertaken in our laboratory, dichlorophene was reported to occur in detectable but not at measurable level in effluent samples analysed (Hill et al., 2010). Up till the time of reporting this work, no single work has evaluated the anti-androgenic activity contribution of these compounds to wastewater samples (influent and effluent).

Triclosan is one of the three widely used biocides which are detected in the wastewater samples analysed. Triclosan is a known multi-purpose anti-microbial agent contained in the compositions of skin care products and cosmetics, the commonly used household products (carpet, sponges, socks and underwear), deoderants products and oral care compositions (toothpastes). In recent times, triclosan has been incorporated in some kitchen earthware products; also, it is the major anti-microbial present in liquid soaps (Perencevich et al., 2001). In the medical environment, it is used as surgical scrubs, hand washes and body washes to control the spread of Methicillin-resistant Staphylococcus aureus (MRSA) bacteria strains (Tierno, 1999; Russell, 2004). Triclosan, a trichlorinated bisphenyl, is a fairly non-volatile hydrophobic compound which blocks the biosynthesis of lipid in bacteria and fungi by antagonising the enzyme enoyl-acyl carrier protein reductase (Heath et al., 1999; McMurry et al., 1998). Even though triclosan has been widely detected in environmental samples such as domestic wastewaters, river sediments, sewage sludge and reclaimed water as well as fish bile, human breast milks and the human plasma due to its lipophilicity (Adolfsson-Erici et al., 2002; Allmyr et al., 2006; Houtman et al., 2004), the information about its endocrine

toxicity is not available. At a concentration of $3.50x10^5$ng/L, triclosan shows acute toxicity to rainbow trout (Adolfsson-Erici et al., 2002; Lindstrom et al., 2002). Although it is not considered to be acutely toxic in mammals, it can cause disturbances to the metabolic system as well as hormone homeostasis (Hanioka et al., 1996; Schuur et al., 1998; Wang et al., 2004; Jacobs et al., 2005). Administration of triclosan via dental care products in humans could lead to accumulation of the compounds in tissues especially when they gain access into the body through the mucosa (moist tissues lining the gateway into the body such as nasal and buccal passages) and intestinal cavity (Lin, 2000; Sandborgh-Englund et al., 2006). It is also possible for triclosan to bioaccumulate in the body through the skin despite the level of metabolism that may occur during the dermal absorption (Moss et al., 2000). Taking into account that endocrine disruption studies of triclosan are limited, determination of its anti-androgenic effects in animals would be extremely necessary.

Gatidou and colleagues (2007) reported that the anti-androgenic activity of triclosan occurred in average concentration of 280ng/L, 430ng/L and 11840ng/g in filtered municipal wastewater influent, effluent and sewage sludge respectively. In addition, the hospital and university samples analysed recorded concentrations of 9070ng/L and 790ng/L respectively (*ibid.*). McAvoy and colleagues (2009) reported field concentrations range of 3800-16600ng/L for influent and 200-2700ng/L for effluent. In this study, the concentration of triclosan in influent and effluent samples recorded is 25402.40ng/L and 131.470ng/L respectively. Two influent samples collected from rural wastewater treatment plants in Australia recorded triclosan concentrations as low as 805ng/L and 791ng/L (Ying and Kookana, 2007). Studies have also reported the occurrence of triclosan in effluent between 40ng/L and 2700ng/L (McAvoy et al., 2009; Reiss et al., 2002; Singer et al., 2002; Sabaliunas et al., 2003).

Chlorophene and chloroxylenol are two other antibacterial compounds discovered in both influent and effluent fractions to be anti-androgenic. Chlorophene is widely used as disinfectants for cleaning hospitals and homes (Zhang and Huang, 2003). The percentage chlorophene contribution to the total anti-androgenic activity of the wastewater samples in my study is 0.12% (3790.03ng/L) for influent and 0.21% (312ng/L) in effluent. Chlorophene is the only anti-androgenic compound that recorded relative potency of two digits (13.3 relative to flutamide) in this work. Chlorophene concentration detected in influent and effluent samples taken from Cilfynydd

Wastewater Treatment Plant measured in the region of 114 ng/L and 29ng/L respectively (Kasprzyk-Hordern et al, 2008). Cilfynydd Wastewater Treatment Plant is a treatment plant situated in southern Wales receiving predominantly domestic wastewaters from a population of about 110,000. In another study involving five sewage treatment works in Spain, the average concentration of chlorophene detected was greater than 1000ng/L (Bueno et al., 2007). Similarly, chloroxylenol (also known as para-chloro-meta-xylenol) is another chlorinated phenolic compound that is actively used in disinfectants and antiseptics especially toilet cleansing. In addition, it has been used as preservative in pharmaceuticals and cosmetic products. Loraine and Pettigrove (2006) detected chloroxylenol in reclaimed wastewater as well as dry-season and wet-season influent samples at an average concentration of 285ng/L, 3550ng/L and 1610ng/L respectively. In this study, 1032.45ng/L of chloroxylenol was discovered in influent, 6.28ng/L was found in the effluent sample.

Resin acids are a group of diterpenoid carboxylic acids found in some coniferous tree species. Resin acids can broadly be classified into abietanes and pimaranes (Mohn, 1995). Abietanes can be distinguished from pimaranes by the isopropyl group at C-13 as against vinyl and methyl groups at C-13 of the latter. Wang and co-workers (1995) reported that lodgepole pine wood resin acids consist of abietic acid, dehydroabietic acid, neoabietic acid, palustric acid, isopimaric acid, pimaric acid and levopimaric acid. Sandaracopimaric acid is another pimarane discovered in wood (Martin et al., 1999). Most of these wood acids and their metabolites are discovered in wastewater samples. It is equally reported that wastewater receiving river sampled downstream bleached kraft pulp and paper mill industry contains some of these resin acids (Zender et al., 1994). Although, only abietic acid, isopimaric acid and pimaric acid were detected in my wastewater samples, other wood resin acids may be present in negligible concentration (i.e. concentrations <LOD). In the influent fractions studied, the percentage contribution of abietic acid, isopimaric acid and pimaric acid to the total wastewater AA is 0.25%, 0.23% and 0.14% respectively. Also, it must be stated that the quantity of the individual resin acids detected in the effluent fractions was below the limit of detection which has further been reflected by the effluent profile (Chapter Three). Individual resin acids have shown acute toxicity in aquatic fish such as rainbow trout and the acute lethal concentration occurred within the range of $4.00x10^5$-$1.10x10^6$ng/L (Chung et al., 1979; Leach and Chung, 1982).

Another chemical compound detected in wastewater is a common plasticizer n-butylbenzenesulfonamide (NBBSA). It is used for manufacturing sulfonyl carbamate herbicide, polyamide and copolyamide plastic industry (Lee et al., 1995; Huppert et al., 1998). Although account of its aquatic toxicological effects is presently unavailable, acute exposure of Wistar rats can induce chronic adverse effects in their motor activities (Lee et al., 1995). In addition, laboratory studies have revealed that it could show acute neurotoxocity by interfering with choline acetyltransferase in animals (Nerurkar et al., 1993; Lee et al., 1995) and juvenile New Zealand white rabbits (Strong et al., 1991). Presently, the production and use of NBBSA have been discontinued in Germany (Huppert et al. 1998). A number of published works have revealed that NBBSA occurs in a wide range of environmental samples such as seepage and leakage water from landfills ($4.20x10^5$–$7.10x10^5$ ng/L), runoffs from agricultural field irrigated with treated wastewater (350-6000ng/L) and wastewater influent samples (260-2600ng/L) (Schwarzbauer et al., 2002; Paxeus and Schroder, 1996; Soliman, 2003; Paxeus, 1996; Kasprzyk-Hordern and Dinsdale, 2008). In the samples analysed, a concentration of 4441.03ng/L was detected in influent samples while 274.46ng/L was found in effluent. A study reported an NBBSA concentration range of 351-6000ng/L in effluent wastewater (Pederson et al., 2005). Its occurrence in wastewater treatment works has been linked to its composition in plastics used in domestic environment.

Bisphenol A (BPA) is a known environmental contaminant found in wastewater effluents. It is used for producing polycarbonates (Krishnan et al., 1993) and epoxy resins from which products such as inner coatings for canned food and drinks (Brotons et al., 1995), baby-milk bottles and dental sealants (Olea et al., 1996), powder paints and optical lenses (Steinmetz et al., 1998; Gatidou et al., 2007; Fromme et al., 2002) are made. Epoxy resins are prepolymers with each molecule formed from two or more epoxide groups. Thermoset plastic is formed when the resins react with curing agents. Some of the properties of the cross-linked product (thermoset plastic) formed include toughness, versatility, high chemical resistance and elastic strength (Vilchez et al., 2001). The can inner linings react with the food in it and induce estrogenic effect (Kim et al., 2001). It is also used as raw materials in plastic and polymer industry (Fukazawa et al., 2001). In addition, it is a precursor/starting material in the production of adhesives, automobile lenses, window glazing, building materials, compact disks, thermal paper, paper coatings and as electrical encapsulation and electronic components

(Fürhacker et al., 2000; Pulgar et al., 2000). It may be discharged accidentally as fugitive dust during processing, packaging and transportation in a closed system (Staples et al., 2000). Bisphenol A has been quantified in various environmental samples which include river sample (9-780ng/L), sediments (0.018-0.190mg/kg), sewage sludge (0.004-1.363mg/kg) and wastewater samples (21-702ng/L) (Fromme et al., 2002). Due to inconclusive experimental work as at the time of putting together this report, the concentration of BPA in influent and effluent samples could not be determined.

It must be stated that the calibration plots of some compounds showed poor goodness of fit (or lack of fit) due to a wide range of reasons. It is possible for some compounds which produce a linear calibration plot at low concentration range with positive gradient also to generate a linear calibration plot at high concentration range with negative gradient or vice versa. When such occurs, there is the likelihood that some plot points would fall at random away from the projected line on the calibration graphs. The calibration graphs derived under this condition cannot be expected to produce a perfectly accurate estimate of such compound. Moreover, inappropriate choice of internal standard could contribute adversely to the level of inaccuracy that may likely arise from such estimation. Another reason for the poor goodness of fit is the sensitivity of various columns deployed for the analyses of the compounds in wastewater fractions and the standards. As would be expected, the level of analysis undertaken on the GC-MS can reduce their sensitivity (due to bleeding) and hasten their rapid burn-out. Variability in the column sensitivity (random error) could arise which will affect the estimated results from the data generated. In this study, various statistical measurements of errors and level of confidence in these results were estimated. No doubt, the various sources of variation explained in this discussion section will contribute to the process that generates the calibration curves and the poor goodness of fit. Beside these limitations, it is also necessary to emphasise that the least square regression approach is not suitable to effectively and accurately determine the goodness of fit and linearity of experimental data, especially using the R^2. Whilst the values generated as R^2 in the regression line could sometimes be as high as 1, it is possible for the fitted data to spread evenly around the regression line without any of the data points falling on the regression line. The correlation coefficient, as well as the value of R^2, generated in such instance will be misleading. Although none of my regression models

exhibits such data geometry, it will still be inappropriate to say that the measurement errors arising from lack of fit of the data points to the regression lines have been effectively accounted for by the regression models (AMC, 1994). It simply implies that the regression model can be grossly inaccurate in predicting the future outcomes of the two variables (dependent and independent). On the account of such development, there is the possibility that the outcome of the measurements and estimations in this Chapter will be affected either positively or negatively.

All the compounds identified in this study have contributed immensely to the growing knowledge of steroid receptor antagonist compounds in wastewater samples especially under *in vitro* conditions. However, their contributions to the overall anti-androgenic activity in fractions of the wastewater extracts analysed was relatively little (1.69-2.00%) compared to what are yet to be discovered. There are two possible reasons that could be responsible for this result. It is possible that the compounds contributing to the total anti-androgenic activity in each of these fractions is yet to be identified. It is also possible for compounds already identified which were commercially unavailable for testing to be partly responsible for the AA discrepancy. Another possible reason for this could be additive or synergic effect (Graumann et al., 1999). It is plausible for compounds which recorded anti-androgenic activity below the detection limit to add up their effect (*ibid.*). It is possible that some highly potent androgen receptor antagonists (e.g. dioxin congeners) were not detected in the GC-MS and therefore their contributions were not included in the overall estimation of anti-androgenic activity in the fractionated samples. It is also possible for the multiple contaminants with AA to act as a mixture effect, either by concentration addition or by synergistic effect to enhance the receptor AA of some fractions in the AYAS. In addition to the difficulty associated with the identification of compounds occurring in low concentration, producing a model for quantifying additive or synergic anti-androgenic effects of multiple compounds, especially in instances where cumulative effects are being expressed at sub-LOD concentrations, may be difficult. For that reason, most cumulative effects are either not accounted for or inaccurately estimated. Furthermore, the fact that the majority of the compounds identified came from the non-polar section and, to some extent, moderately polar area of the profile suggests that most of the compounds yet to be identified (i.e. about 98% unknown) may be localised in the polar section of the profile.

4.5. Conclusion

A number of chemicals present in Horsham wastewaters have androgen receptor inhibitory affinity. Although these chemicals emerged from different sources, and possess different structural formula, they have demonstrated the potential of binding antagonistically to androgen receptors using an *in vitro* AYAS assay. These results have shown a level of human androgen receptor promiscuity, in that a wide range of chemically active molecules can bind to the androgen receptor. In addition to screening the anti-androgenic xenobiotics from the collections of environmental chemicals detected, the AYAS assay was also used successfully to define the concentration range for which a compound is said to be active and non-toxic at the same time. It was further used to determine the potency of various xenobiotics tested for anti-androgenic activity relative to the AA of flutamide. The quantity and the percentage contribution of each of these anti-androgenic xenobiotics were evaluated in fractions and wastewater samples collectively based on their relative potency. In conclusion, the various forms of environmental anti-androgenic chemicals detected in Horsham wastewaters have been found in other wastewater samples in United Kingdom, Europe and the Americas. What appears significant in my study is that the *in vitro* anti-androgenic activity of most of the identified compounds is being reported for the first time and clearly shows the complex arrays of chemicals with potential endocrine disrupting activity present in wastewater samples.

CHAPTER FIVE

General Discussions and Conclusion

5.0 Introduction

This study has established that wastewater influents and effluents are repositories of known and unknown environmental anti-androgens. It has also shown that some chemicals associated with regular human consumption possess hormonal properties and that continued interaction with such xenobiotics-indoor and outdoor-could result in grave reproductive endpoints such as intersex, feminisation and masculinisation. Given that the knowledge about the fate and biological activity of emerging and new environmental chemicals is presently poor, it has also raised concern that continued release of poorly treated environmental samples such as effluents, known to contain unidentified offending chemicals, into various receiving environments may pose severe health consequences. It has further reinforced the potential danger involved in distributing recycled wastewaters for drinking and irrigation purposes across the world when the current chemical removal technology seems ineffective for this purpose.

5.1. Recovery Methodology of Xenobiotic Anti-androgens on SPE and HPLC.

Comprehensive study of the receptor activity of xenobiotic anti-androgens contained in the wastewater samples was undertaken with a SPE recovery methodology and a modified HPLC fractionation program (in Section 2.2.4). The revised SPE methodology produced over 96% recoveries of the test compounds which were selected to cover a broad range of polarities. Although several cartridges with different sorbent composition were tested, the HLB cartridge recoveries were generally satisfactory (>94%) for all test compounds used. The TIE procedure was modified by replacing the methanol-water solution with less polar acetonitrile-water solution as the sample injecting solution into the HPLC. There was a general improvement to the recovery of the total anti-androgenic activity (>78.6%) in the HPLC fractions of influent and effluent extracts which emphasises the success of the new methodology.

5.2. Xenobiotic Anti-androgens in Wastewater Treatment Works

This study has demonstrated that a wide range of offending chemicals which have androgen receptor antagonistic affinity are not completely removed from wastewater influents at the treatment works. Some studies on wastewater effluents have also identified that endocrine disrupting chemicals which possess steroid receptor affinity for estrogens, anti-estrogens and androgens were not removed during treatments at sewage treatment works. Generally, the level of chemical removal from wastewater influent varies from one treatment works to another. This is largely due to variability in chemical elimination technology deployed from one treatment works to another (Bandelj et al., 2006; Conroy et al., 2007; Johnson and Sumpter, 2001; Kim et al., 2006; Ternes et al., 1999b). Also, this study has shown that removal of various contaminants at the treatment works cannot proceed at the same proportion. This may be attributed to the difference in physicochemical properties of the various compounds involved. Elimination of toxic chemicals at the WwTP is generally known to proceed through physical, chemical and biological processes. It must also be emphasised that removal efficiencies of WwTPs are also dependent on the physicochemical properties of the compounds and the treatment technology employed amongst other factors (such as population of community served by the treatment works, the temperature of operation, season and climatic variable e.g. rainfall and variation in the composition input from the contributing sources) (Bound and Voulvoulis, 2005). The processes for removing some proportion of non-polar organic and inorganic contaminants in these wastewater samples are largely successful while also noting that most dissolved organic compounds are not removed (Tabak et al., 1981; Alcock et al., 1999; Ternes et al., 1999a; Ternes et al., 1999b; Drewes et al., 2002; Snyder et al., 2004; Joss et al., 2005). Also, after undergoing oxidation or degradation at the works, some compounds would transform to a receptor active moiety which could stubbornly persist and, to some extent, influence the overall activity of the emerging effluents (Richardson et al., 2005). In this study, no specific compound in that category has been identified, but that has not foreclosed the non-existence of such compounds (given that some group of contaminants were removed from the influent profile which culminated in the cluster of anti-androgenic activity around the polar section of the effluent profile) (Chapter Three). This study has further revealed that the anti-androgens in wastewaters are spread across a wide range

of polarities. Incomplete removal of some chemicals and their metabolites can explain why effluent samples emerging from treatment plants, as well as wastewater receiving rivers, demonstrate toxicity. Actually, it will be inappropriate to conclude that trickling filters used was successful in removing a wide range of environmental chemicals at the Horsham treatment works. This is simply because the bulk of compounds identified so far came from non-polar fractions only. As conspicuously seen, the most active compounds occur in the polar section of the profiles many of which are yet to be identified.

5.3. Profiles of Anti-androgens in Wastewater Treatment Works.

Wastewater profiles (influent and effluent) in Chapter Two and Three indicate the occurrence of a wide range of receptor-active xenobiotic anti-androgens in the WwTP samples. The fact that bioactive HPLC fractions are numerous in this study suggests that xenobiotic compounds capable of demonstrating receptor antagonism after binding are preponderant. Androgen activity, if at all it existed, is not conspicuous in the profiles the reason for which may be attributed to the low occurrence concentration in the samples analysed or masking by higher concentrations of anti-androgens in the same fraction. There are some basic differences noticeable on the polar profiles of both inlet (influent) and outlet (effluent) samples which have been attributed to the biological activity at the wastewater works. Similar profile of bile harvested from effluent exposed trout (from Horsham), investigated in our laboratory using the same protocol as in this work, produced similar profile as the effluent samples in Chapter Three (Rostkowski et al., 2011). This preliminary works with fish bile suggests that most xenobiotic anti-androgens identified in effluent profile were bioconcentrated in fish.

5.4. Confirmation of Anti-androgenic Compounds in Wastewater Treatment Works.

This study identified for the first time twelve xenobiotic anti-androgenic compounds in wastewater samples (influent and effluent) which can interact with steroid receptors. The category of xenobiotic compounds identified and confirmed to occur in wastewater influent samples (and some in effluent samples) includes wood resin acids (abietic acid, isopimaric and pimaric acids), antibacterial agents (chlorophene, chloroxylenol, dichlorophene and triclosan), flame retardants (TCPP and

TBEP), polycarbonate material (BPA), musk fragrance (galaxolide) and plasticiser (NBBSA). With exception of galaxolide, triclosan, BPA, TCPP isomer and abietic acid which have been identified as anti-androgens in previous studies (Chen et al., 2007; Evans, 2008; Roh et al., 2010; Schreurs et al., 2005; Sohoni and Sumpter, 1998; Weiss et al., 2011), all other compounds identified in this work are being reported to have androgen receptor antagonist *in vitro* for the first time. Mostly, the identified compounds (with exception of resin acids and NBBSA) could be traced to the compositions of general consumer products. Given that Horsham wastewater influent comprises over 95% contribution from domestic sources (Evans, 2008), it is likely that these compounds emanated from domestic usage of consumer products. The findings in this study corroborate the published discoveries which recognised that these arrays of compounds can occur in domestic wastewater effluent (Rudel et al., 1998; Stasinakis et al., 2008; Miege et al., 2009).

Bioaccumulation of most endocrine disrupting compounds in the tissues of aquatic animals and humans can trigger dose-response effects at a certain threshold concentration. At sub-lethal concentration, fish species exposed to bioactive chemicals that are present in wastewater samples can develop biological effects, such as intersexuality (Jobling et al., 1998). Although additive effects associated with environmental samples can stand as the logical basis for explaining such behaviour, it is also possible for highly potent bioactive compounds to induce such biological effects at concentration below detection limit. It is difficult to estimate the implications of environmental bioavailability of some anti-androgens in their metabolite state given that their fate during transition may likely not be known. Several compounds can form metabolites which are more potent than their parent compounds so also the intermediates during transition which may even be much potent. For the fact that chemical metabolism can also be induced in the animals' system will partly widen the diverse possibilities of chemical transformation in the environment. For that reason, it will be difficult to explain which state of xenobiotic anti-androgen will be much potent and whether the singular impact of a xenobiotic anti-androgen will account for the intersex phenomenon and other interrelated reproductive effects.

Another important point that could be taken from this study is that chemicals leaving the WwTPs may be relatively low in concentration in the receiving

environment. The variability in the final environmental concentration as related to the point source will be affected by seasonal activities (Loraine and Pettigrove, 2006). It is discovered that concentration of effluent released into the receiving rivers will be diluted during the rainy season and hence the concentration of the compounds in it becomes low further. Conversely, concentration of possible pollutants in the same effluent becomes relatively high when no rain falls indicating how seasonal climatic effects can impact on the environmental level of some chemical toxicity and bioactivity. Currently, the danger in distributing treated water for drinking and irrigation purposes is as grave as converting the sludge from treated water as fertiliser given that much are still needed to be done to improve on the treatment technologies presently in use. In addition, the current chemical methodology for screening treated drinking water alone cannot address the growing persistent cases of emerging contaminants. Biologically-based risk assessment assays must be developed for evaluating chemical toxicological end-points such as endocrinotoxicity, immunotoxicity, carcinogenicity and neurotoxicity. These all-purpose assays must be designed to accommodate measurement of multiple chemical effects. This intended assay could also be used by water industry for certifying the safety of water available for public consumption.

5.5. Further Work

Evaluation and screening of endocrine disrupters have been effectively achieved through *in vitro* bioassay-directed mechanisms, many of which have proved very useful in predicting molecular disturbances and endpoints. In the category of these belongs the recombinant yeast transcription assay used in this study. Although AYAS has its short-comings, it is significantly sensitive and effective for determining the status of an environmental chemical in the midst of a complex mixture. However, it has been discovered that no single assay, especially *in vitro* assay, can be sufficient alone to determine the biochemical and molecular responses of genes to chemical interference/intervention. It is in the light of this that an *in vivo* bioassay method would be required to determine the full implications of this chemical binding on gene expression. Three-spined stickleback assay has been used as effective genetic sex biomarker for determining the effects of androgens and anti-androgens as endocrine disrupting agents (Katsiadaki et al., 2002). Stickleback has the potentials of surviving both in sea and freshwater environments. It can also exhibit distinctive reproductive

behaviours (nest-building and courtship), one of which is the production of glue protein (otherwise known as spiggin) used for quantifying the proportion of chemicals involved in endocrine disruption. It has also been reported that the high egg survival rate coupled with a very low fecundity are twin factors that have made it a better choice than other fish (*ibid.*). Above all, it has a very short life span which is necessary for comprehensive experimental studies. On that account, the *in vivo* stickleback androgen assay should be used to test the anti-androgenic compounds identified from the *in vitro* studies.

Following the discovery that the presence of both estrogens and anti-androgens has led to the formation of intersex in some fish species (Jobling et al., 2009), it is not clear whether either of them can induce this effect independent of another. Due to lack of clarity about separate individual chemical effect on this reproductive abnormality, the mixture effects of the anti-androgenic compounds identified in this study should be tested in order to understand the exact chemical conditions for which such biological anomaly would occur.

Following limitation of *in vitro* analytical approach and the uneconomical use of *in vivo* approach, for evaluating risk and toxicological effects of xenobiotics, a microarray analysis is a novel pre-clinical methodology and a high throughput approach for analysing simultaneous expression of virtually all known genes within a single analytical run. The broad spectrum of existing *in vivo* bioassays and biomarkers which are developed for evaluating chemical effects (through developmental and reproductive end-points) in wildlife and laboratory animals are short in providing a measurement of simultaneous multi-genetic end-points. Moreover, they are inadequate to predict long-term toxicological risk assessment for clinical tests and trials. Microarray analysis tends to provide a computational platform for toxicological measurements.

The result analysis in this study clearly indicated that about 2% androgen receptor antagonists contributing to the TAA of wastewater samples were recovered. The study further revealed that large proportion of AA yet to be recovered occurred in the polar and semi-polar regions of the profiles. In order for the most potent compounds responsible for androgen receptor antagonism in wastewater samples to be identified, further analysis must be directed at identifying polar anti-androgens in wastewaters.

5.6. Summary

This research work has accomplished all the objectives outlined in Chapter One. The examination and analysis of influent and effluent profiles of steroid-receptor anti-androgens, stated in objective 1, were carried out fully in Chapter Three (p.90) using the newly developed method and partly in Chapter Two (p. 54). The structural identification and measurement of steroid-like xenobiotic anti-androgens (Objective 2) was accomplished partly in Chapter Three (p.90) and Four (p.133). The Objective 3 which entails determination of relative potency of the steroid-like xenobiotic anti-androgens was fulfilled in Chapter Four (p. 133).

5.7. Conclusion

A wide range of home-use chemicals which possess binding ability to the extent of modulating steroid-receptor antagonism are present in wastewater effluents and influents as a result of human activities at both indoors and outdoors environments. Given that domestic wastewaters are centrally collected for treatment at WwTPs, and the discovery that anti-androgenic chemical removal at WwTPs are partial, the receiving environments of effluents are contaminated on continuous basis. Due to their physicochemical properties, they undergo biotransformation in the environment and induce some biological effects in aquatic animals. The concept of recycling wastewaters as an alternative source of drinking and irrigation water has to be re-evaluated following partial removal of potential demasculinising chemicals at wastewater treatment works. There is a need for developing and improving clean-up technologies that would eliminate a wide range of biologically active environmental chemicals present in treated wastewaters that may pose a threat to human and wildlife health.

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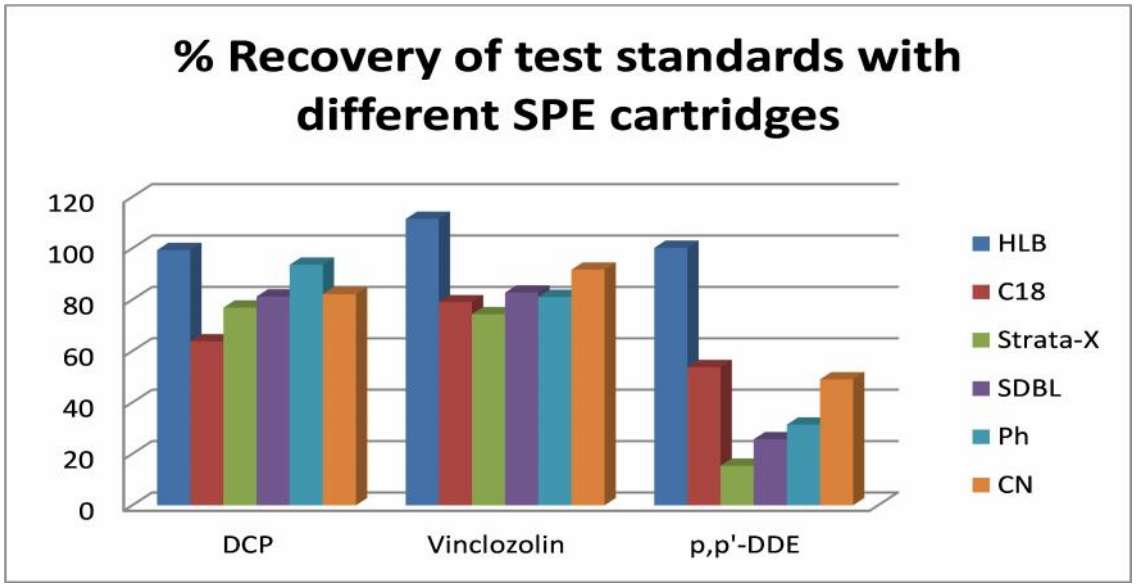
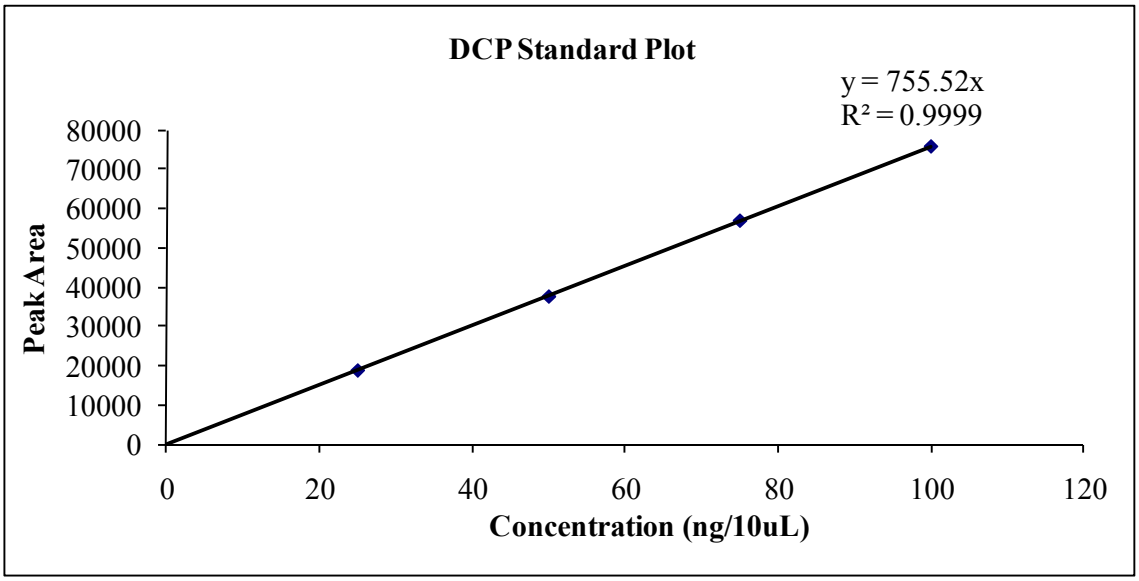
APPENDICES

APPENDIX A: Percentage recovery of test standards comprising dichlorophene (DCP), vinclozolin (Vz) and p,p-DDE on different SPE cartridges and recovery plots of some test compounds on HLB cartridges.

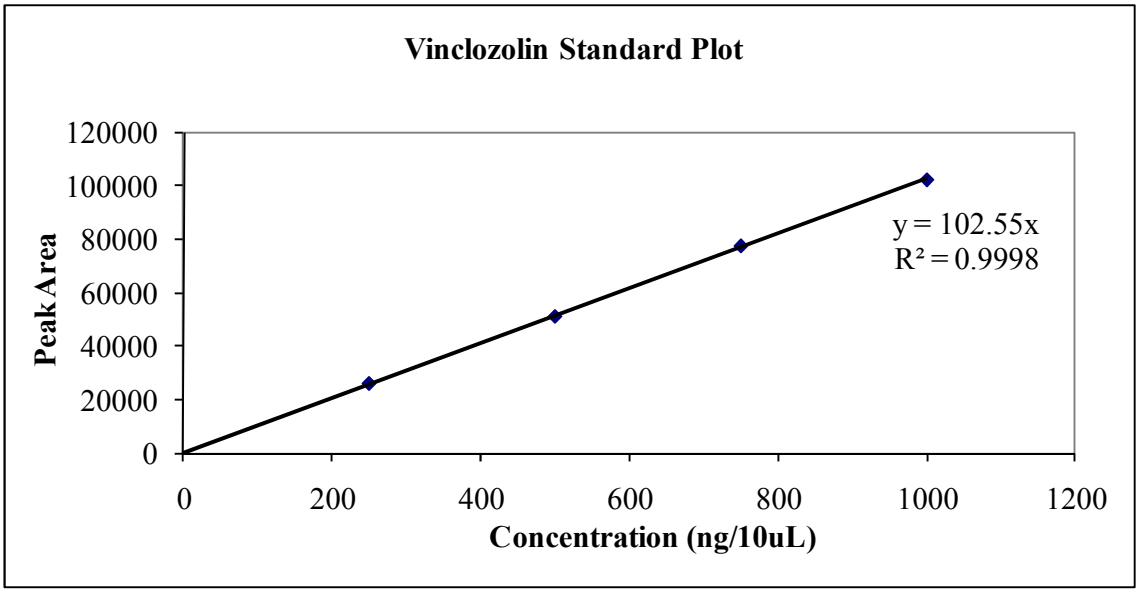
Appendix A.1: Showing % recovery of test standards with different SPE cartridges.



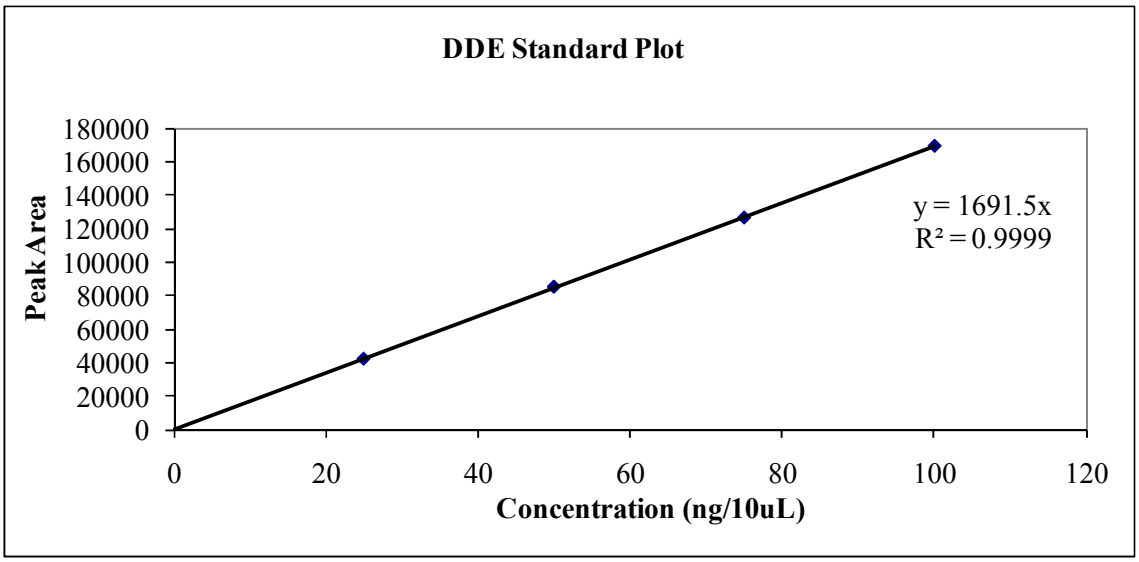
Appendix A.2: Recovery plot of dichlorophene (DCP) standard on HPLC.



Appendix A.3: Recovery plot of vinclozolin standard on HPLC



Appendix A.4: Recovery plot of p,p-DDE standard on HPLC

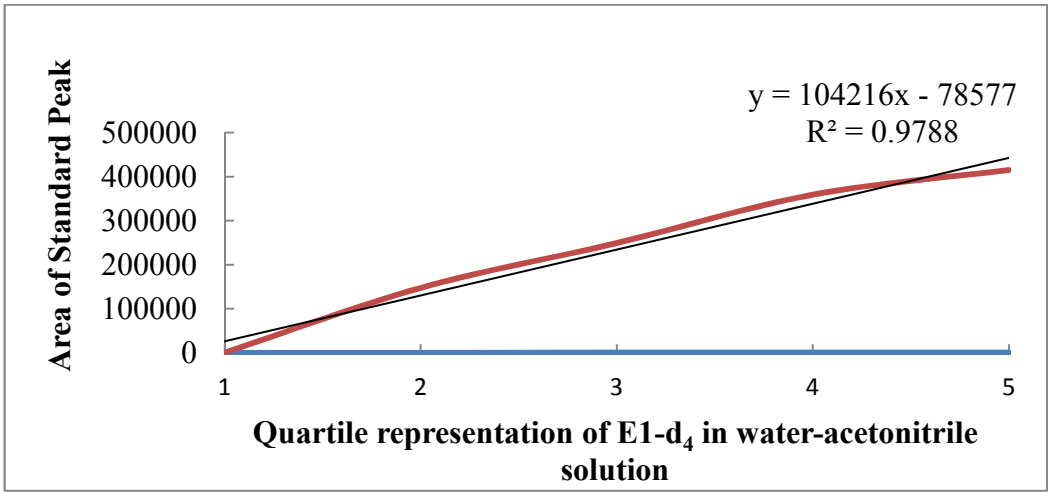


Appendix B: The projection of standard loss in acetonitrile-water solution when evaporated using speed vacuum concentrator during sample derivatisation for GC-MS analysis.

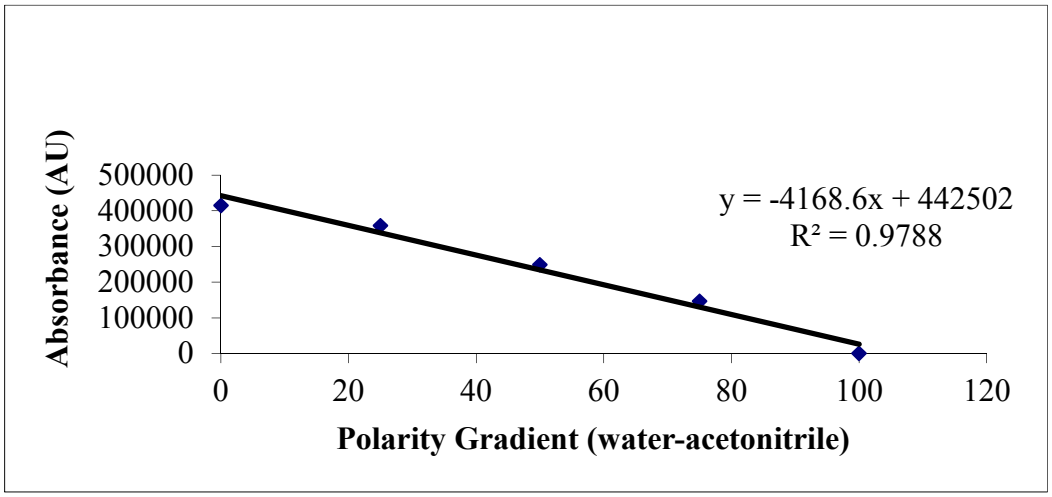
Appendix B.1: Recovery of standard in water-acetonitrile gradient injection

% Water	Corresponding Area	Mass of E1-d_4
100	0	1.50
75	147177	2.25
50	249063	3.00
25	358892	4.50
0	415221	6.00

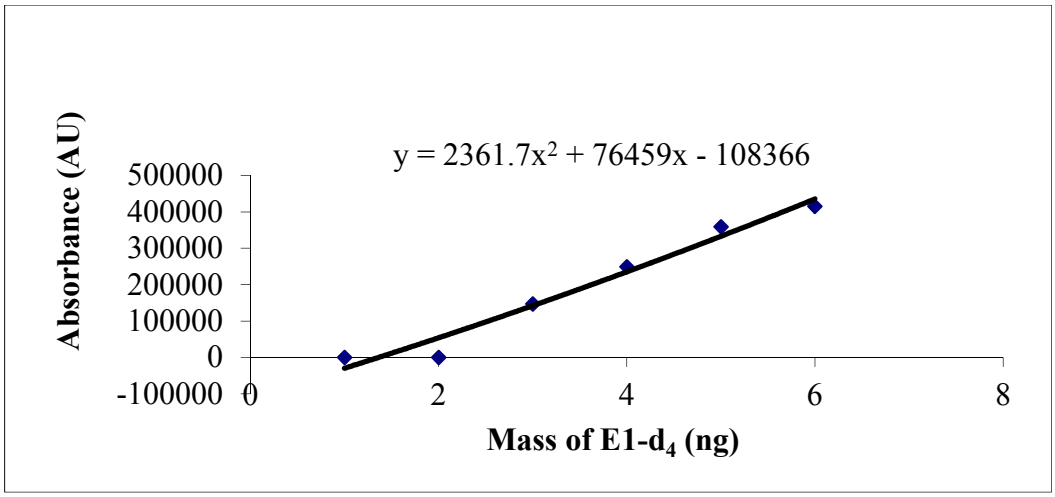
Appendix B.2: Projection of standard loss during work-up as estimated on the HPLC



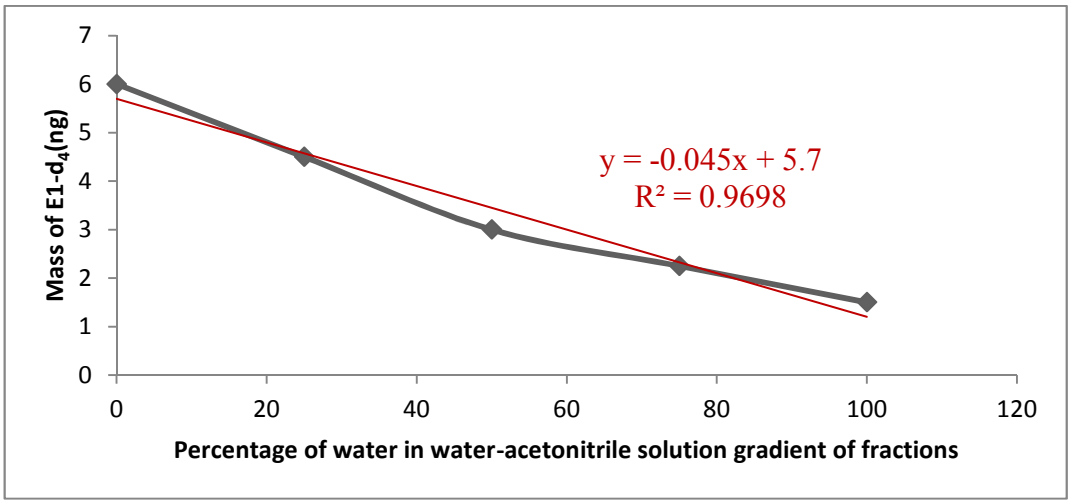
Appendix B.3: Estimation of sample concentration as influenced by polarity factor.



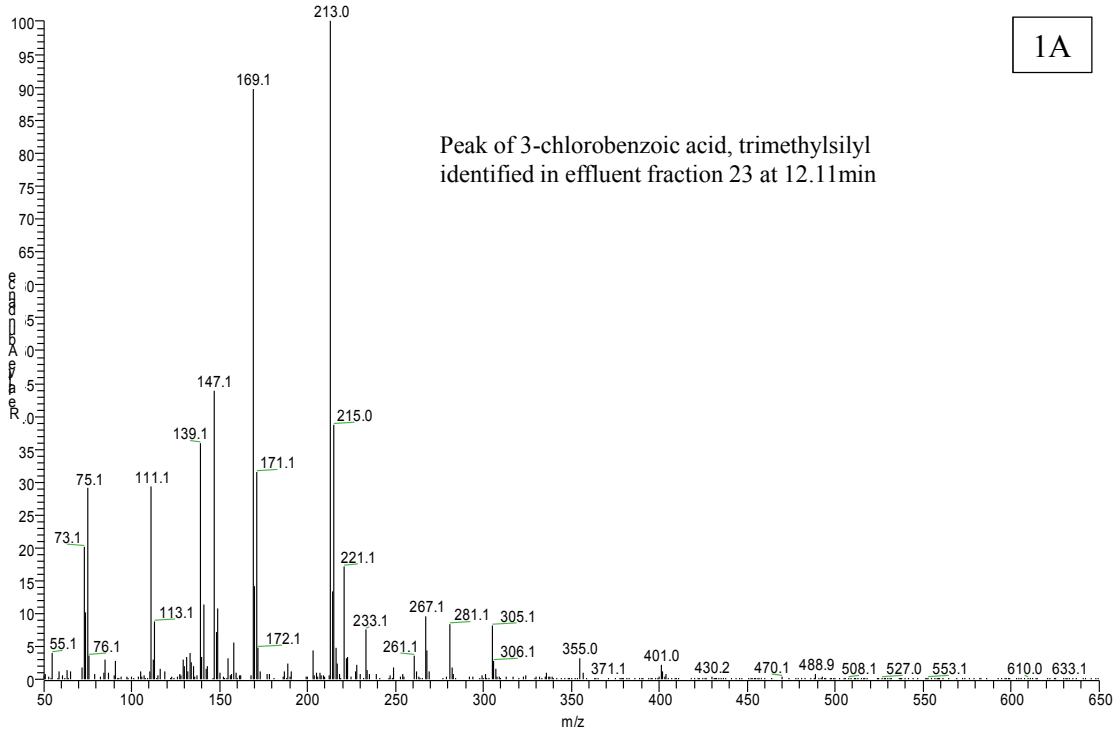
Appendix B.4: Mass of E1-d_4 recovered when the solution ranged from 0-100% acetonitrile (or 100-0% water).

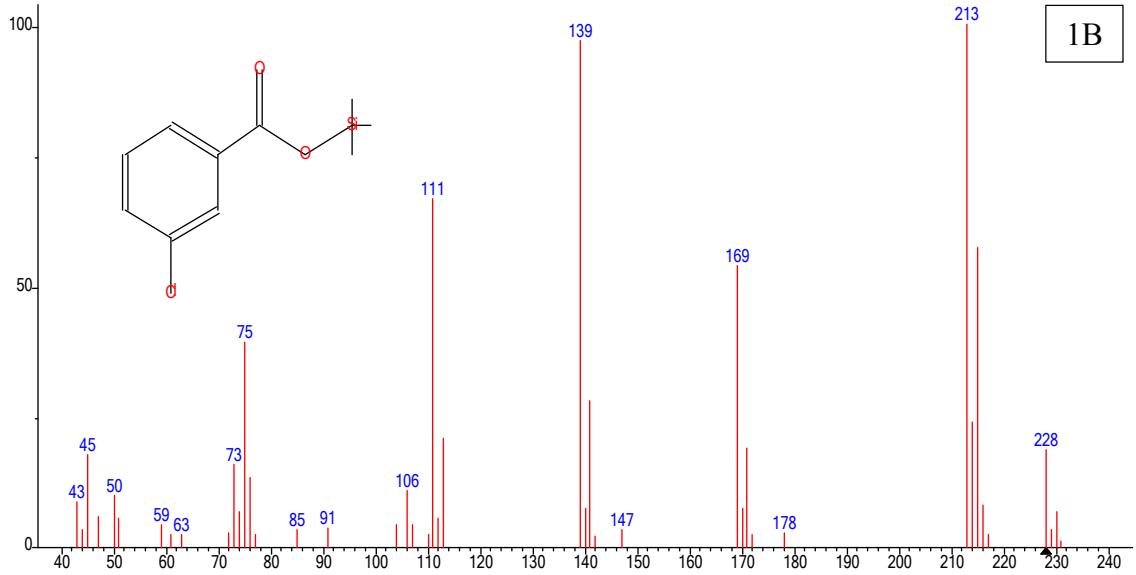


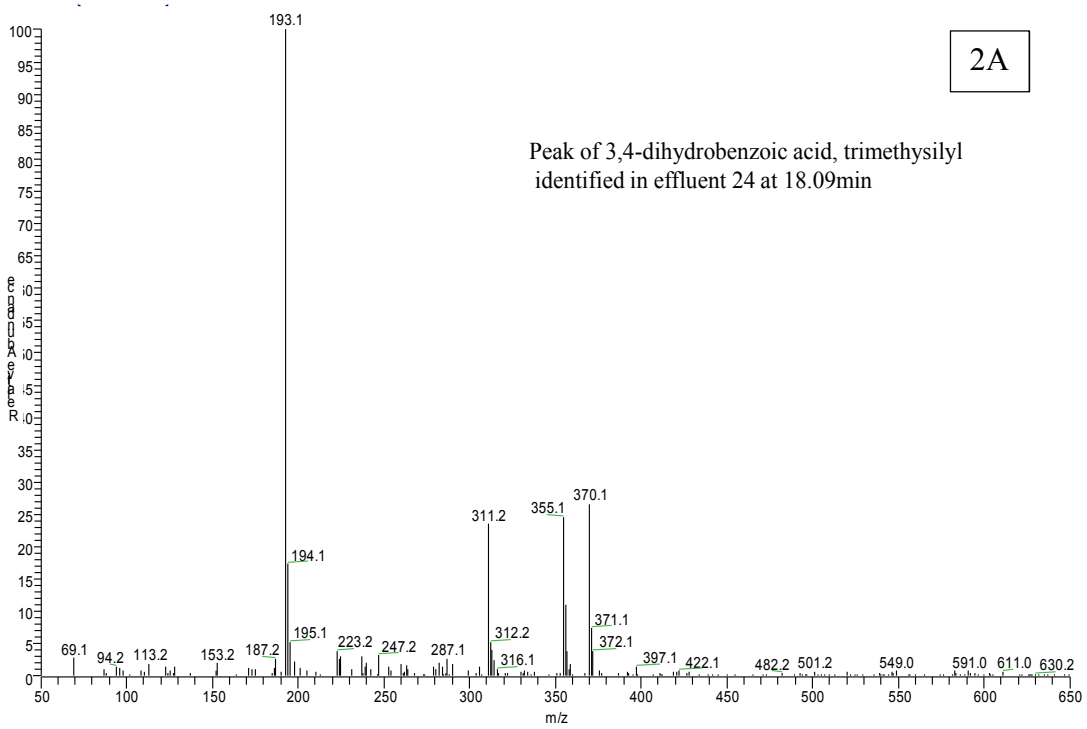
Appendix B.5: Projection of E1-d_4 recovery on GC-MS as the percentage of water in the sample fractions increases.

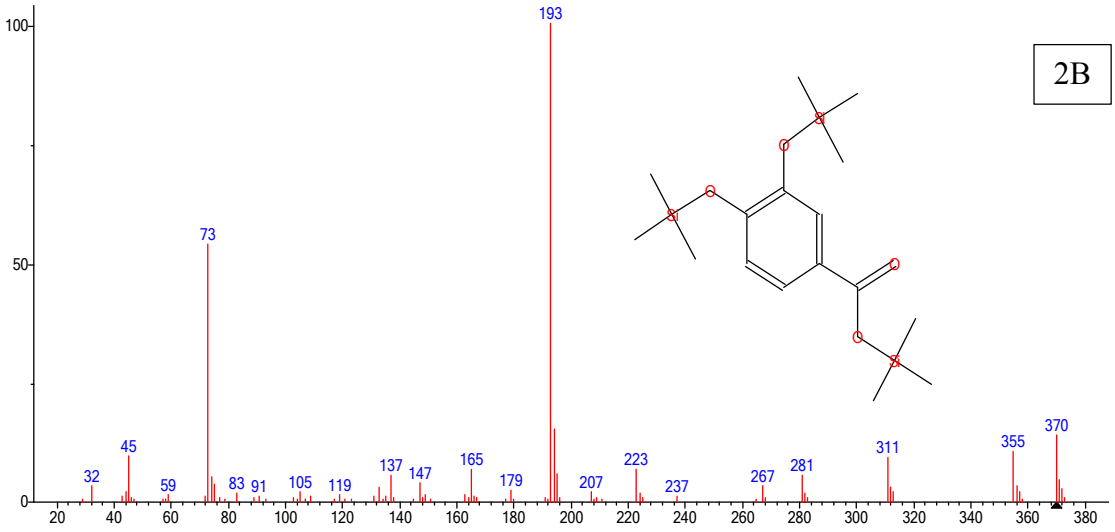


Appendix C: NIST and sample chromatograms of compounds identified in effluent and influent fractions. The chromatogram pair is labelled A and B.



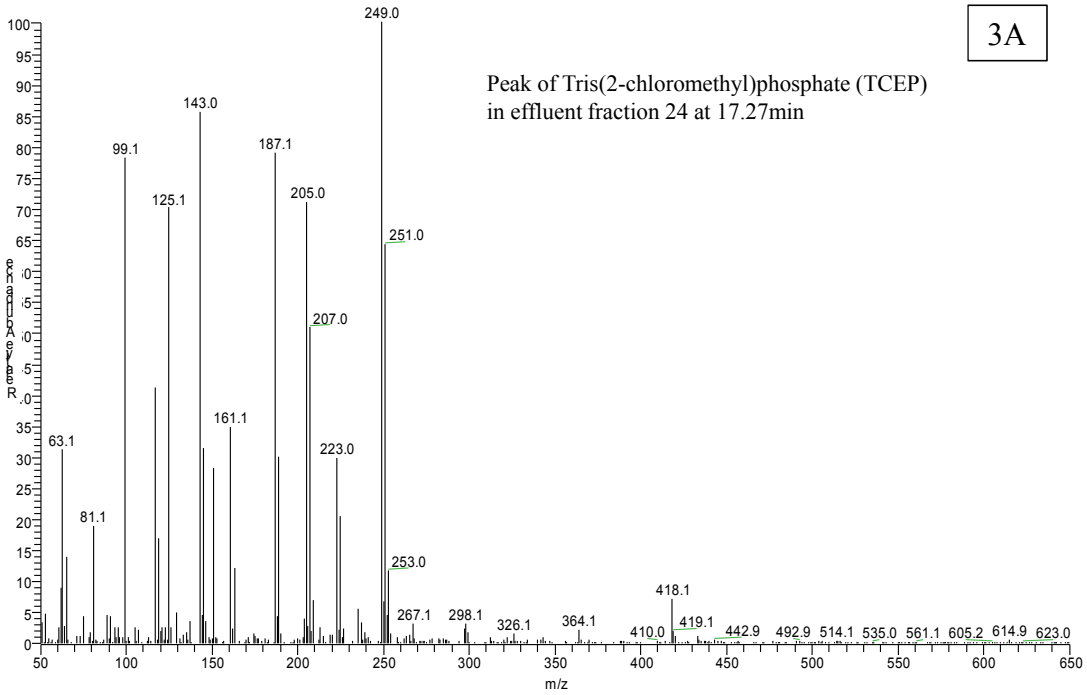



2A
Peak of 3,4-dihydrobenzoic acid, trimethysilyl identified in effluent 24 at 18.09min
193.1
194.1
195.1
311.2
312.2
316.1
355.1
370.1
371.1
372.1
69.1
94.2
113.2
153.2
187.2
223.2
247.2
287.1
397.1
422.1
482.2
501.2
549.0
591.0
611.0
630.2
m/z

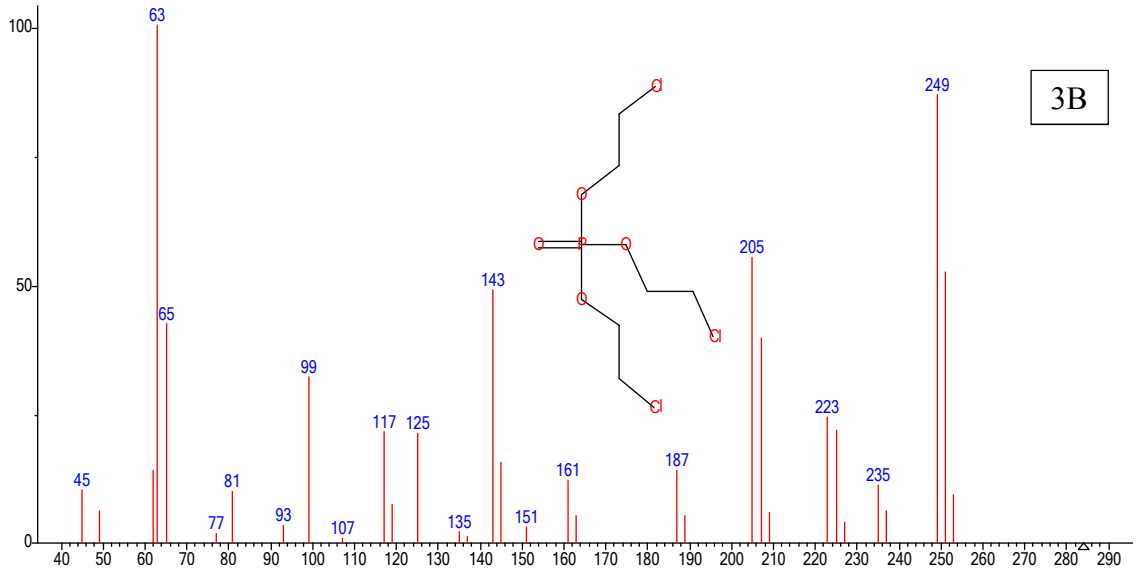

2B
193
73
45
32
59
83
91
105
119
137
147
165
179
207
223
237
267
281
311
355
370

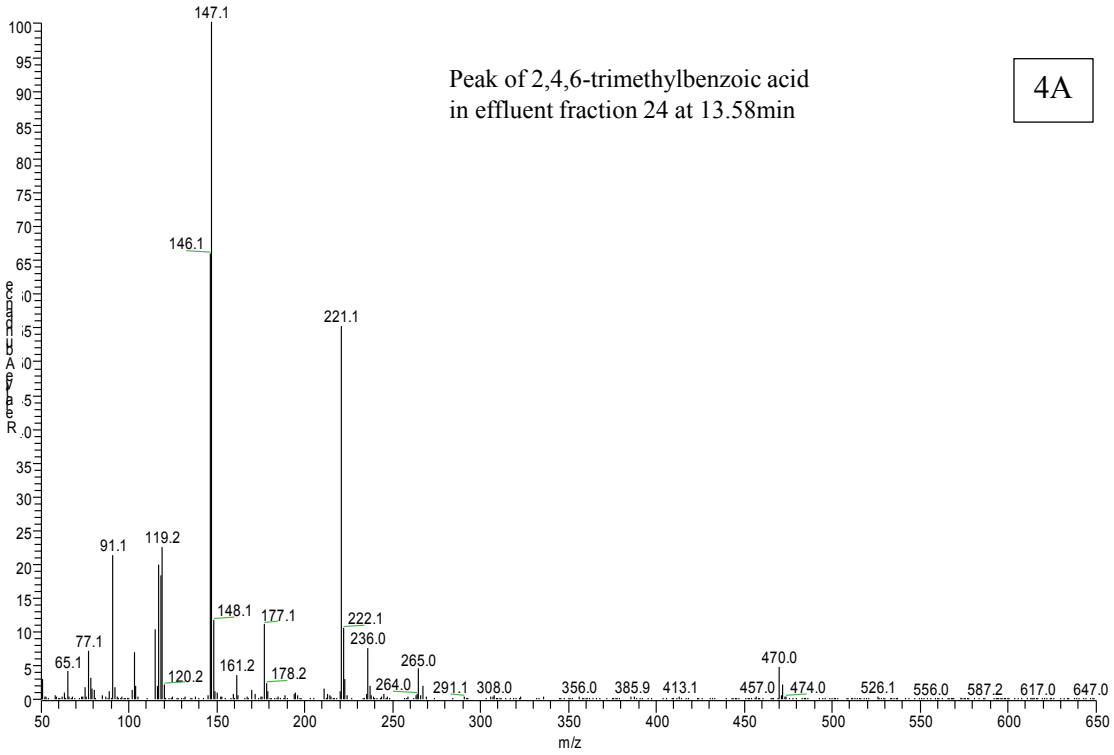
3A

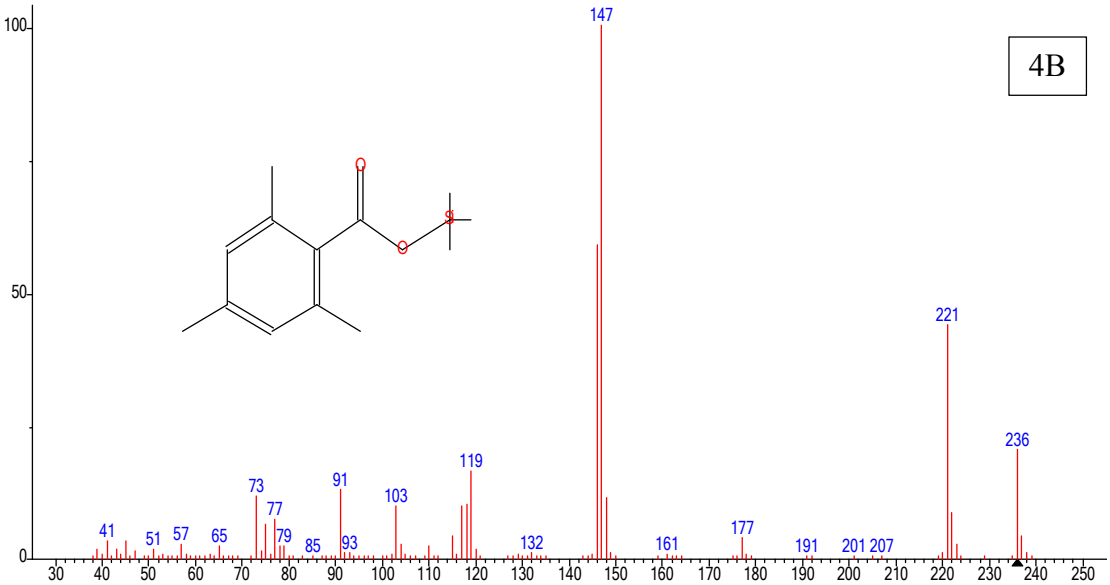
Peak of Tris(2-chloromethyl)phosphate (TCEP)
in effluent fraction 24 at 17.27min

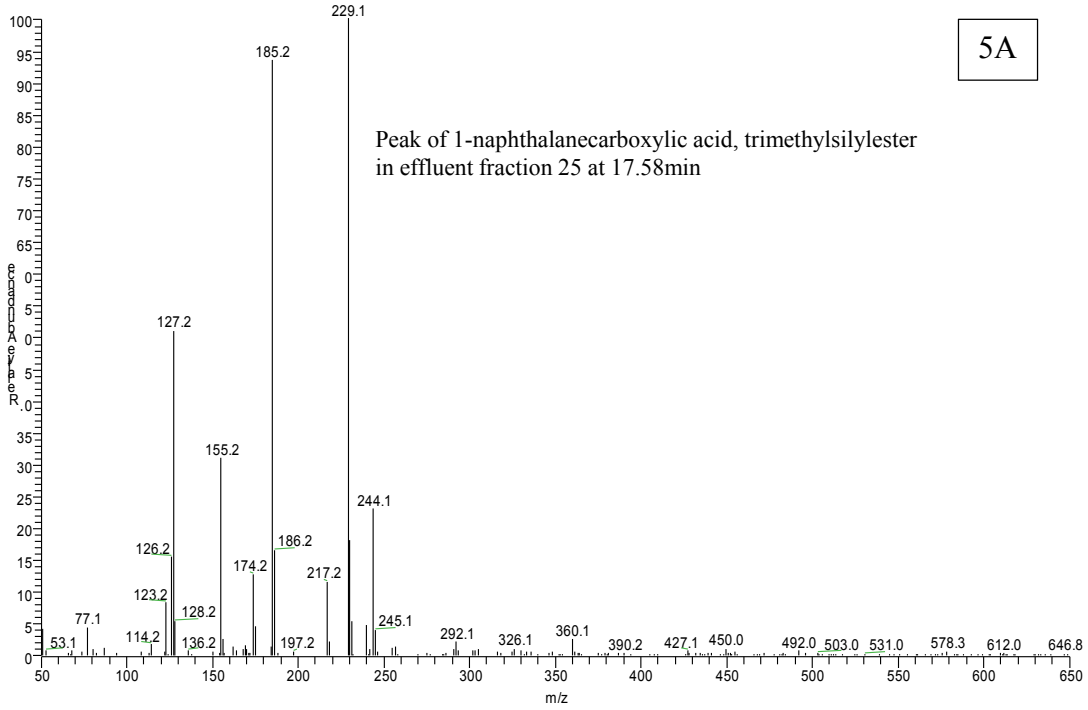


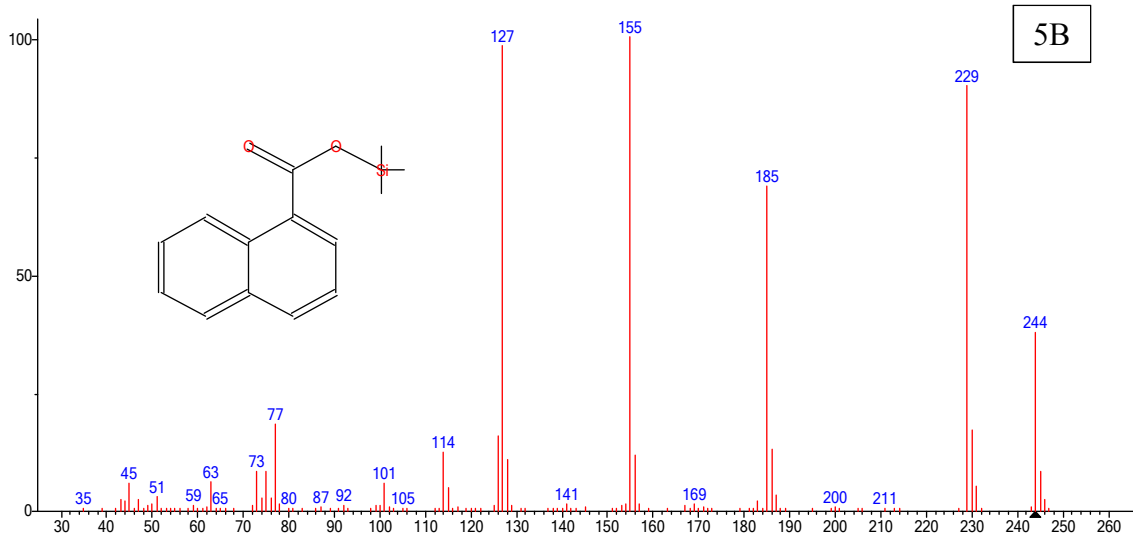
3B

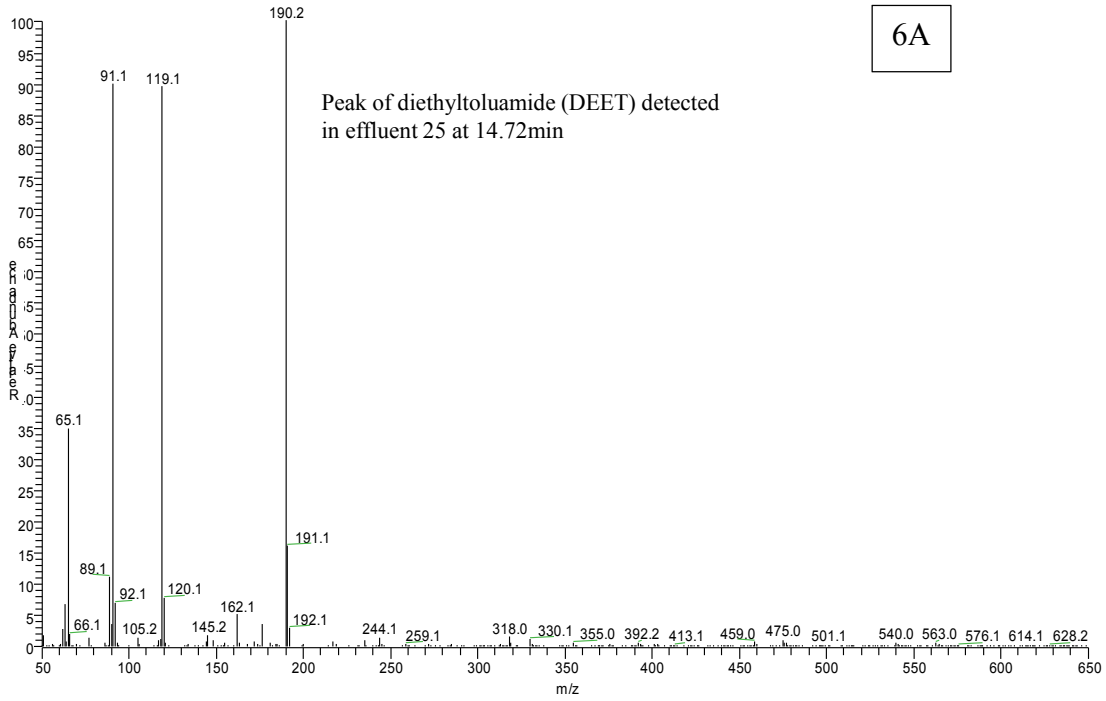


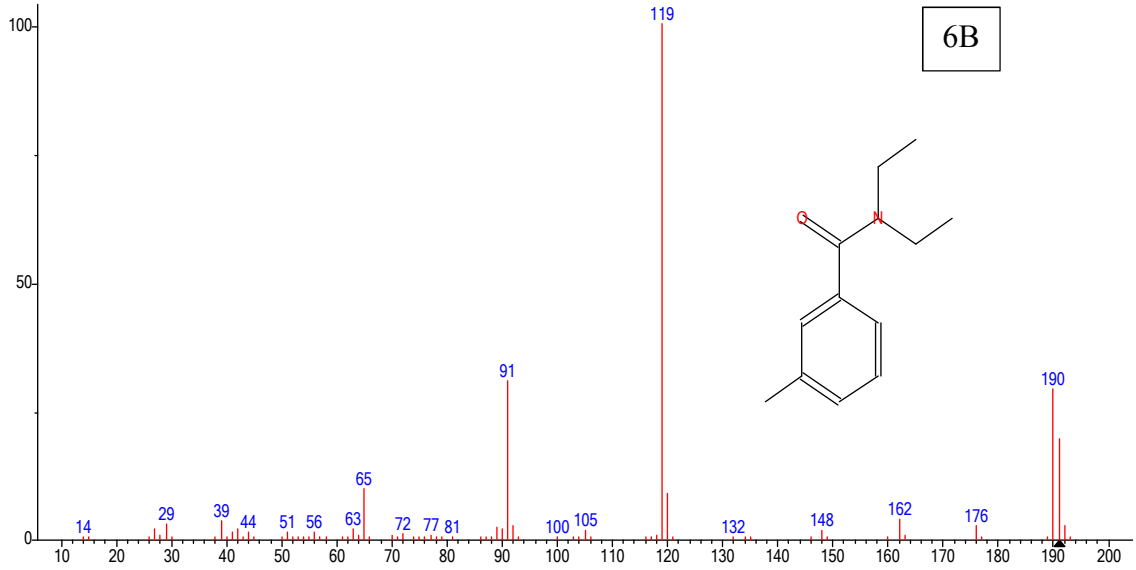

Peak of 2,4,6-trimethylbenzoic acid
in effluent fraction 24 at 13.58min
4A
Abundance
m/z
147.1
146.1
221.1
91.1
119.2
148.1
177.1
222.1
236.0
77.1
65.1
120.2
161.2
178.2
265.0
264.0
291.1
308.0
356.0
385.9
413.1
457.0
470.0
474.0
526.1
556.0
587.2
617.0
647.0

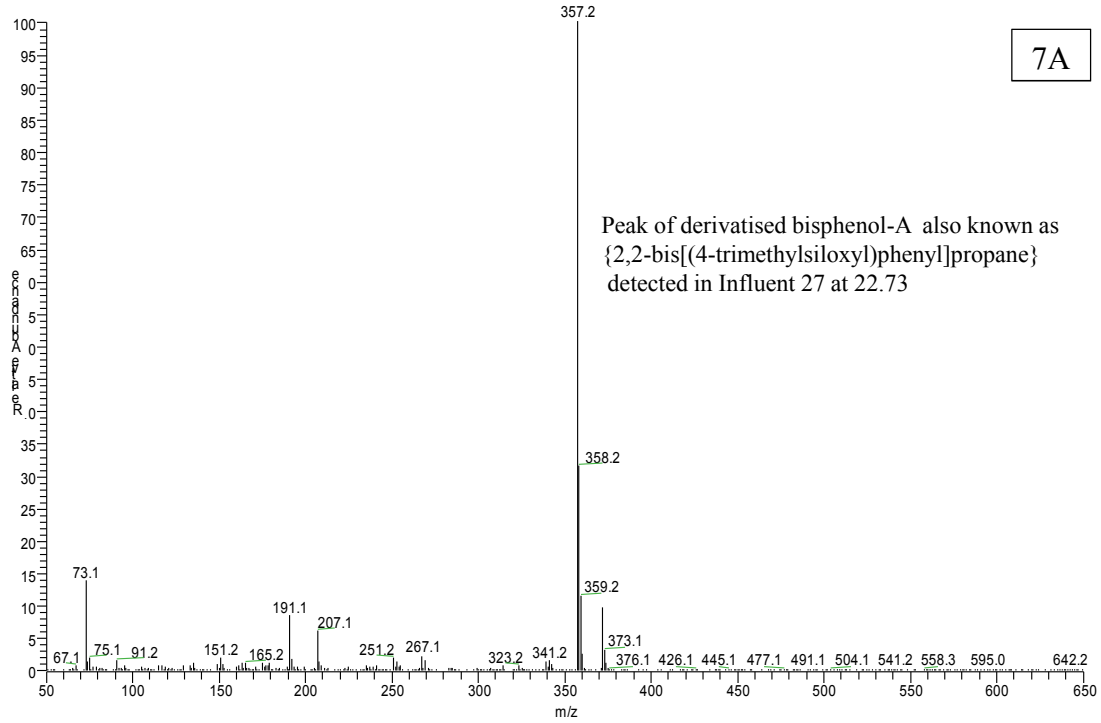

4B
147
221
236
119
73
91
77
103
41
51
57
65
79
85
93
132
161
177
191
201
207

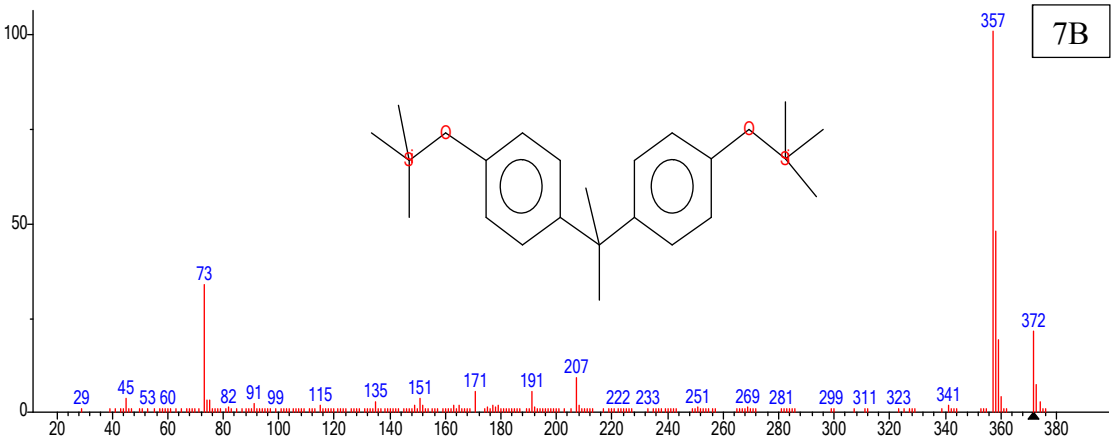

5A
Peak of 1-naphthalanecarboxylic acid, trimethylsilylester
in effluent fraction 25 at 17.58min
Abundance
m/z
229.1
185.2
127.2
155.2
244.1
186.2
126.2
174.2
217.2
123.2
128.2
77.1
245.1
53.1
114.2
136.2
197.2
292.1
326.1
360.1
390.2
427.1
450.0
492.0
503.0
531.0
578.3
612.0
646.8

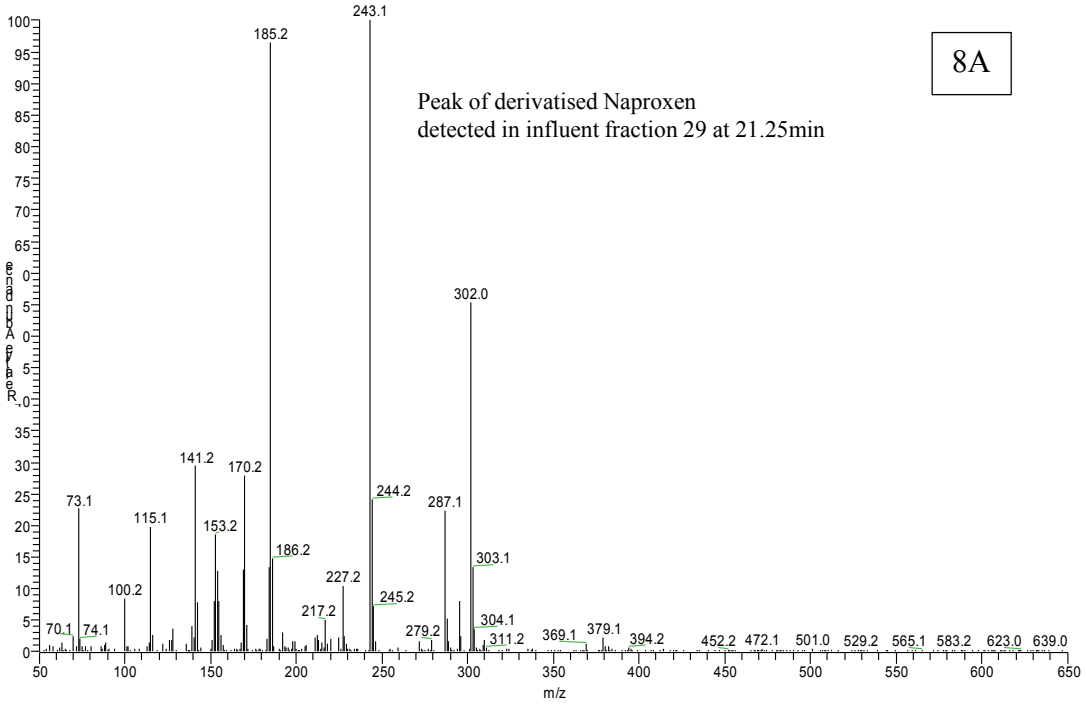

5B
127
155
229
185
244
77
114
73
45
63
51
59
65
80
87
92
101
105
141
169
200
211
35
Si

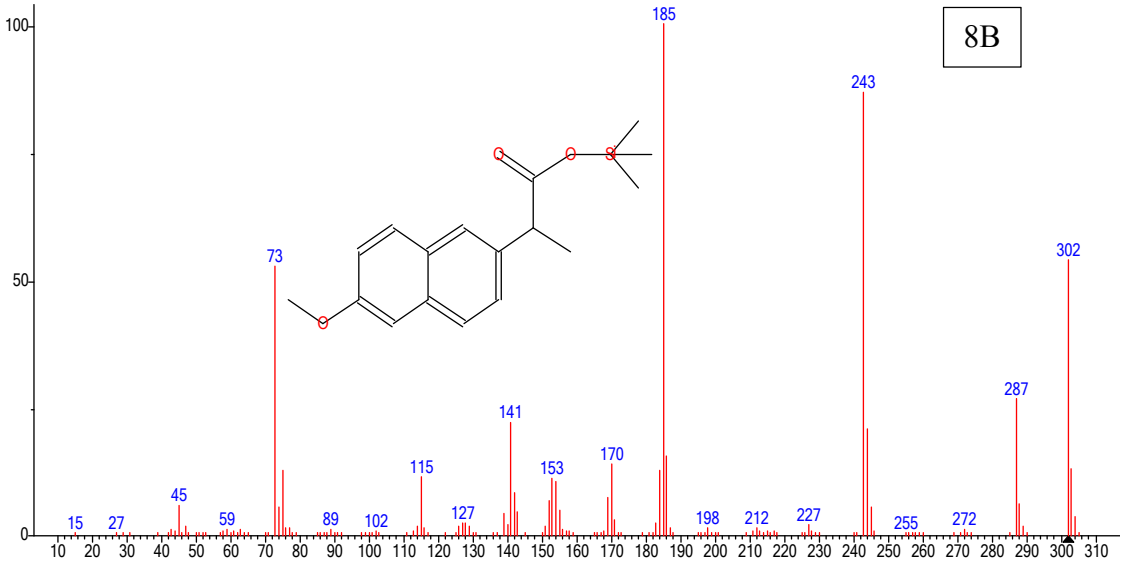

6A
Peak of diethyltoluamide (DEET) detected
in effluent 25 at 14.72min
Abundance
m/z
190.2
91.1
119.1
65.1
191.1
89.1
92.1
120.1
162.1
66.1
105.2
145.2
192.1
244.1
259.1
318.0
330.1
355.0
392.2
413.1
459.0
475.0
501.1
540.0
563.0
576.1
614.1
628.2

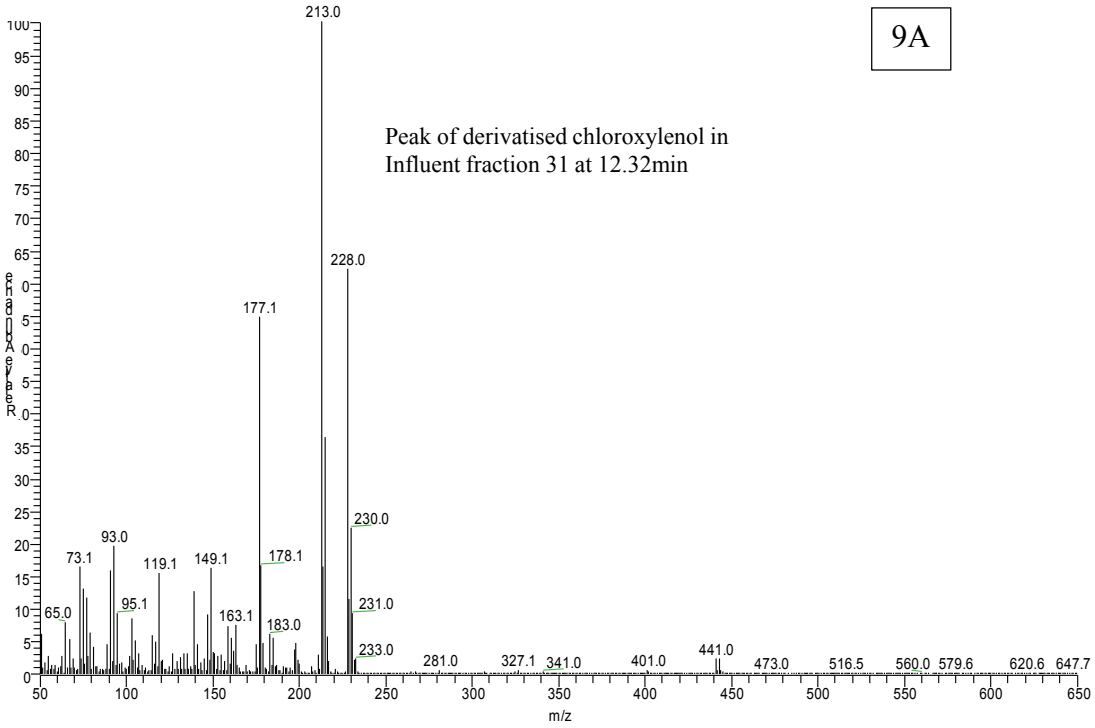

6B
119
91
190
65
14
29
39
44
51
56
63
72
77
81
100
105
132
148
162
176
O
N


7A
Peak of derivatised bisphenol-A also known as {2,2-bis[(4-trimethylsiloxyl)phenyl]propane} detected in Influent 27 at 22.73
Abundance
m/z
357.2
358.2
359.2
73.1
191.1
207.1
373.1

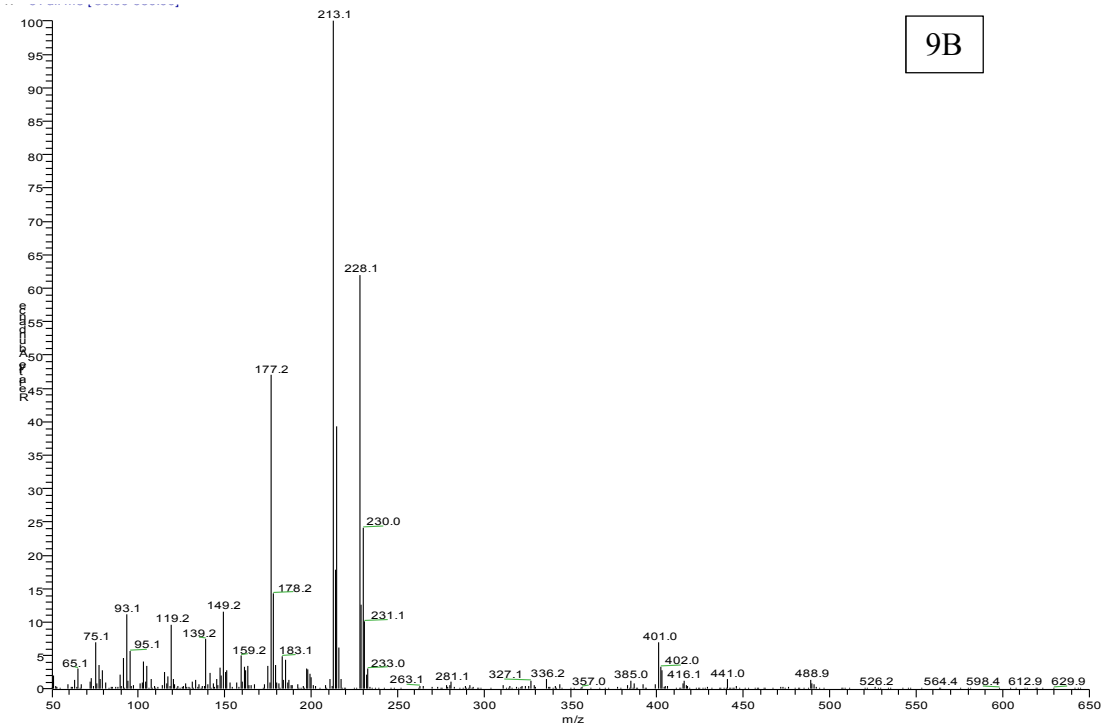

7B
357
372
73
207
191
171
151
135
115
99
91
82
53
45
29
222
233
251
269
281
299
311
323
341

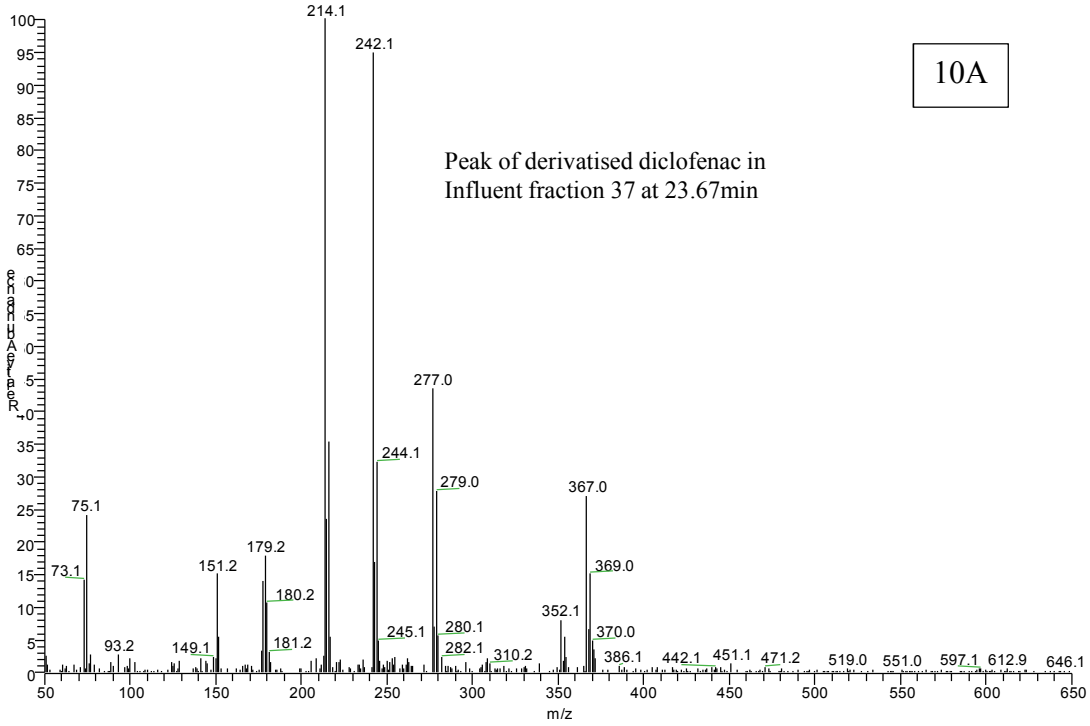

8A
Peak of derivatised Naproxen
detected in influent fraction 29 at 21.25min
Abundance
m/z
243.1
185.2
302.0
141.2
170.2
73.1
244.2
287.1
115.1
153.2
186.2
303.1
227.2
100.2
245.2
217.2
304.1
279.2
311.2
70.1
74.1
369.1
379.1
394.2
452.2
472.1
501.0
529.2
565.1
583.2
623.0
639.0

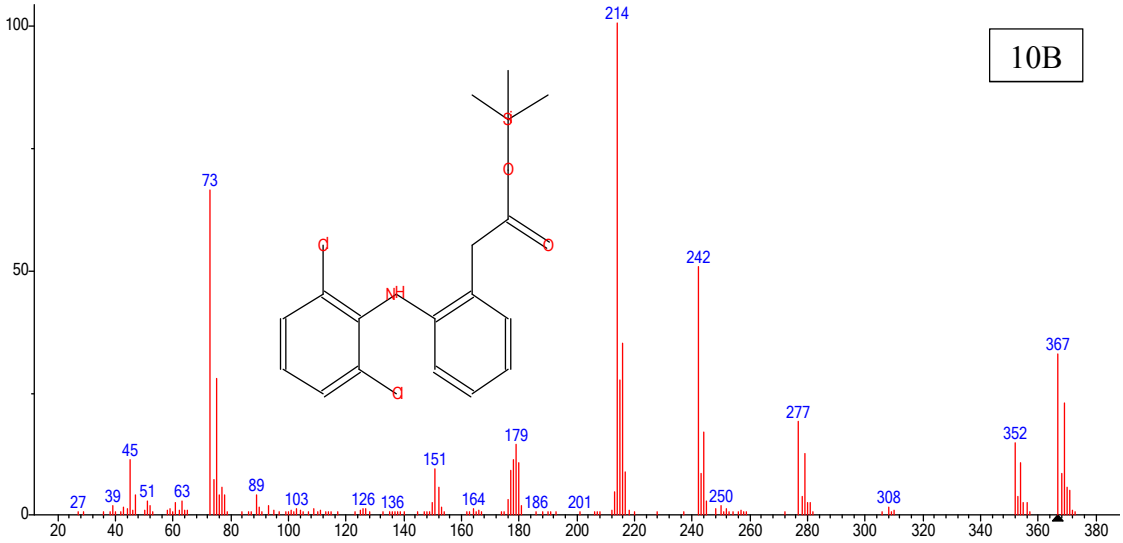

8B
185
243
73
302
287
141
115
153
170
45
15
27
59
89
102
127
198
212
227
255
272

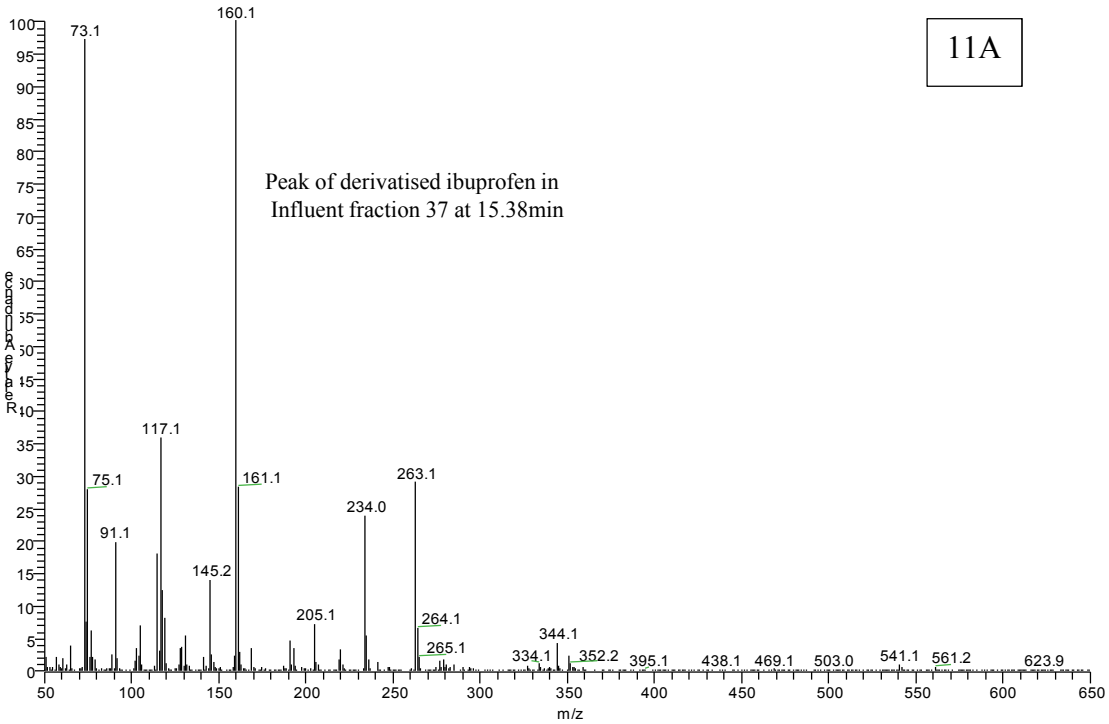


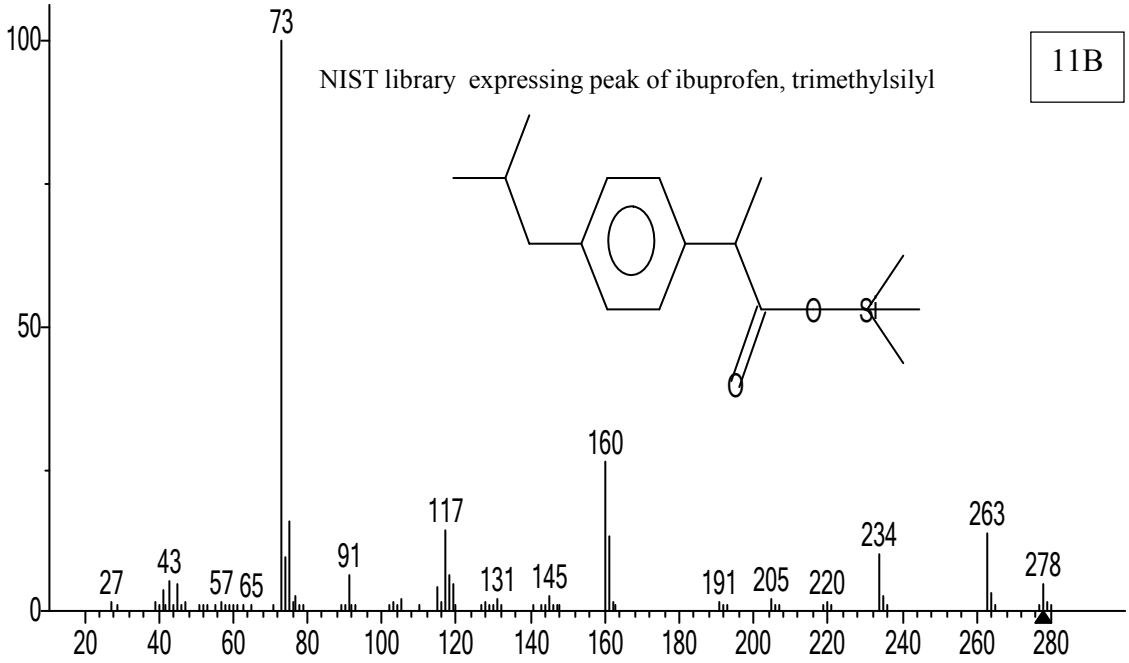
Laboratory standard of derivatised chloroxylenol

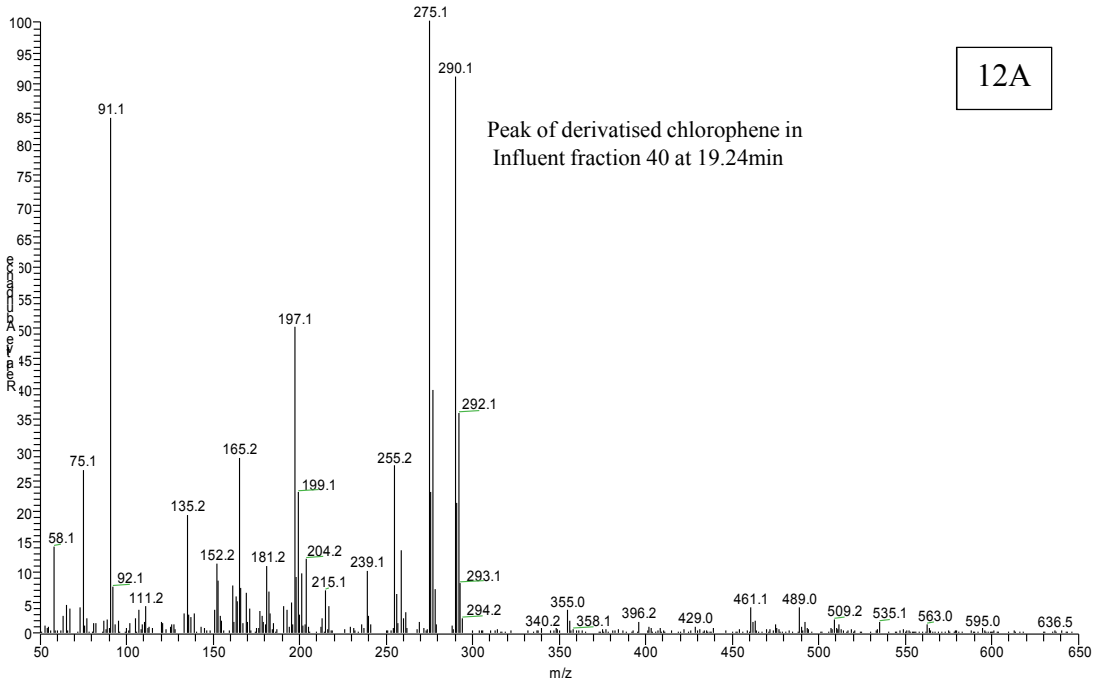



10A
Peak of derivatised diclofenac in
Influent fraction 37 at 23.67min
214.1
242.1
277.0
244.1
279.0
367.0
75.1
73.1
179.2
151.2
180.2
369.0
352.1
245.1
280.1
370.0
93.2
149.1
181.2
282.1
310.2
386.1
442.1
451.1
471.2
519.0
551.0
597.1
612.9
646.1
m/z

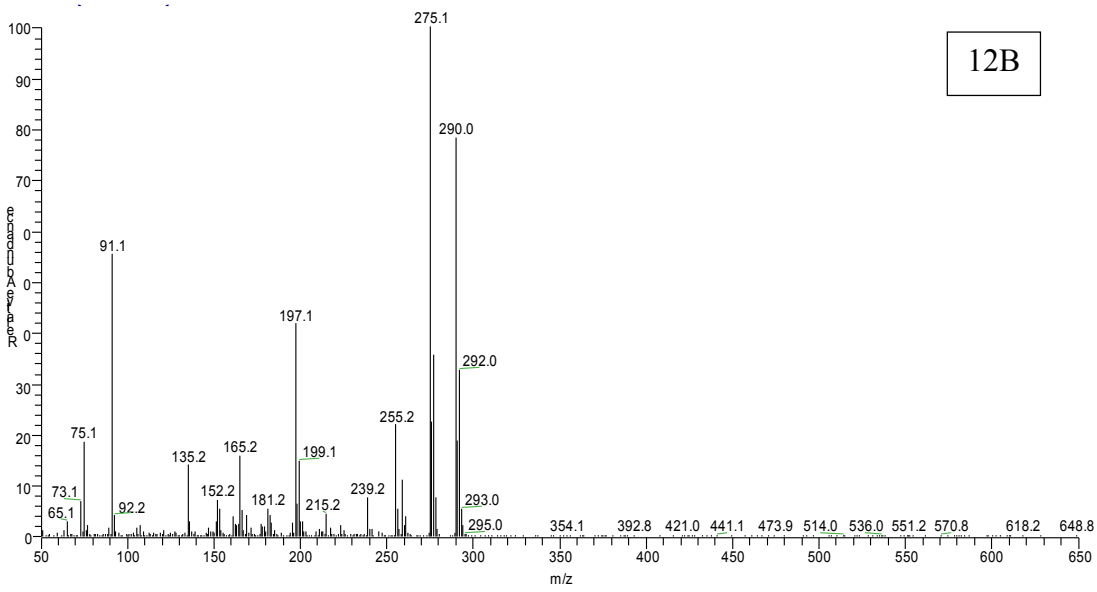

10B
214
73
242
367
277
45
179
151
352
27
39
51
63
89
103
126
136
164
186
201
250
308
Si
O
O
Cl
NH
Cl
m/z


11A
Peak of derivatised ibuprofen in
Influent fraction 37 at 15.38min
73.1
160.1
117.1
75.1
161.1
263.1
234.0
91.1
145.2
205.1
264.1
265.1
344.1
334.1
352.2
395.1
438.1
469.1
503.0
541.1
561.2
623.9
m/z

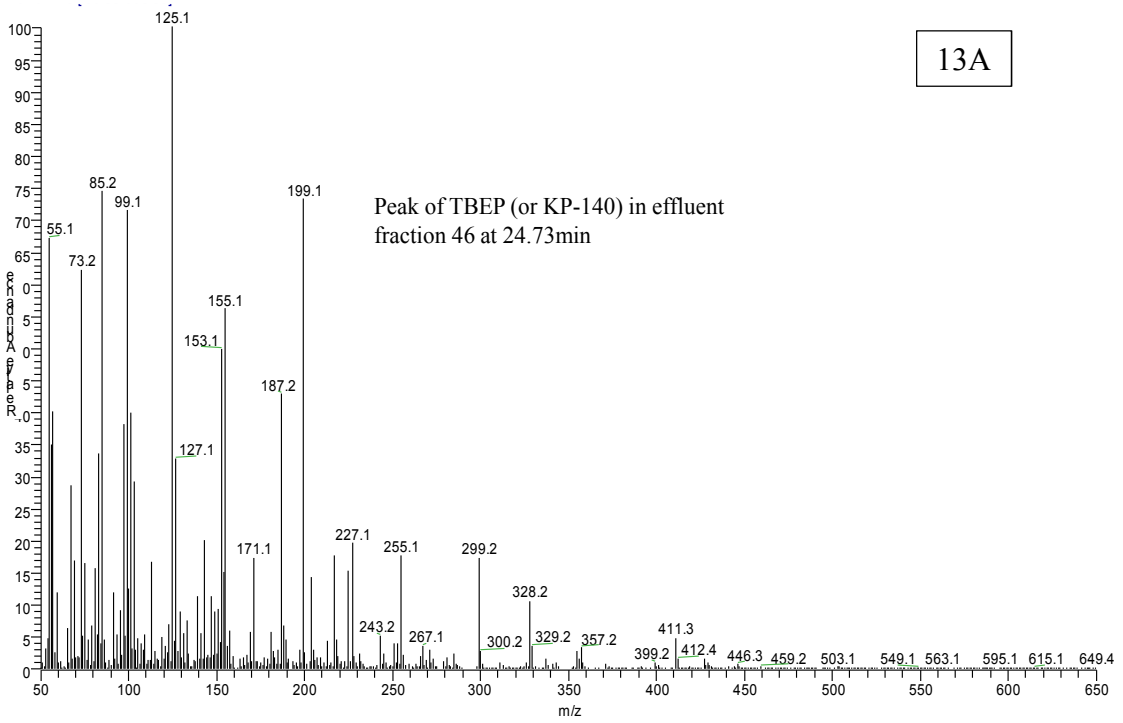

11B
NIST library expressing peak of ibuprofen, trimethylsilyl
73
160
117
91
43
27
57
65
131
145
191
205
220
234
263
278



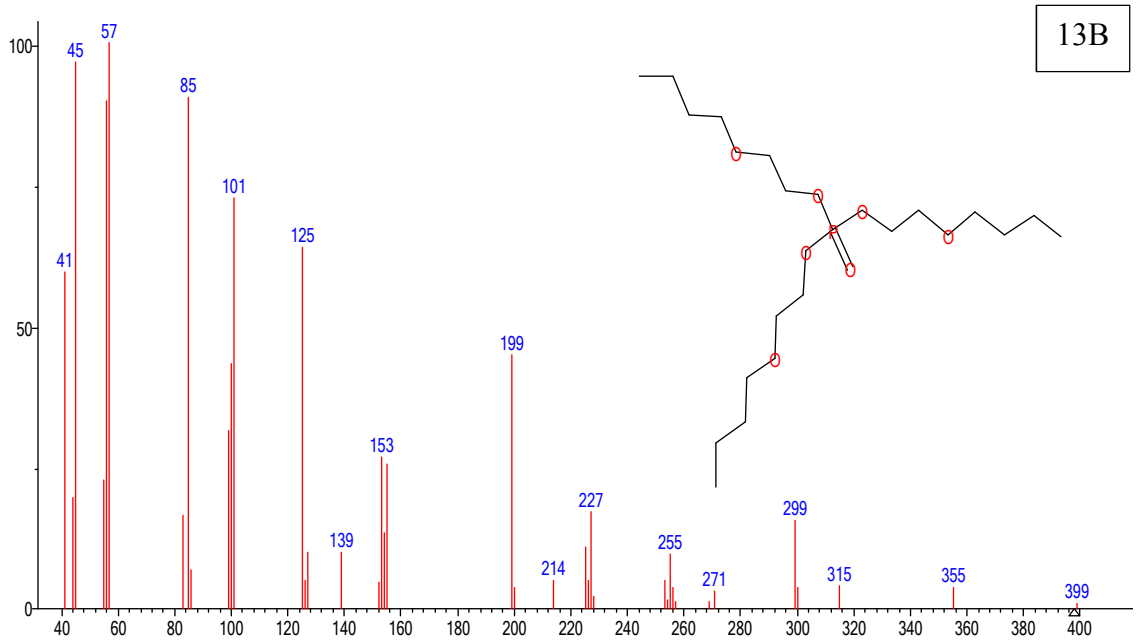
Laboratory standard of derivatised chlorophene



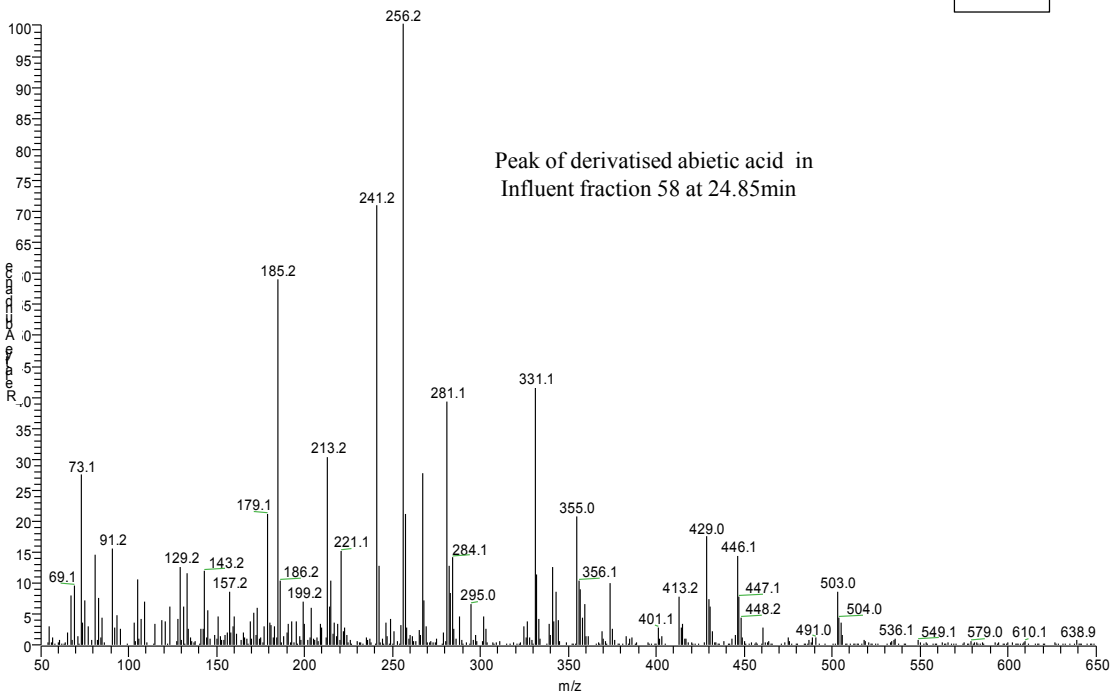
13A


Peak of TBEP (or KP-140) in effluent
fraction 46 at 24.73min
Abundance
m/z
125.1
199.1
85.2
99.1
55.1
73.2
155.1
153.1
187.2
127.1
227.1
171.1
255.1
299.2
328.2
243.2
267.1
300.2
329.2
357.2
411.3
399.2
412.4
446.3
459.2
503.1
549.1
563.1
595.1
615.1
649.4

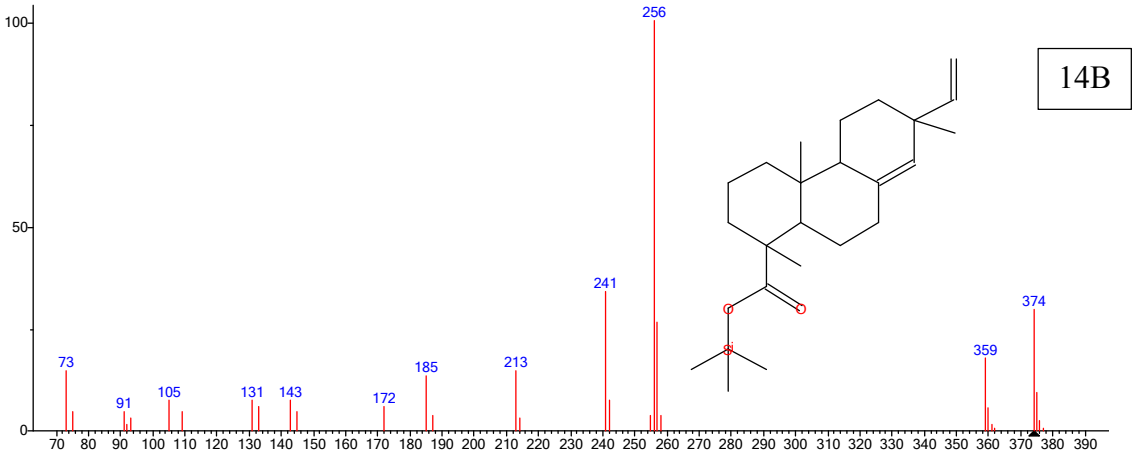
13B

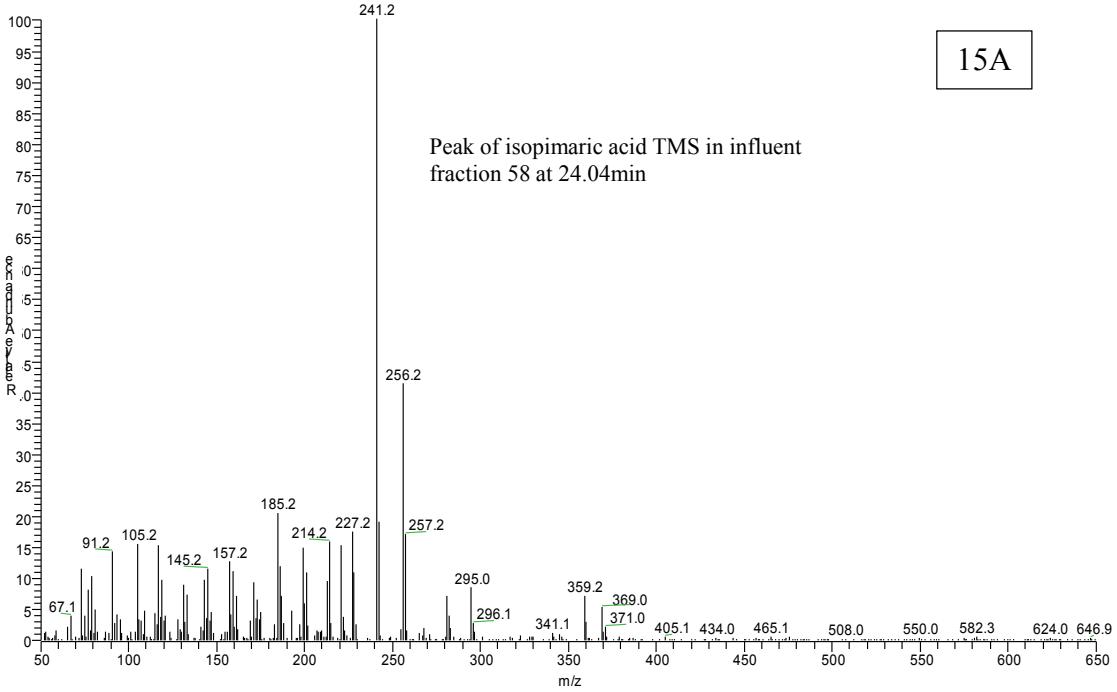

57
45
85
101
125
41
199
153
227
139
214
255
271
299
315
355
399

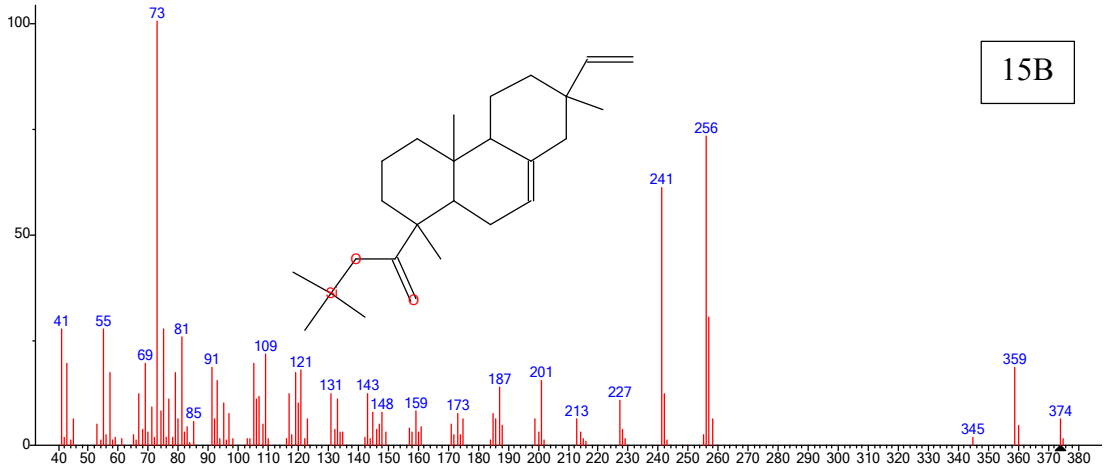
14A


Peak of derivatised abietic acid in
Influent fraction 58 at 24.85min
Abundance
m/z
256.2
241.2
185.2
331.1
281.1
213.2
73.1
179.1
355.0
91.2
221.1
284.1
429.0
446.1
129.2
143.2
186.2
356.1
69.1
157.2
199.2
295.0
413.2
447.1
503.0
401.1
448.2
504.0
491.0
536.1
549.1
579.0
610.1
638.9

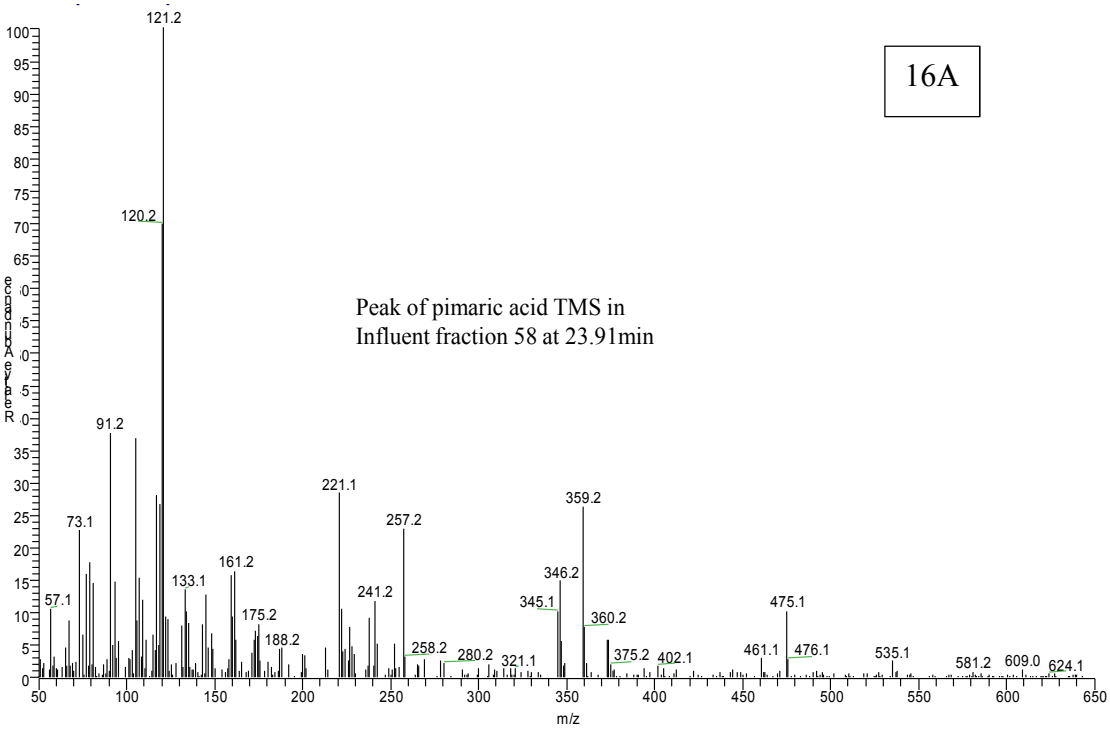
14B


256
241
374
73
359
185
213
105
131
143
91
172

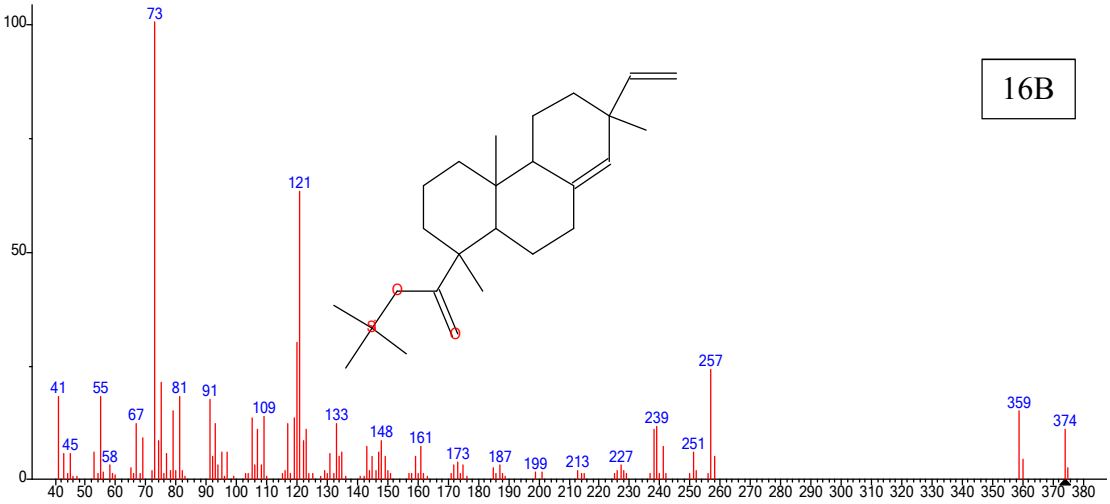

15A
Peak of isopimaric acid TMS in influent fraction 58 at 24.04min
Abundance
m/z
241.2
256.2
185.2
214.2
227.2
257.2
91.2
105.2
145.2
157.2
295.0
359.2
369.0
371.0
67.1
296.1
341.1
405.1
434.0
465.1
508.0
550.0
582.3
624.0
646.9

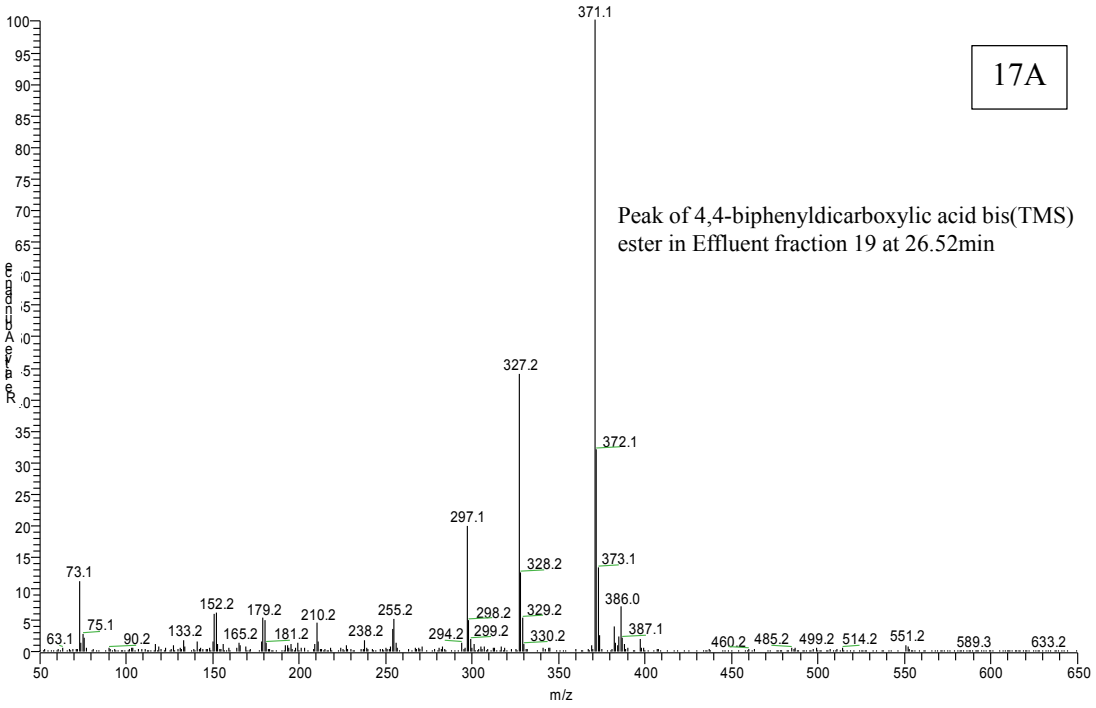

15B
73
256
241
41
55
81
69
91
109
121
131
143
148
159
173
187
201
213
227
345
359
374

16A


Peak of pimaric acid TMS in
Influent fraction 58 at 23.91min
m/z

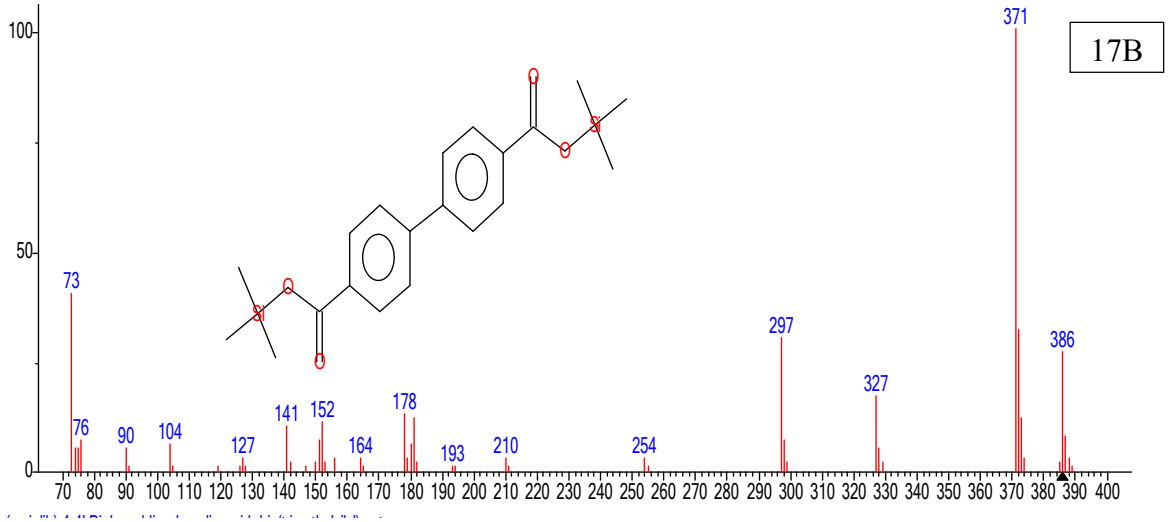
16B



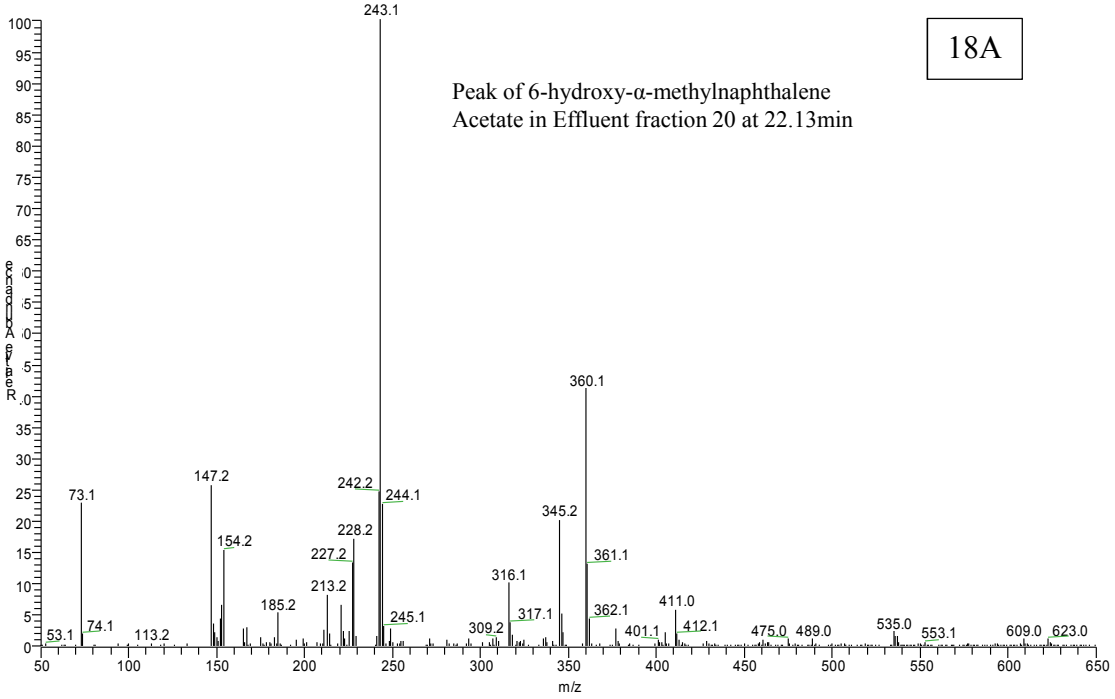


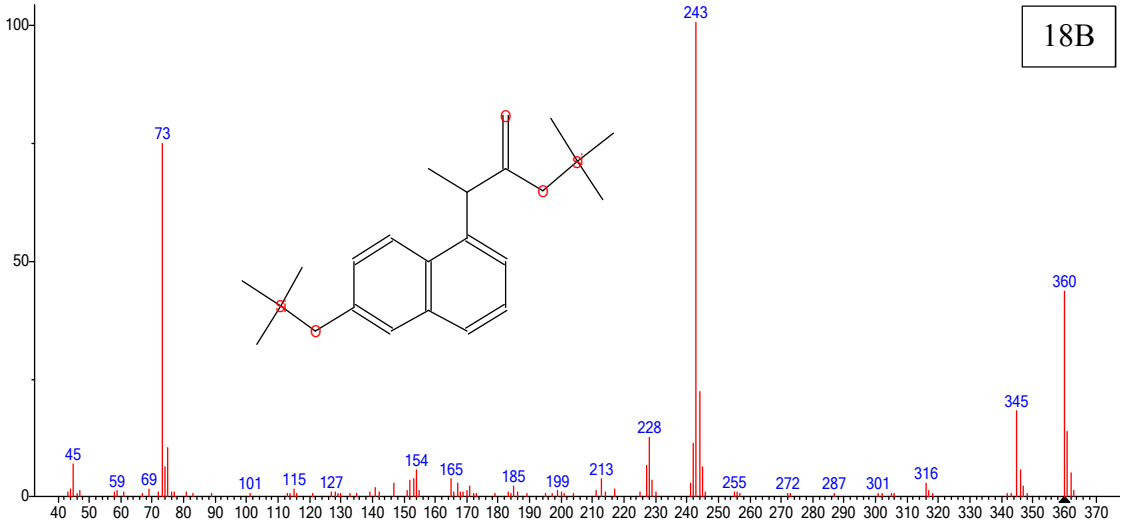
17A

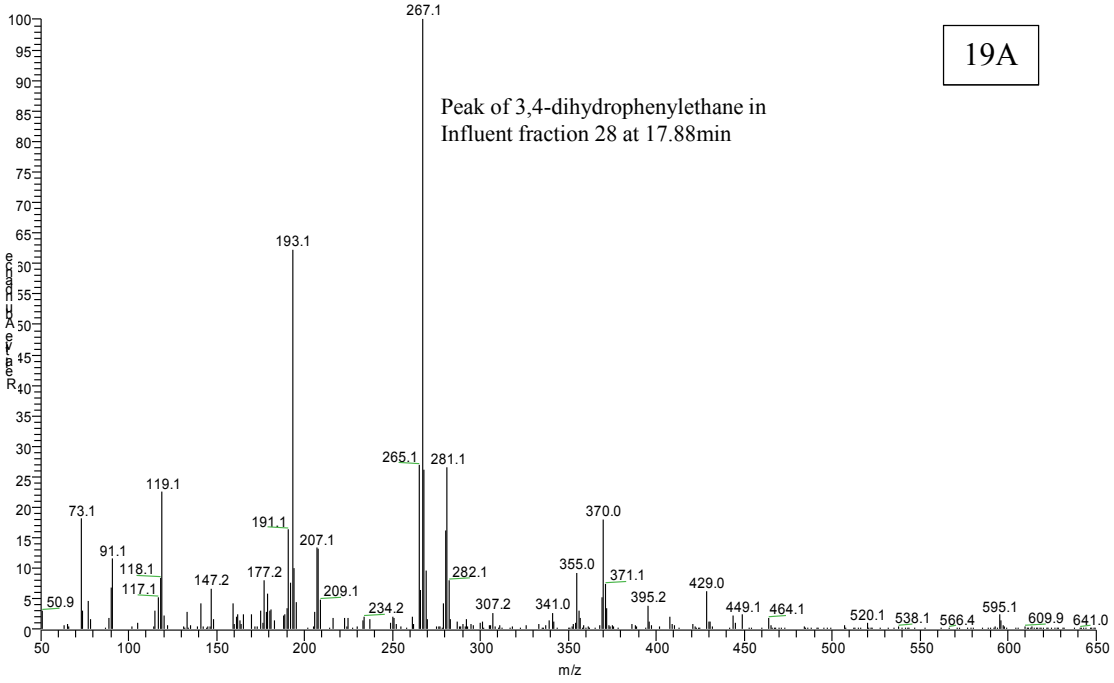
Peak of 4,4-biphenyldicarboxylic acid bis(TMS) ester in Effluent fraction 19 at 26.52min

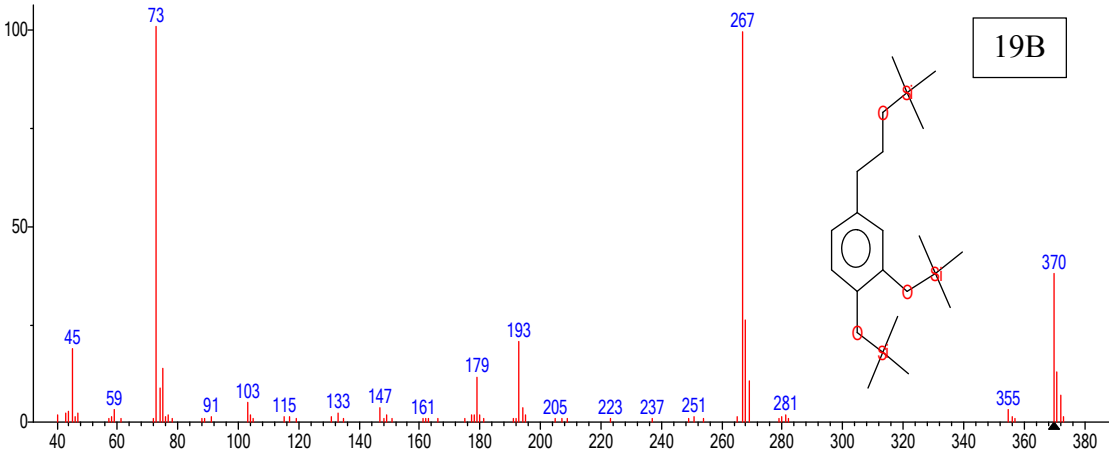


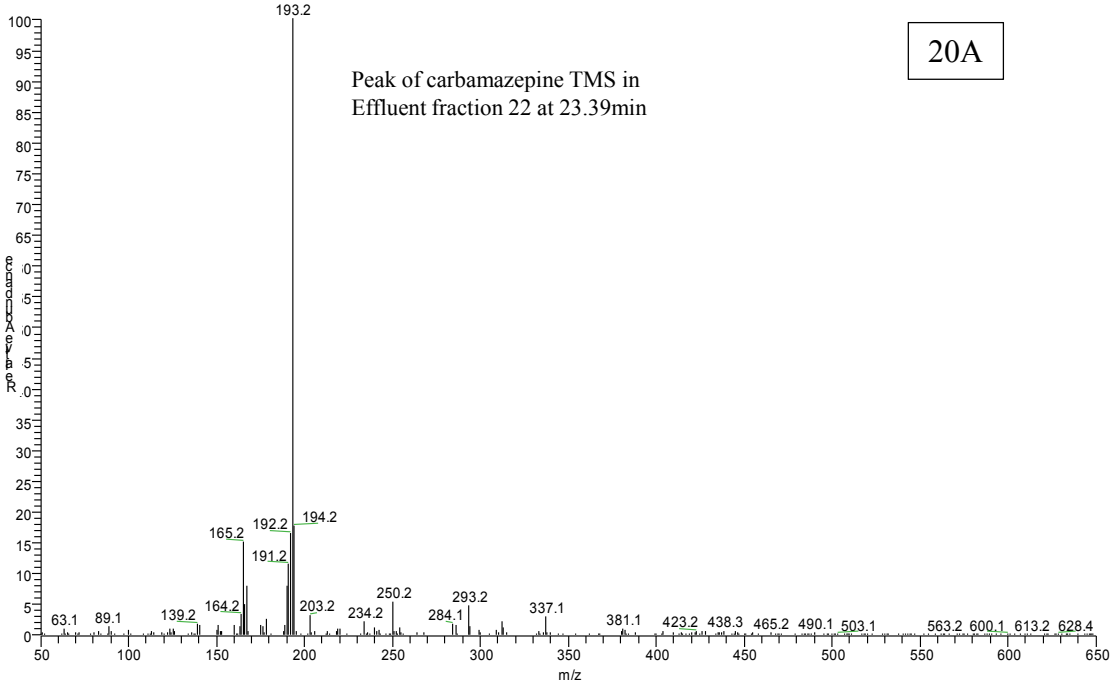
17B

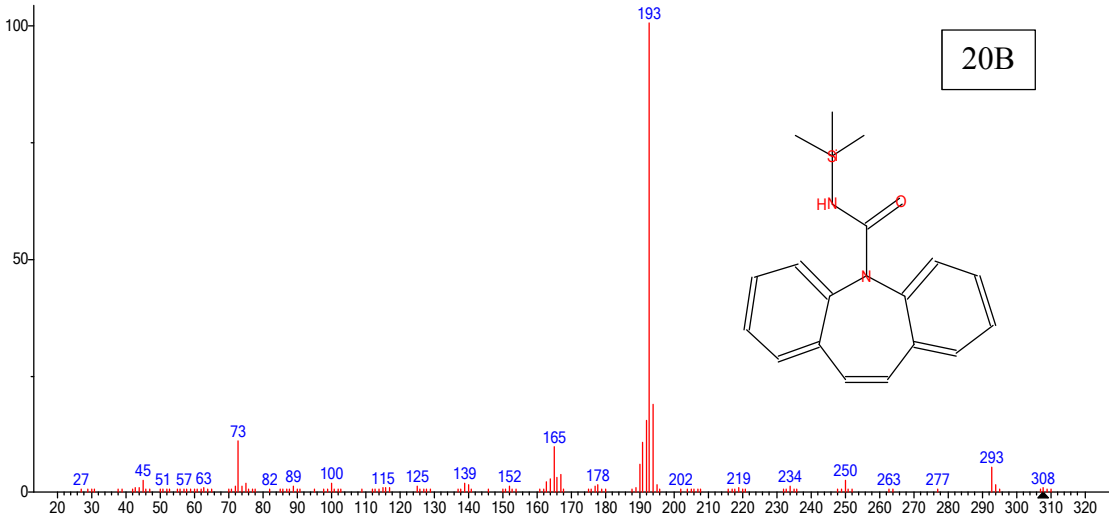

18A
Peak of 6-hydroxy-α-methylnaphthalene
Acetate in Effluent fraction 20 at 22.13min
Abundance
m/z
243.1
360.1
147.2
73.1
242.2
244.1
345.2
228.2
154.2
227.2
361.1
316.1
213.2
185.2
411.0
362.1
245.1
317.1
309.2
401.1
412.1
53.1
74.1
113.2
475.0
489.0
535.0
553.1
609.0
623.0

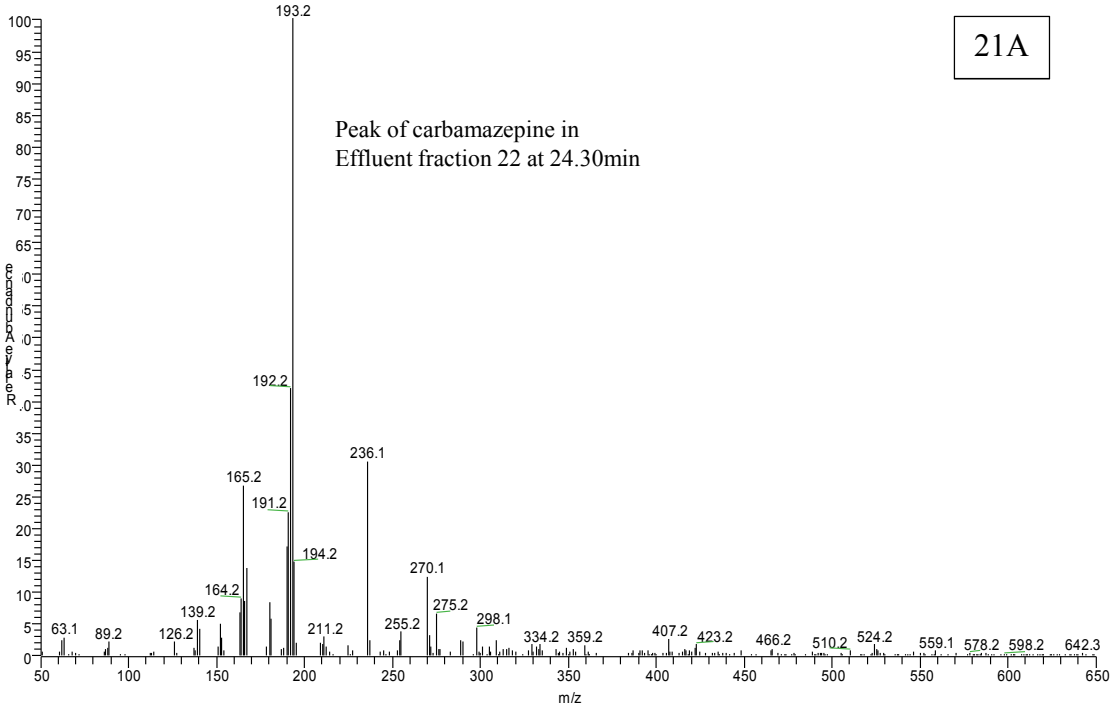

18B
243
73
360
345
228
45
59
69
101
115
127
154
165
185
199
213
255
272
287
301
316

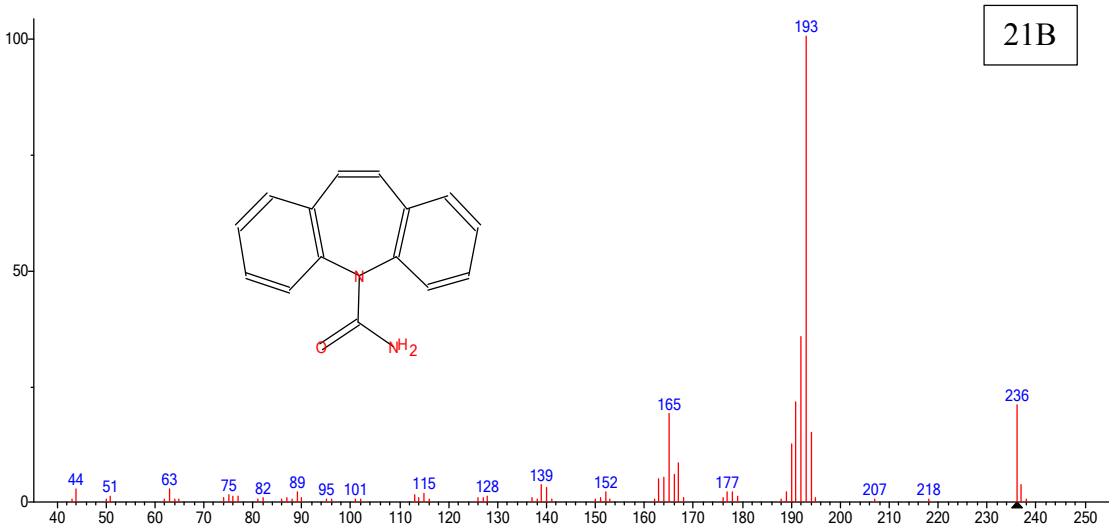

19A
Peak of 3,4-dihydrophenylethane in
Influent fraction 28 at 17.88min
267.1
193.1
265.1
281.1
119.1
73.1
191.1
207.1
370.0
91.1
118.1
117.1
147.2
177.2
209.1
282.1
355.0
371.1
395.2
429.0
234.2
307.2
341.0
449.1
464.1
520.1
538.1
566.4
595.1
609.9
641.0
50.9
m/z


19B
73
267
370
45
193
179
59
91
103
115
133
147
161
205
223
237
251
281
355

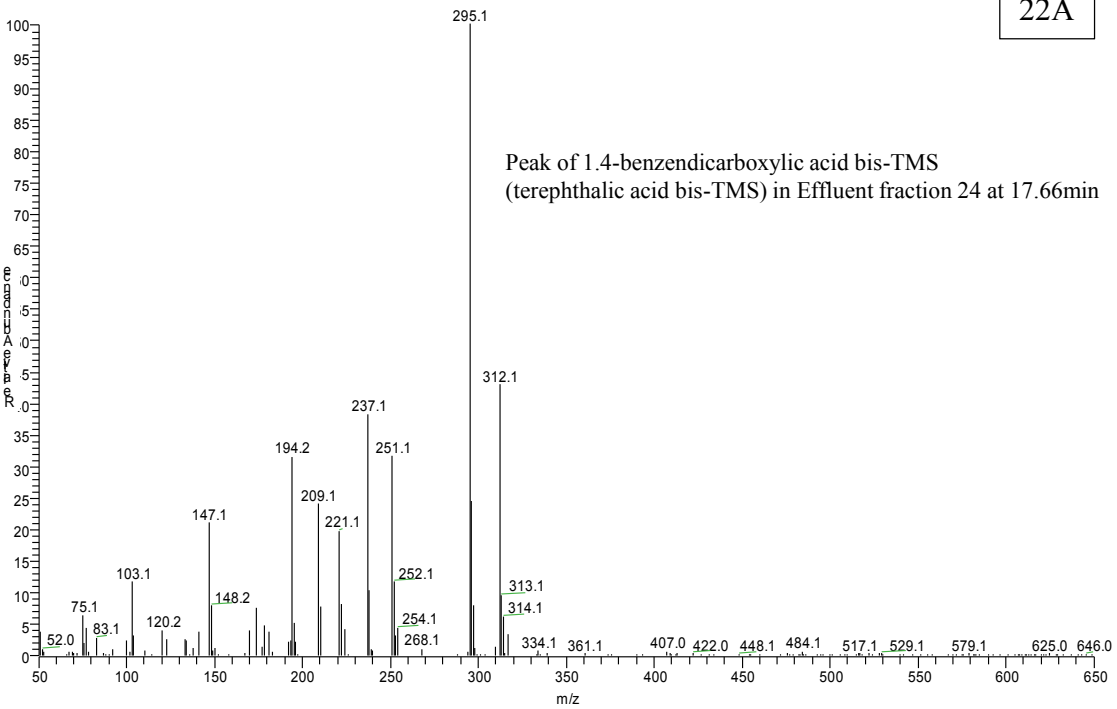

20A
Peak of carbamazepine TMS in
Effluent fraction 22 at 23.39min
193.2
192.2
194.2
165.2
191.2
164.2
203.2
250.2
293.2
234.2
284.1
337.1
63.1
89.1
139.2
381.1
423.2
438.3
465.2
490.1
503.1
563.2
600.1
613.2
628.4
m/z


20B
193
73
165
27
45
51
57
63
82
89
100
115
125
139
152
178
202
219
234
250
263
277
293
308
HN
O
N


21A
Peak of carbamazepine in
Effluent fraction 22 at 24.30min
193.2
192.2
236.1
165.2
191.2
194.2
270.1
164.2
275.2
139.2
298.1
63.1
89.2
126.2
211.2
255.2
334.2
359.2
407.2
423.2
466.2
510.2
524.2
559.1
578.2
598.2
642.3
m/z

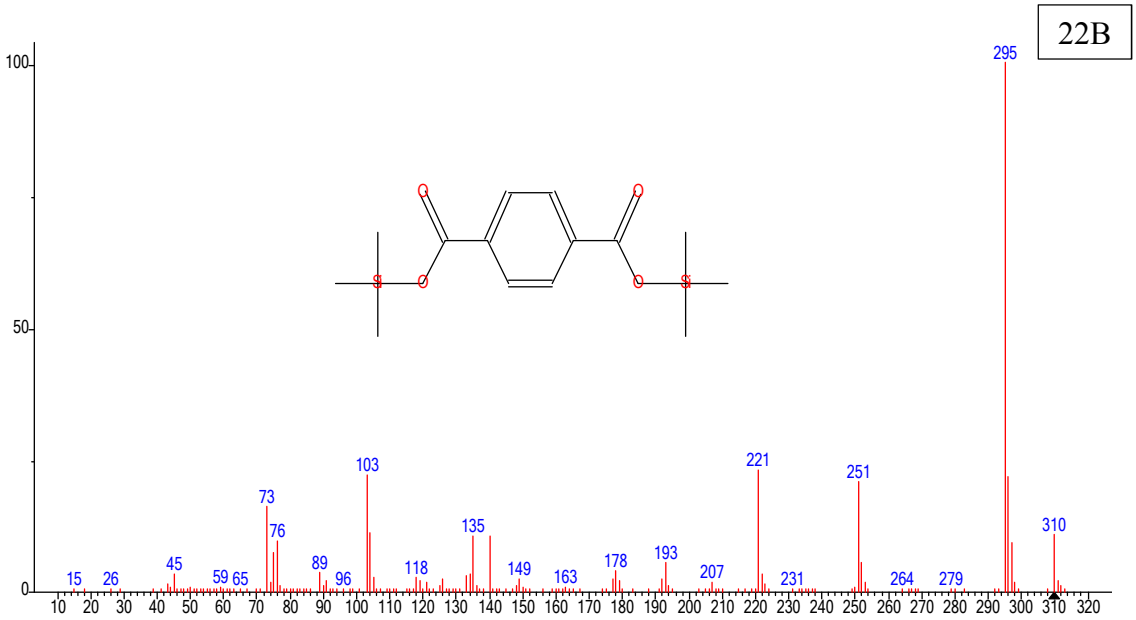

21B
193
165
236
44
51
63
75
82
89
95
101
115
128
139
152
177
207
218
N
O
NH2
m/z

22A

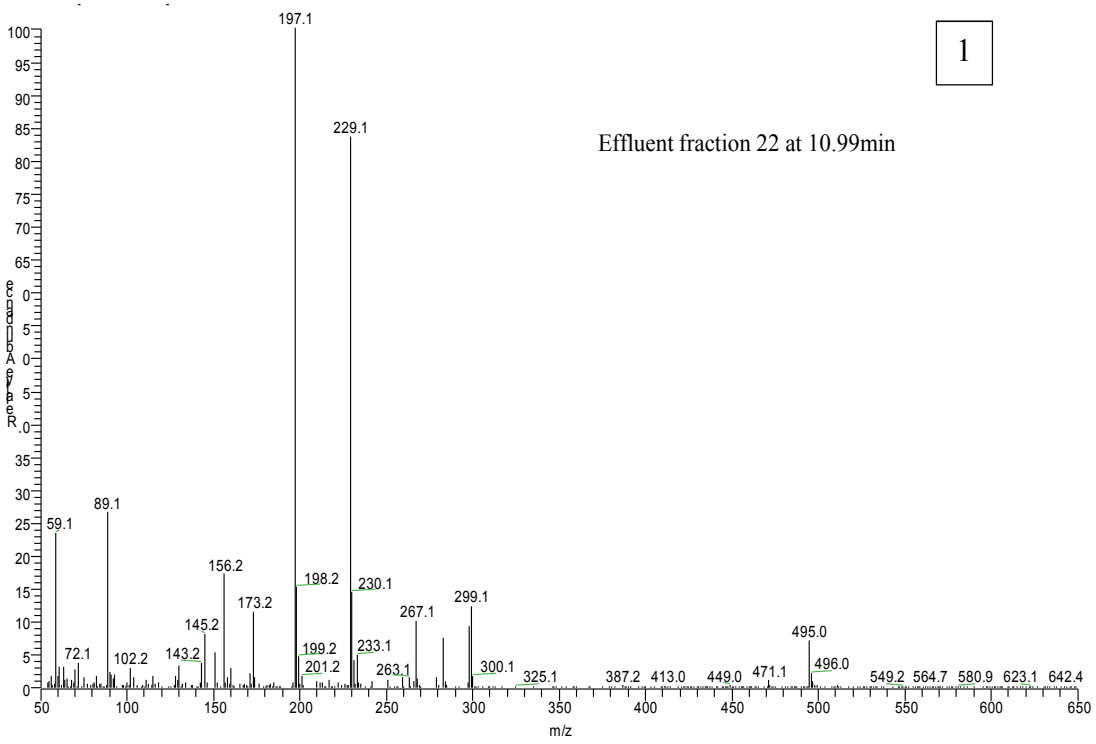


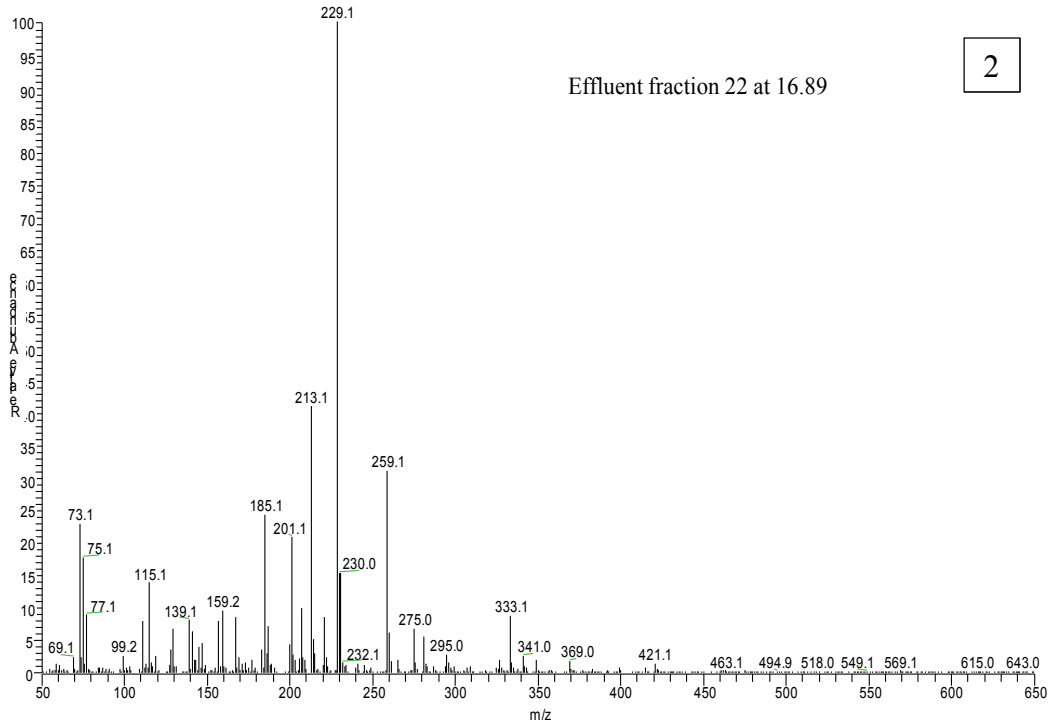
Peak of 1.4-benzendicarboxylic acid bis-TMS (terephthalic acid bis-TMS) in Effluent fraction 24 at 17.66min

22B



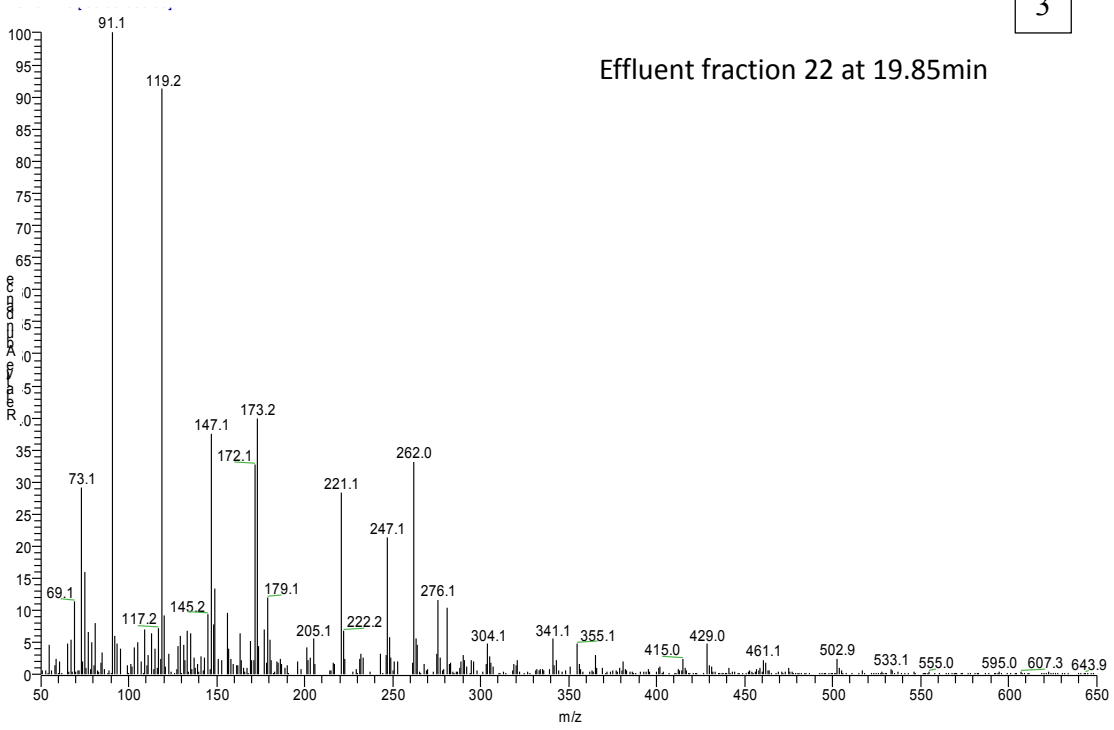
Appendix D: Chromatograms of some unknown compounds that occurred in effluent and influent fractions in high concentrations.





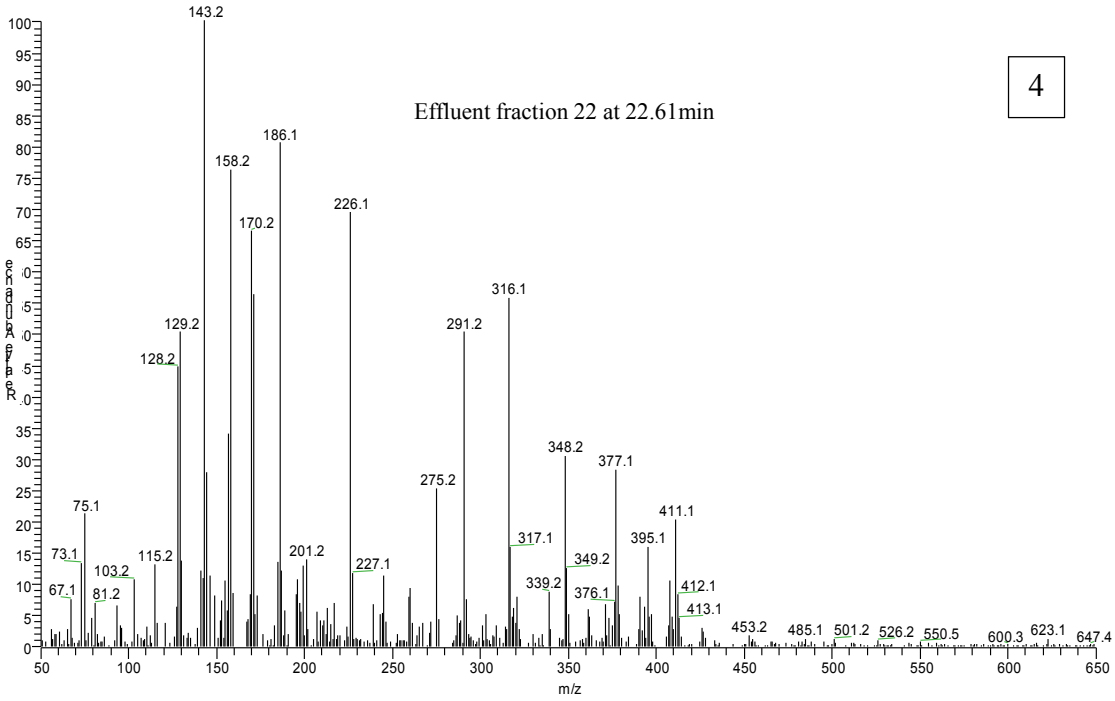
3

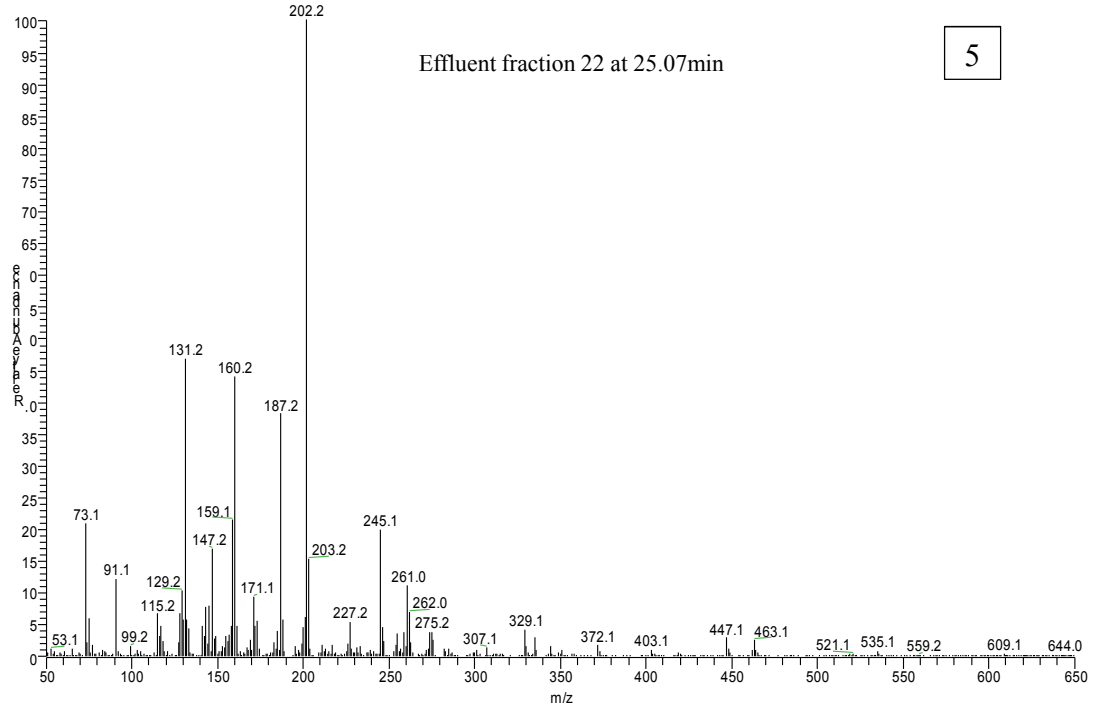
Effluent fraction 22 at 19.85min

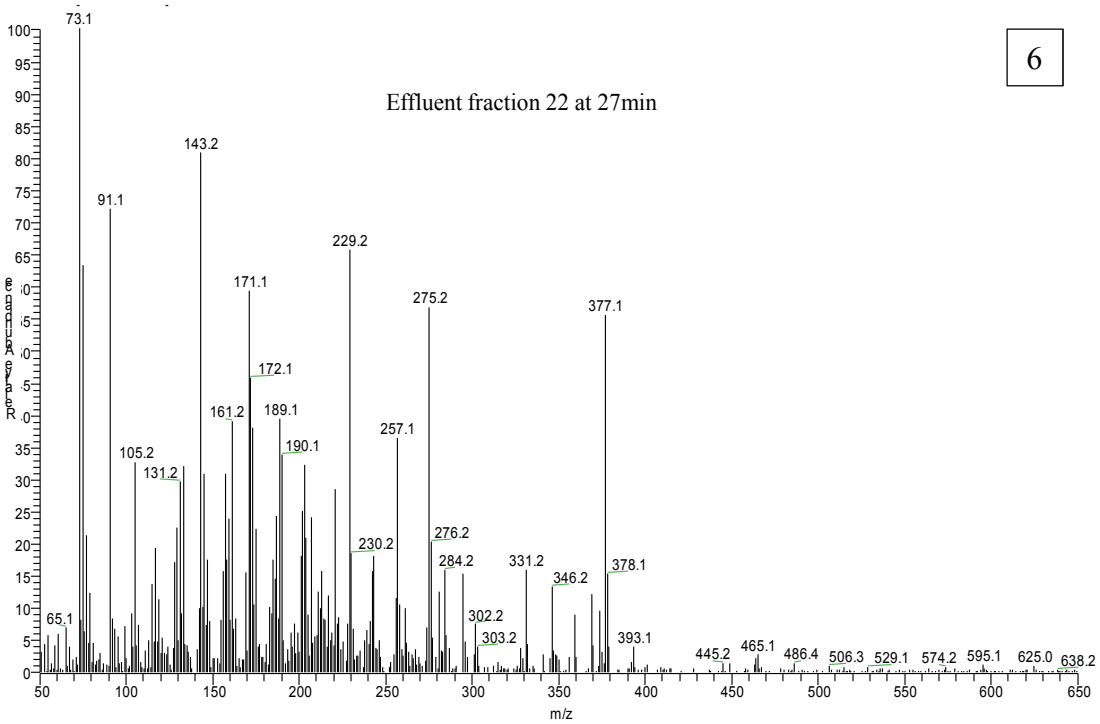


4

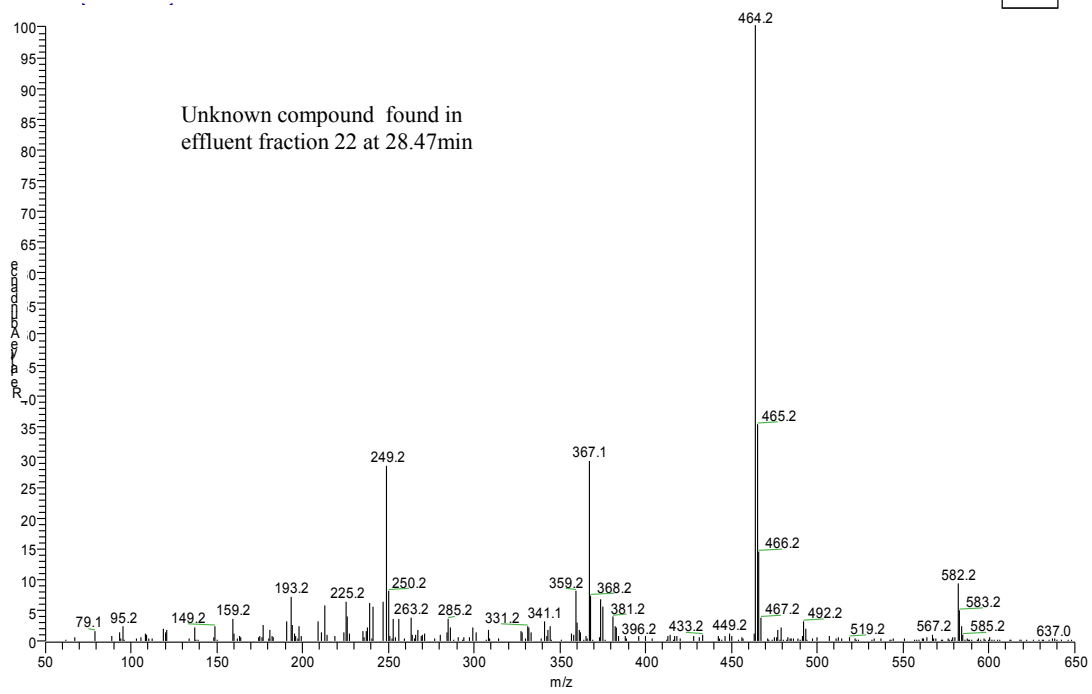
Effluent fraction 22 at 22.61min



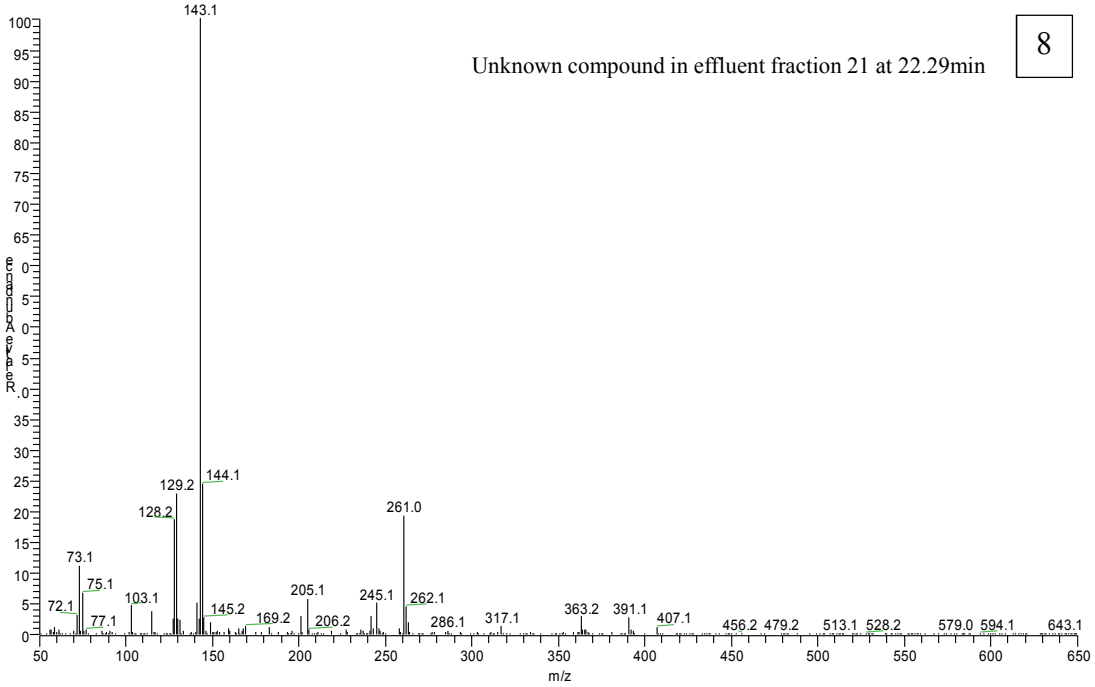

Effluent fraction 22 at 25.07min
5
Abundance
m/z
202.2
131.2
160.2
187.2
73.1
159.1
147.2
245.1
203.2
91.1
129.2
171.1
261.0
262.0
115.2
227.2
275.2
53.1
99.2
307.1
329.1
372.1
403.1
447.1
463.1
521.1
535.1
559.2
609.1
644.0


Effluent fraction 22 at 27min
6
Abundance
m/z
73.1
143.2
91.1
229.2
171.1
275.2
377.1
172.1
161.2
189.1
257.1
105.2
190.1
131.2
276.2
230.2
284.2
331.2
346.2
378.1
65.1
302.2
303.2
393.1
445.2
465.1
486.4
506.3
529.1
574.2
595.1
625.0
638.2

7

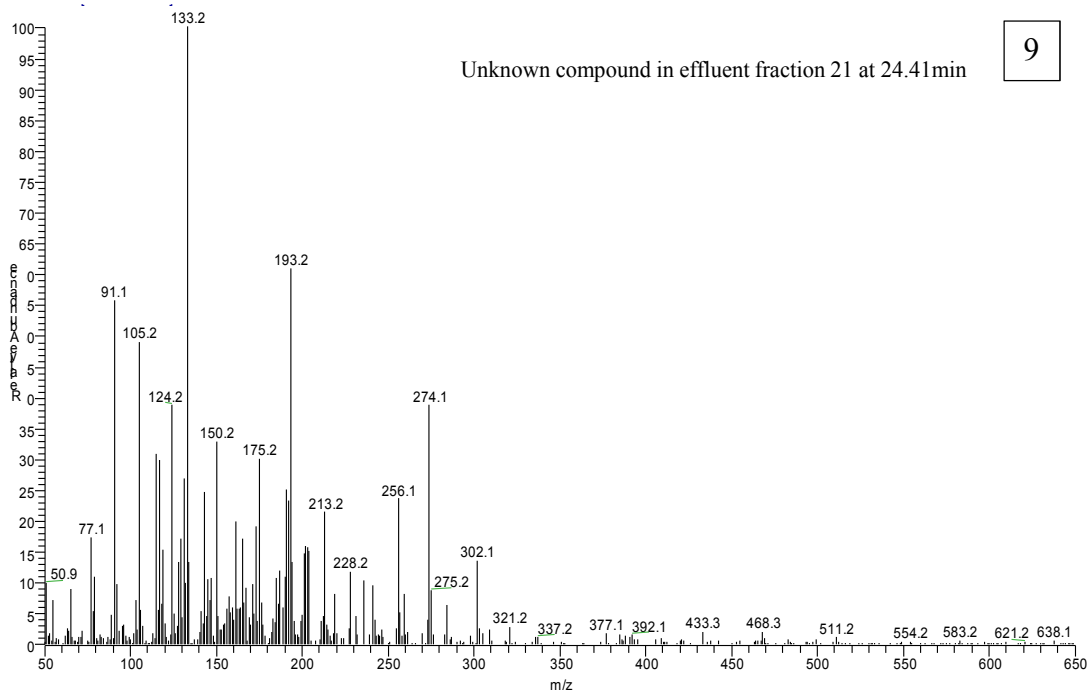

Unknown compound found in
effluent fraction 22 at 28.47min
464.2
465.2
466.2
249.2
367.1
582.2
583.2
585.2
193.2
225.2
250.2
359.2
368.2
381.2
263.2
285.2
331.2
341.1
396.2
433.2
449.2
467.2
492.2
519.2
567.2
637.0
79.1
95.2
149.2
159.2
m/z

8


Unknown compound in effluent fraction 21 at 22.29min
143.1
144.1
129.2
128.2
261.0
73.1
75.1
72.1
77.1
103.1
205.1
245.1
262.1
145.2
169.2
206.2
286.1
317.1
363.2
391.1
407.1
456.2
479.2
513.1
528.2
579.0
594.1
643.1
m/z

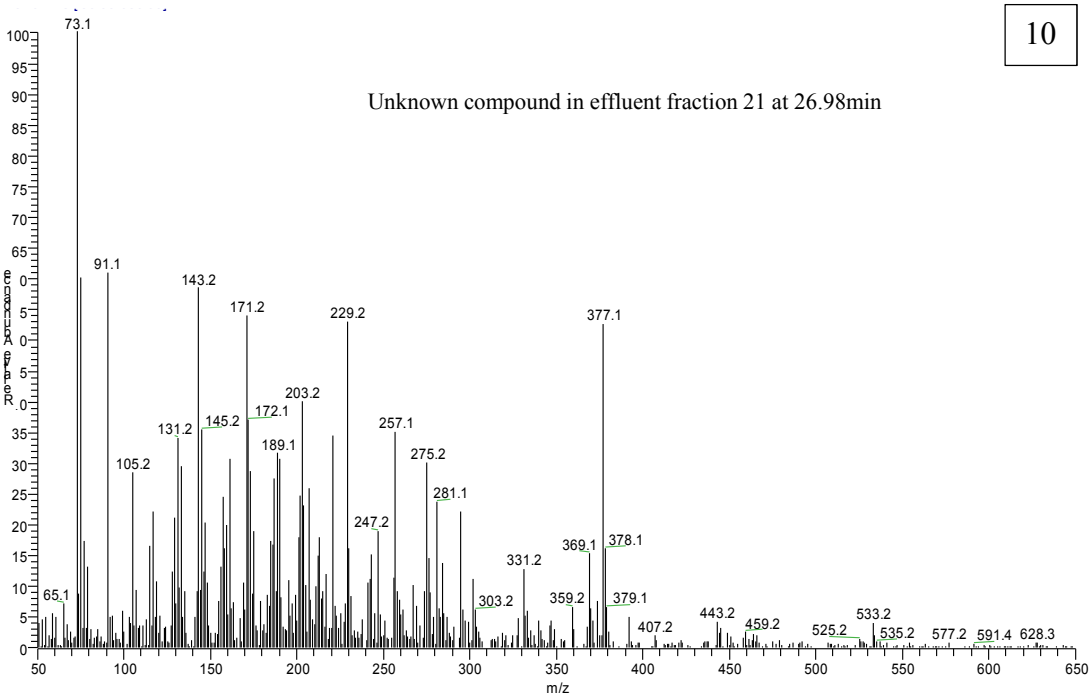
9

Unknown compound in effluent fraction 21 at 24.41min



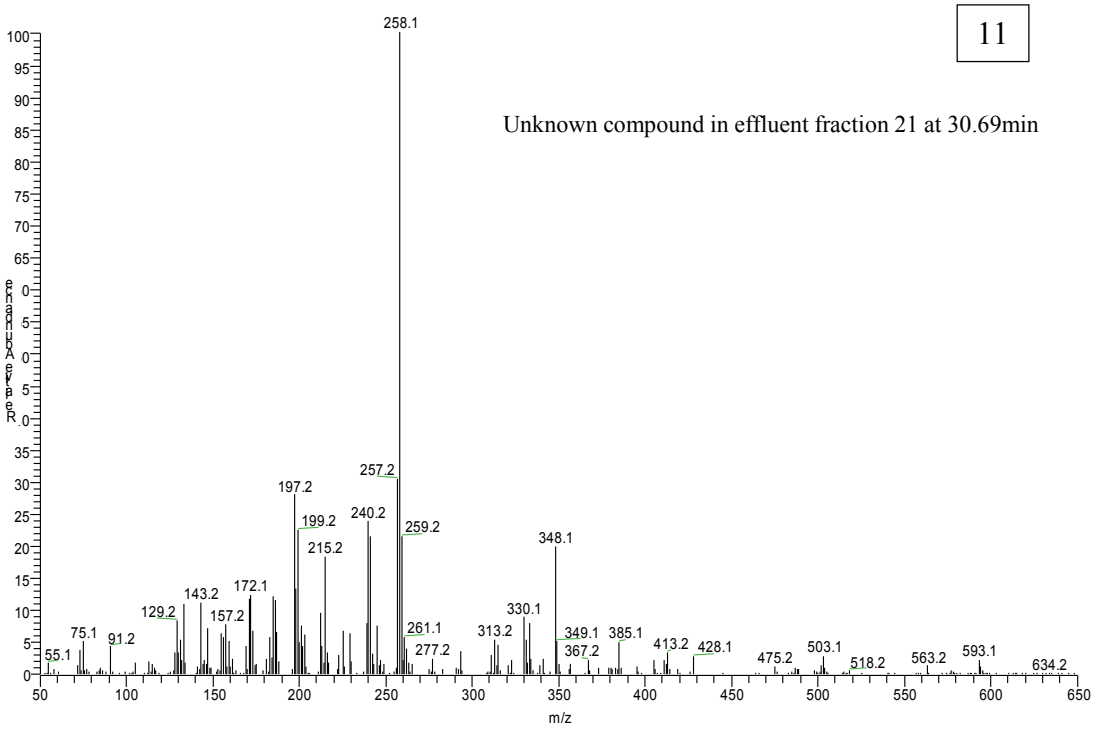
10

Unknown compound in effluent fraction 21 at 26.98min



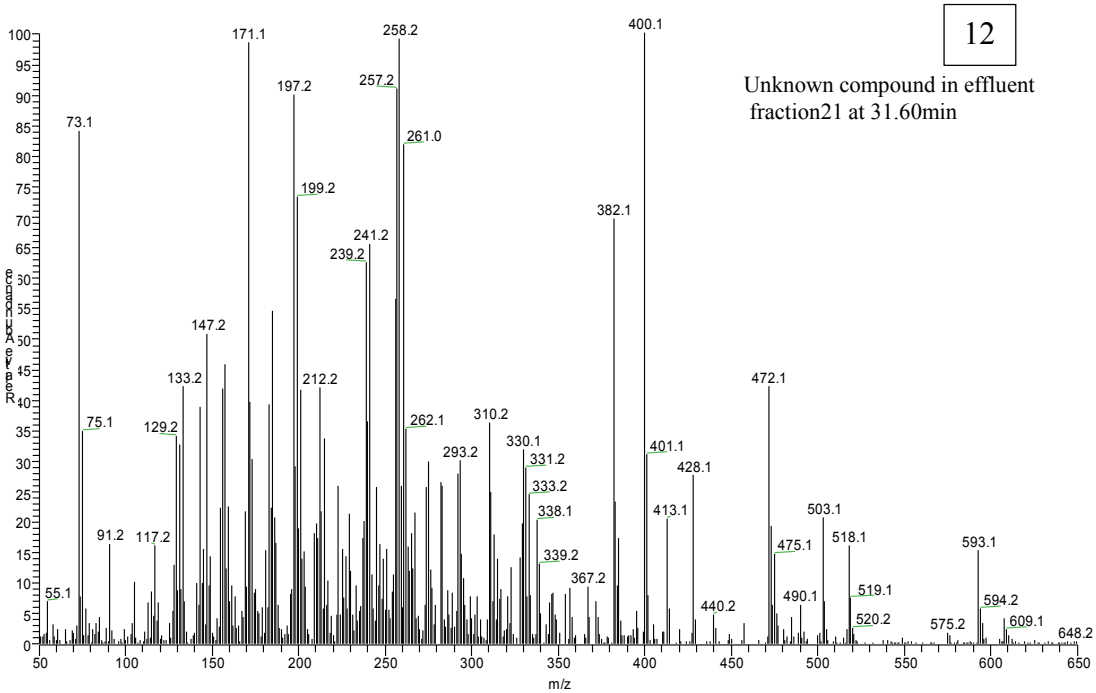
11

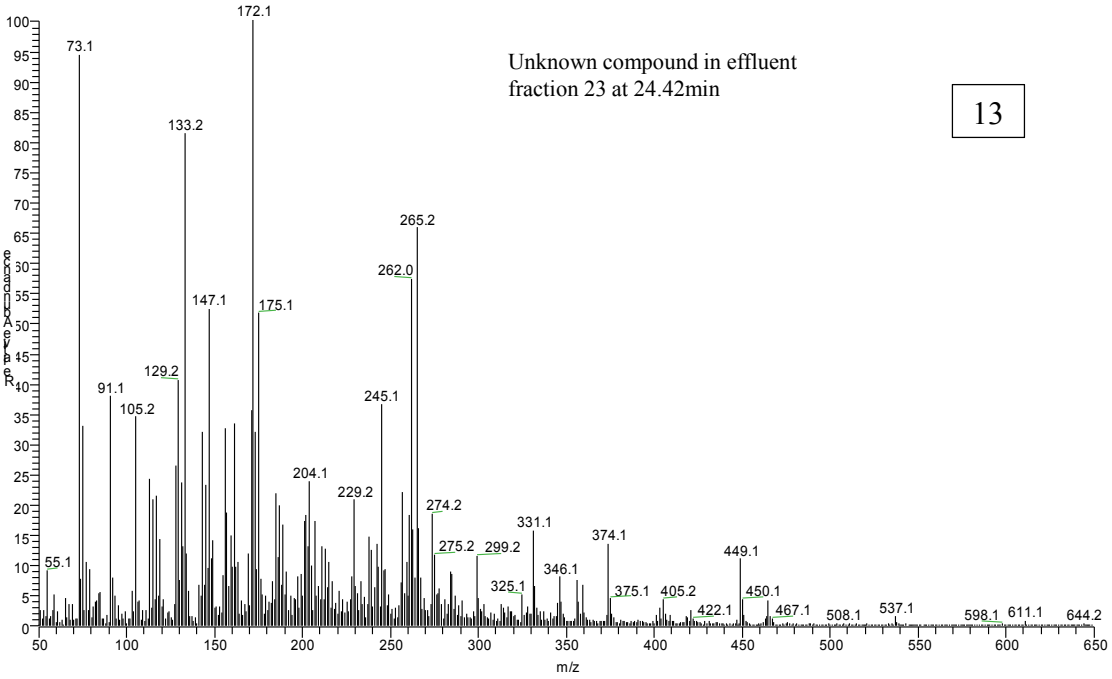
Unknown compound in effluent fraction 21 at 30.69min

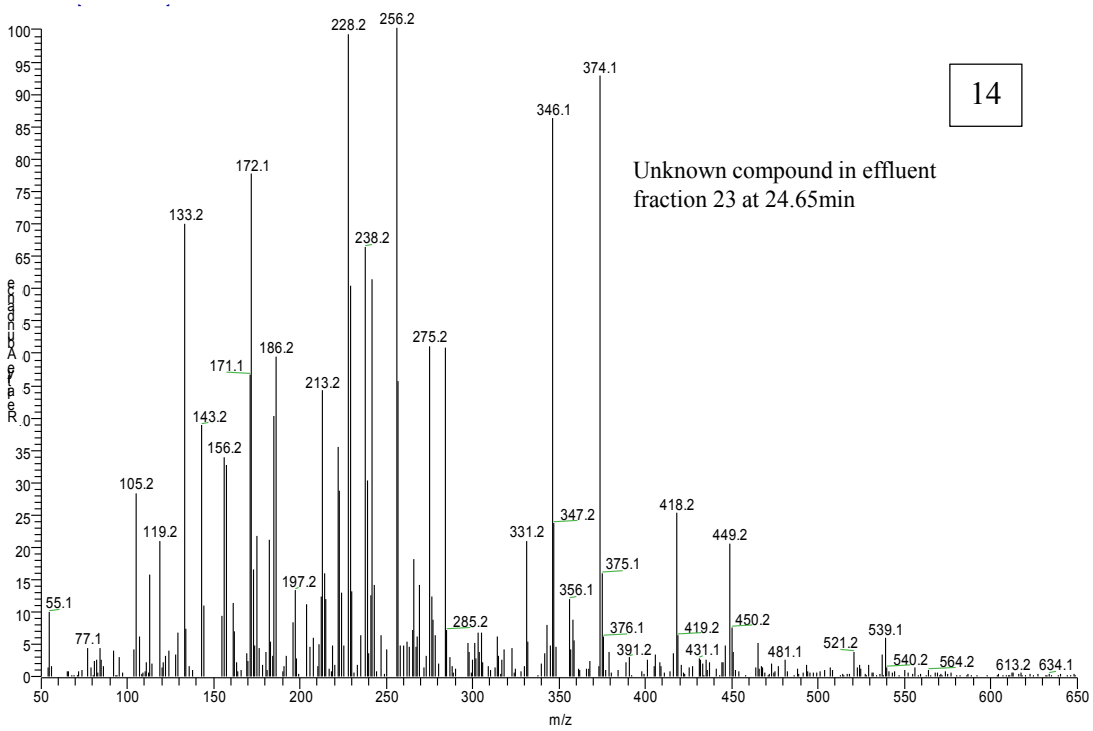


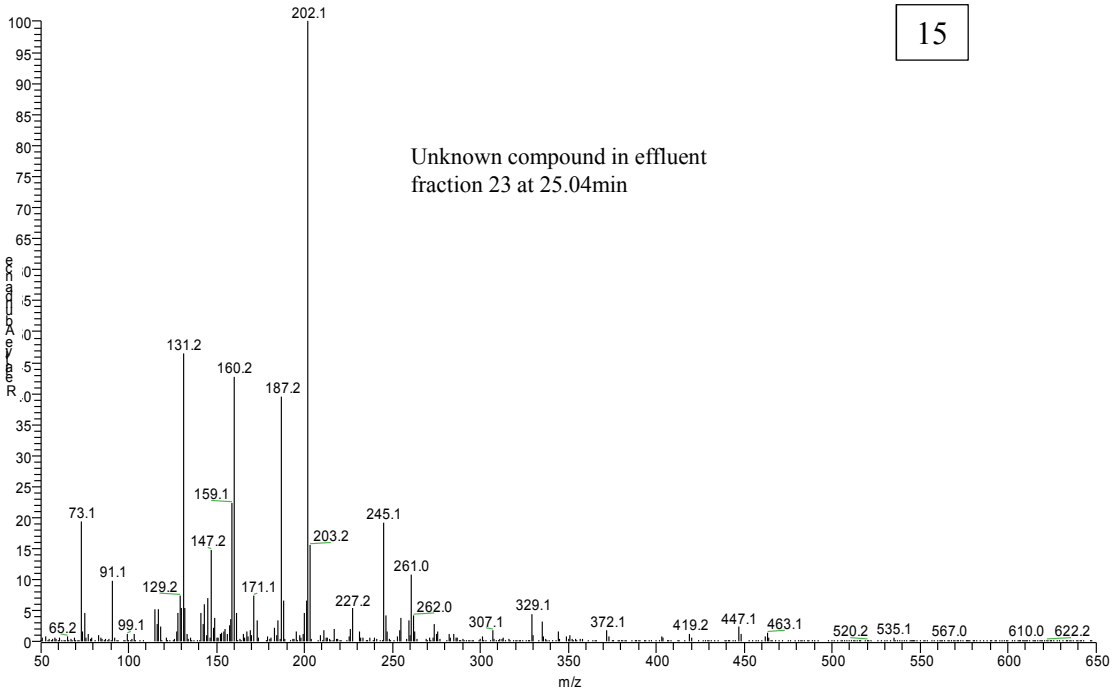
12

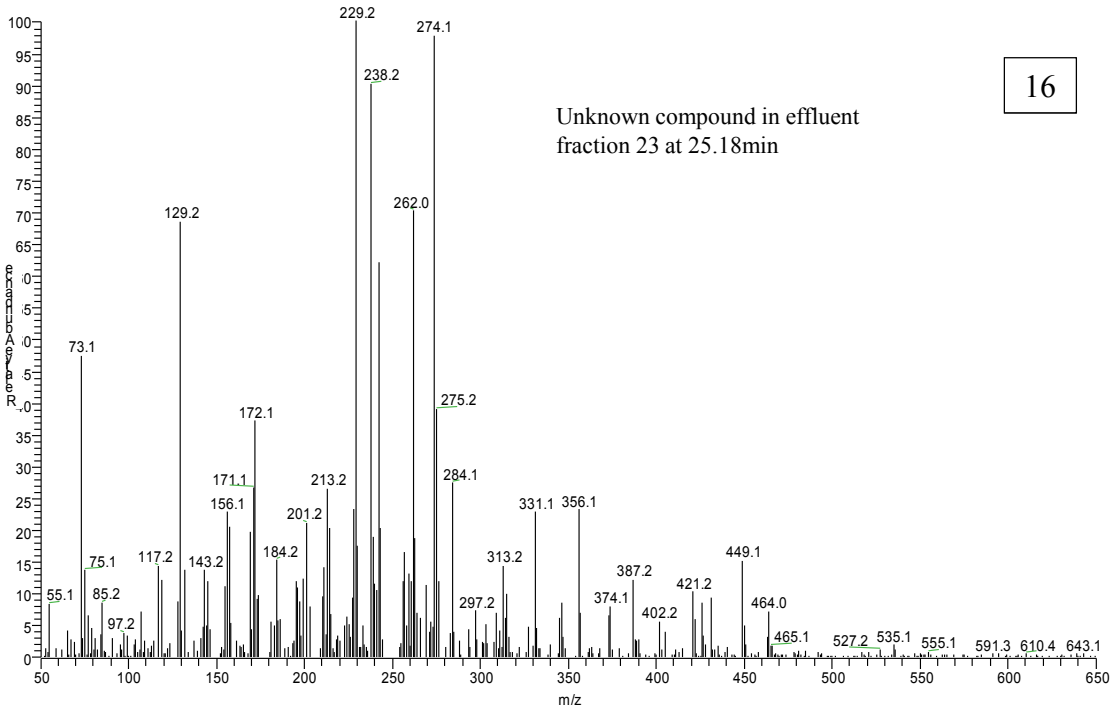
Unknown compound in effluent fraction21 at 31.60min



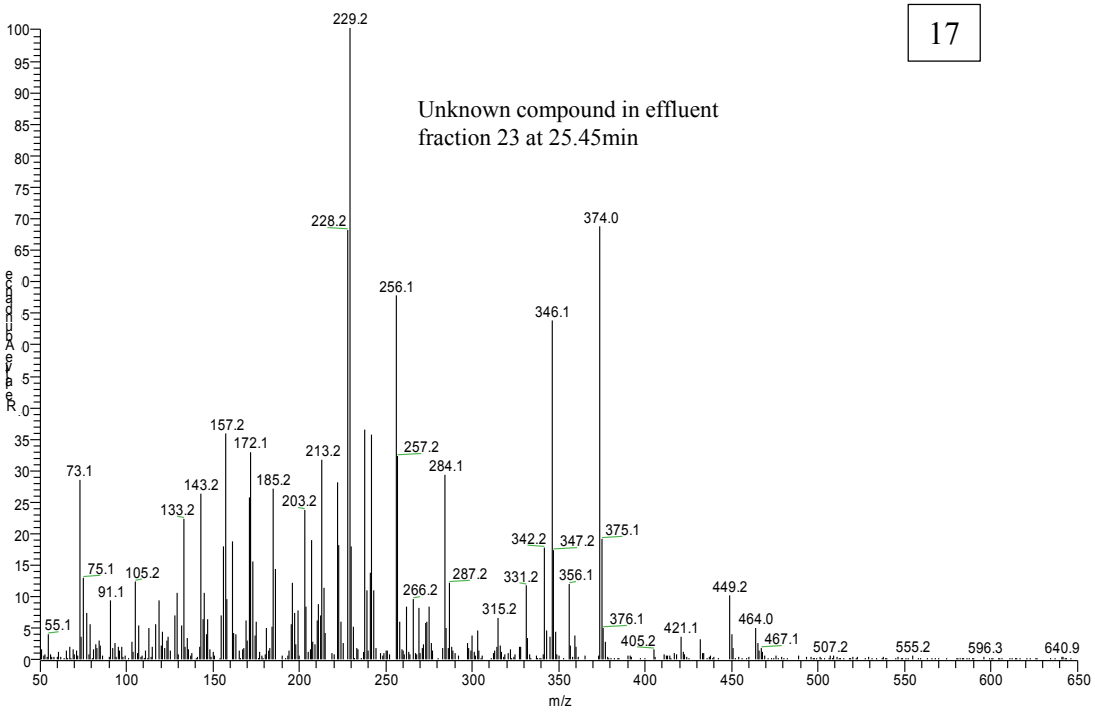

Unknown compound in effluent
fraction 23 at 24.42min
13
Abundance
m/z
73.1
172.1
133.2
265.2
262.0
147.1
175.1
129.2
91.1
105.2
245.1
204.1
229.2
274.2
331.1
374.1
275.2
299.2
449.1
55.1
346.1
325.1
375.1
405.2
450.1
422.1
467.1
508.1
537.1
598.1
611.1
644.2


14
Unknown compound in effluent
fraction 23 at 24.65min
Abundance
m/z
228.2
256.2
374.1
346.1
172.1
133.2
238.2
275.2
186.2
171.1
213.2
143.2
156.2
105.2
119.2
418.2
347.2
331.2
449.2
375.1
197.2
356.1
55.1
285.2
376.1
419.2
450.2
77.1
391.2
431.1
481.1
521.2
539.1
540.2
564.2
613.2
634.1

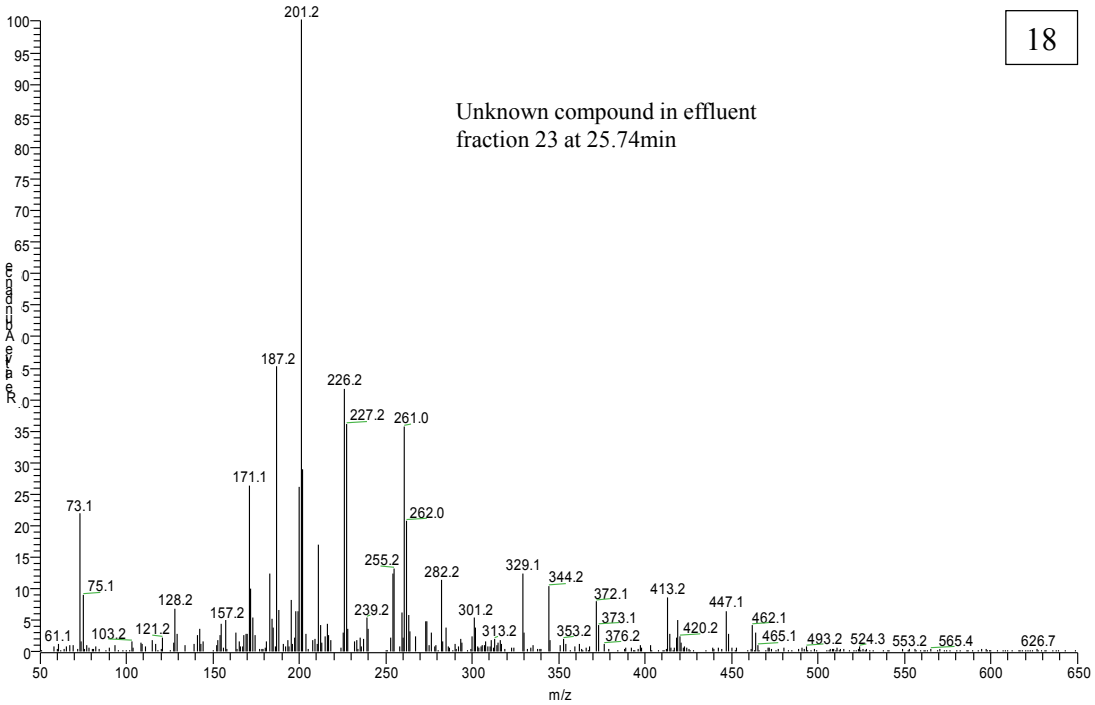

15
Unknown compound in effluent
fraction 23 at 25.04min
Abundance
m/z


16
Unknown compound in effluent
fraction 23 at 25.18min
Abundance
m/z

17

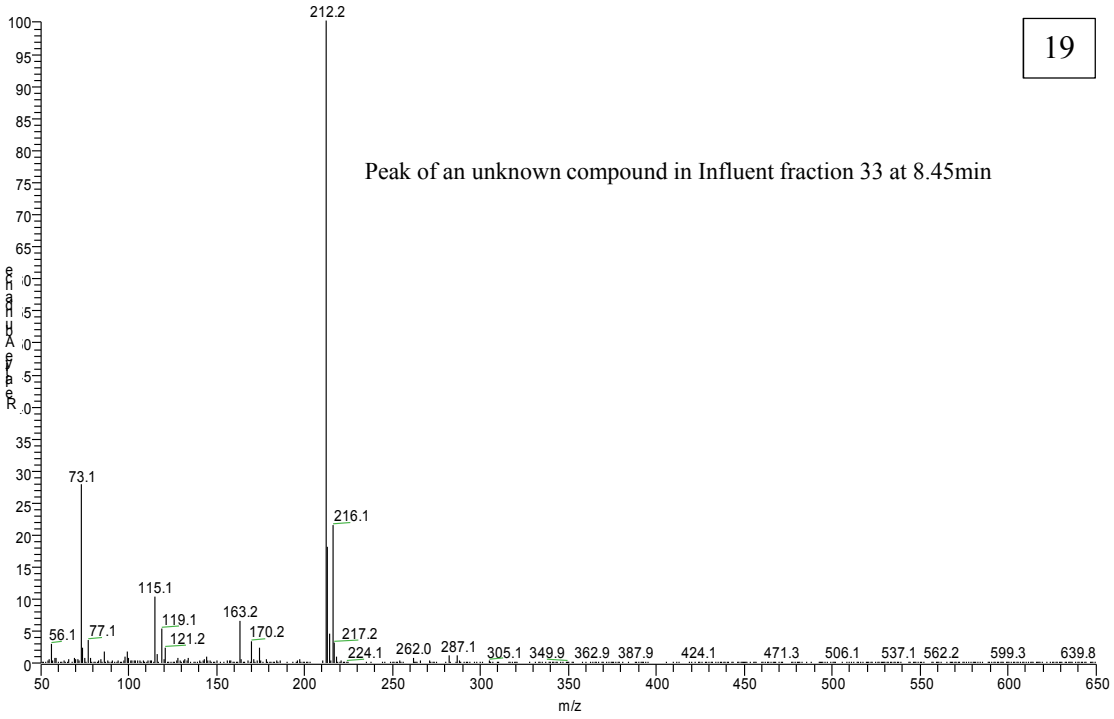

Unknown compound in effluent
fraction 23 at 25.45min
Abundance
m/z
229.2
228.2
374.0
256.1
346.1
157.2
172.1
213.2
257.2
284.1
73.1
143.2
185.2
133.2
203.2
342.2
347.2
375.1
75.1
105.2
91.1
287.2
331.2
356.1
266.2
449.2
315.2
376.1
464.0
55.1
421.1
405.2
467.1
507.2
555.2
596.3
640.9

18


Unknown compound in effluent
fraction 23 at 25.74min
Abundance
m/z
201.2
187.2
226.2
227.2
261.0
171.1
73.1
262.0
255.2
282.2
329.1
344.2
75.1
372.1
413.2
128.2
157.2
239.2
301.2
447.1
373.1
462.1
61.1
103.2
121.2
313.2
353.2
376.2
420.2
465.1
493.2
524.3
553.2
565.4
626.7

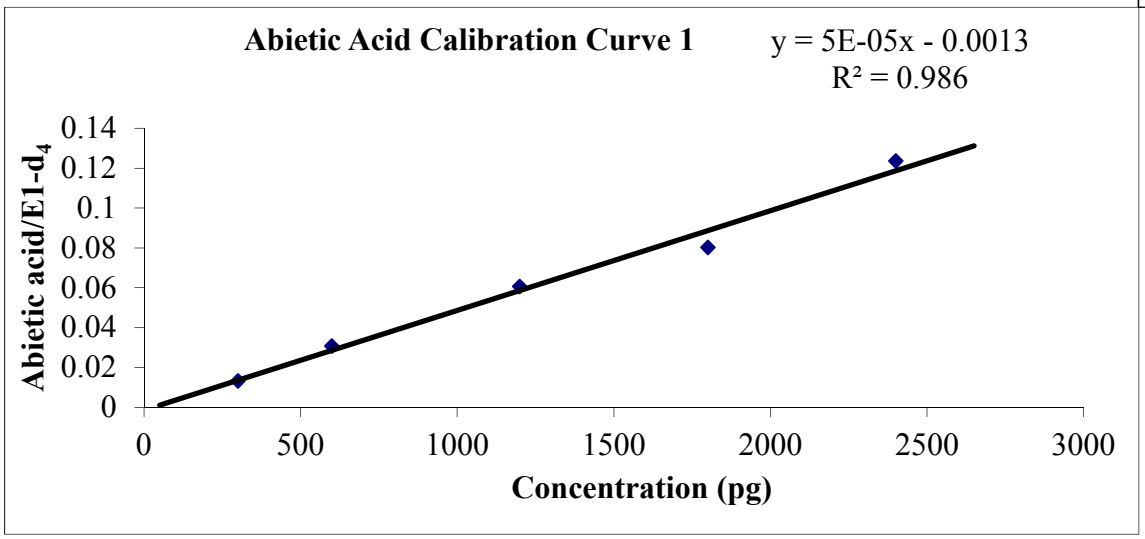
19

Peak of an unknown compound in Influent fraction 33 at 8.45min

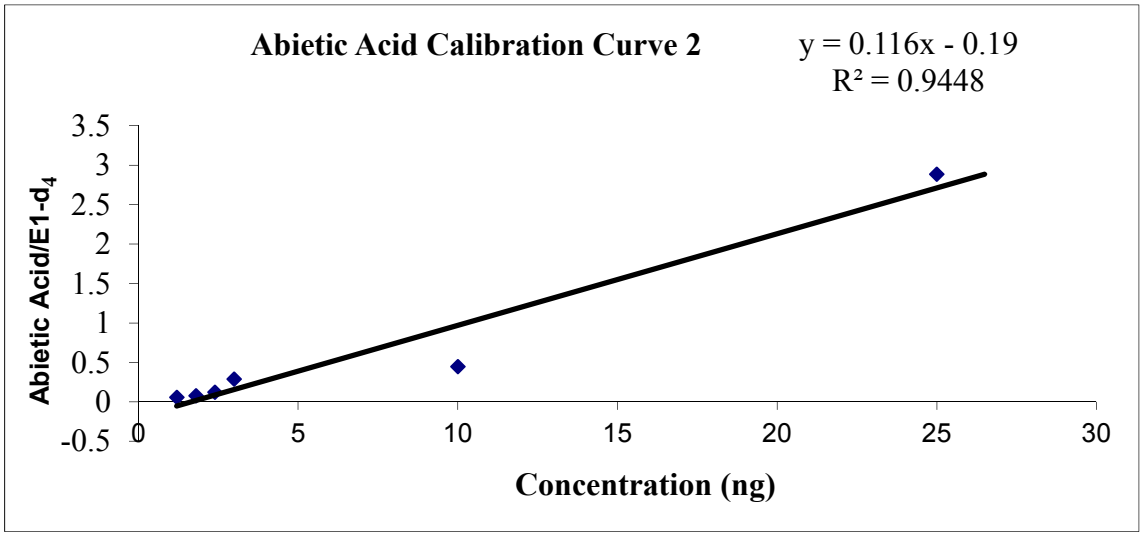


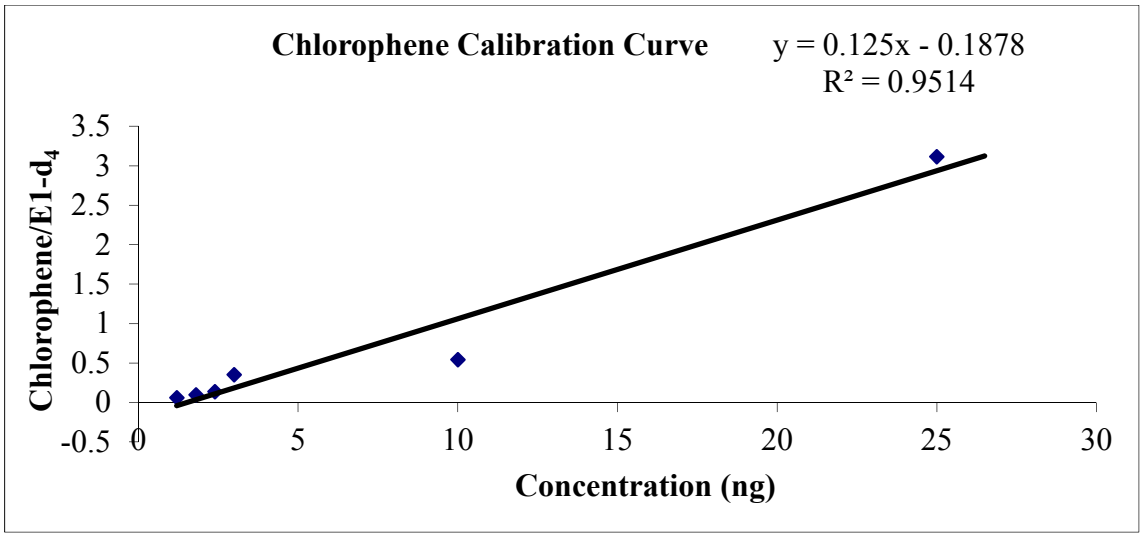
Appendix E: The calibration curves of identified compounds in wastewater samples. These include abietic acid, chlorophene, chloroxylenol, dichlorophene, galaxolide, isopimaric acid, N,N-butylbenzenesulfonamide (NBBSA), pimaric acid, TBEP, TCPP and triclosan.

1A

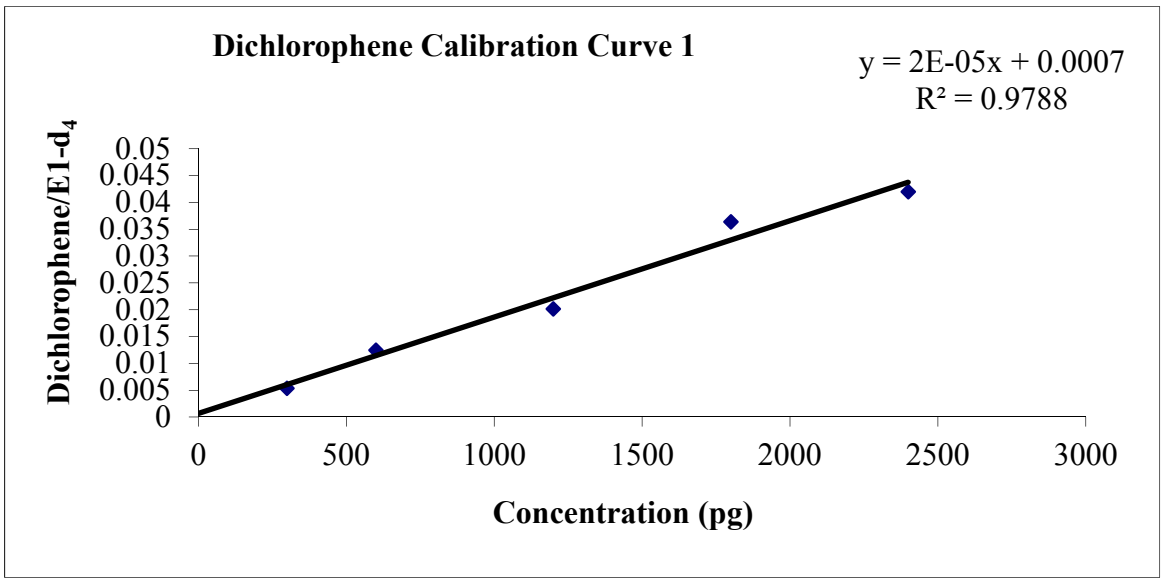


1B

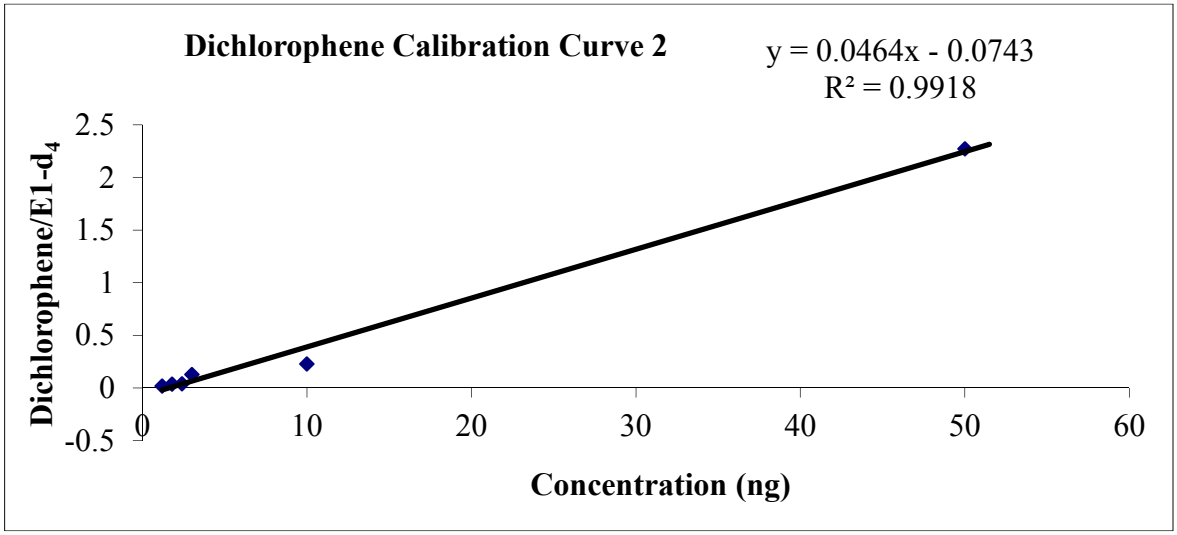



Chlorophene Calibration Curve
y = 0.125x - 0.1878
R² = 0.9514
Chlorophene/E1-d4
Concentration (ng)
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
0
5
10
15
20
25
30

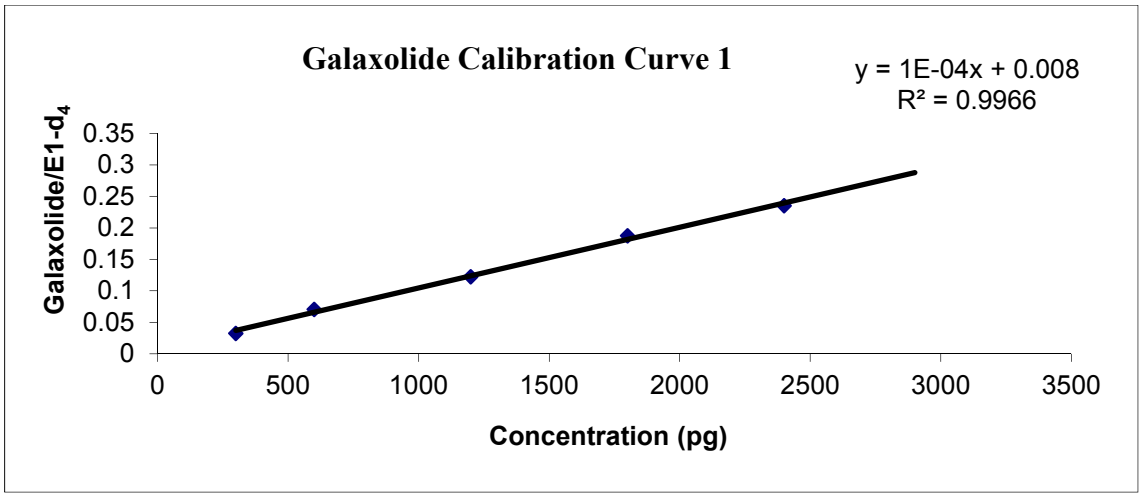
2


Dichlorophene Calibration Curve 1
y = 2E-05x + 0.0007
R² = 0.9788
Dichlorophene/E1-d4
Concentration (pg)
0.05
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
0
500
1000
1500
2000
2500
3000

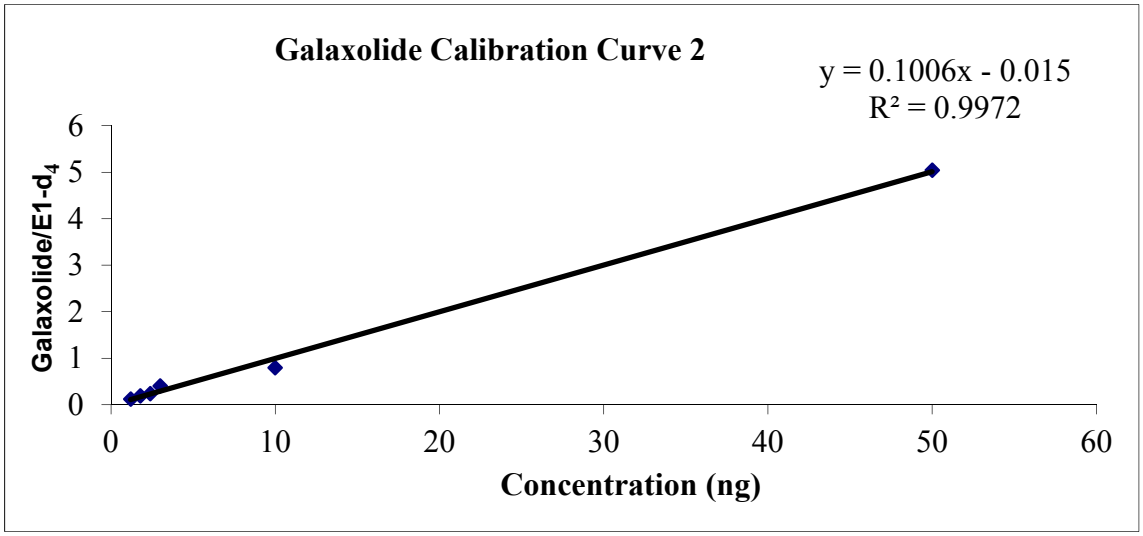
3A


Dichlorophene Calibration Curve 2
y = 0.0464x - 0.0743
R² = 0.9918
Dichlorophene/E1-d4
Concentration (ng)
2.5
2
1.5
1
0.5
0
-0.5
0
10
20
30
40
50
60

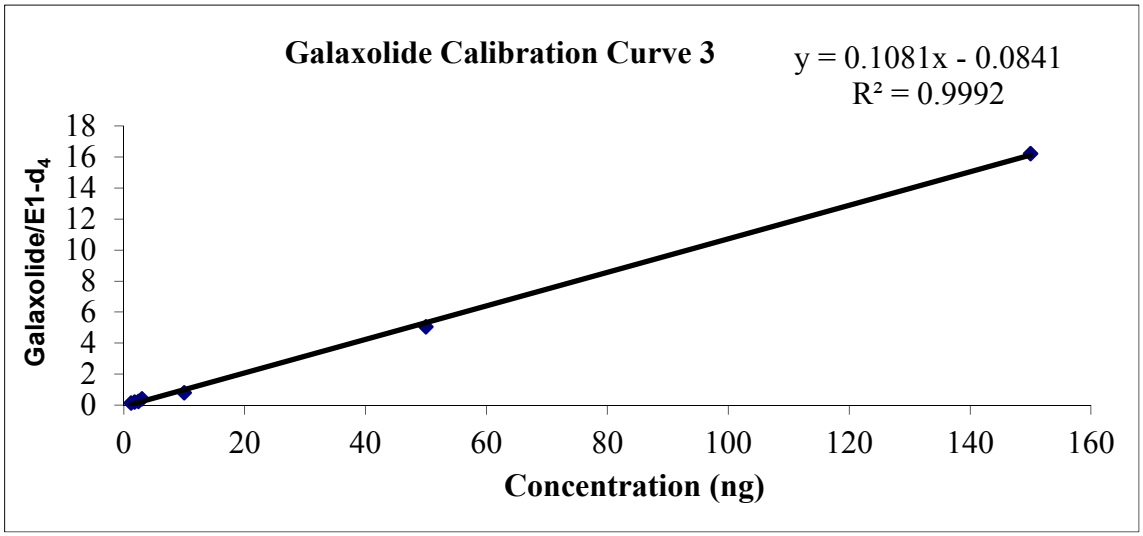
3B


Galaxolide Calibration Curve 1
y = 1E-04x + 0.008
R² = 0.9966
Galaxolide/E1-d4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
500
1000
1500
2000
2500
3000
3500
Concentration (pg)

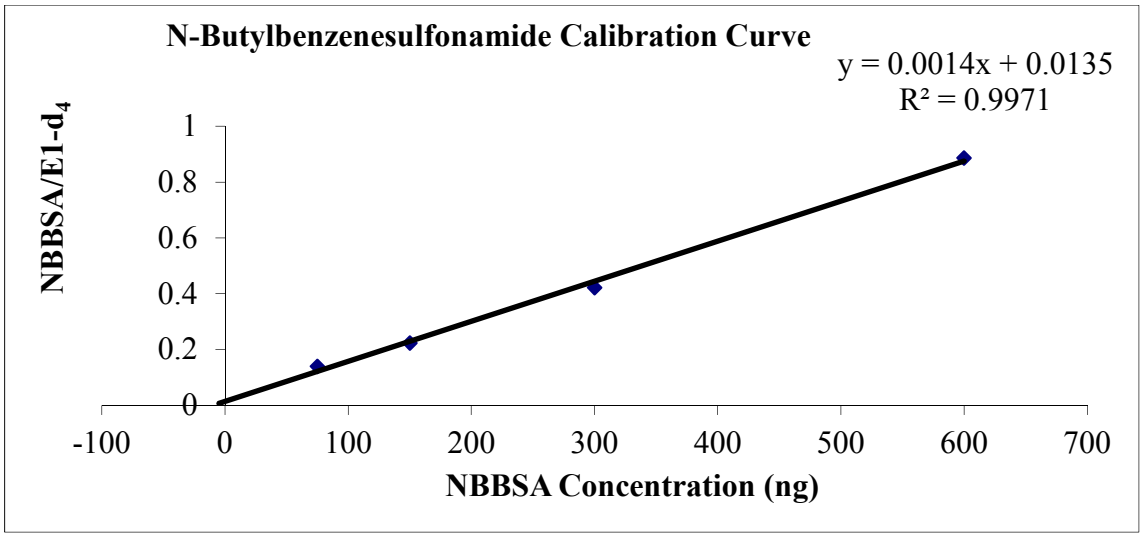
4A


Galaxolide Calibration Curve 2
y = 0.1006x - 0.015
R² = 0.9972
Galaxolide/E1-d4
6
5
4
3
2
1
0
0
10
20
30
40
50
60
Concentration (ng)

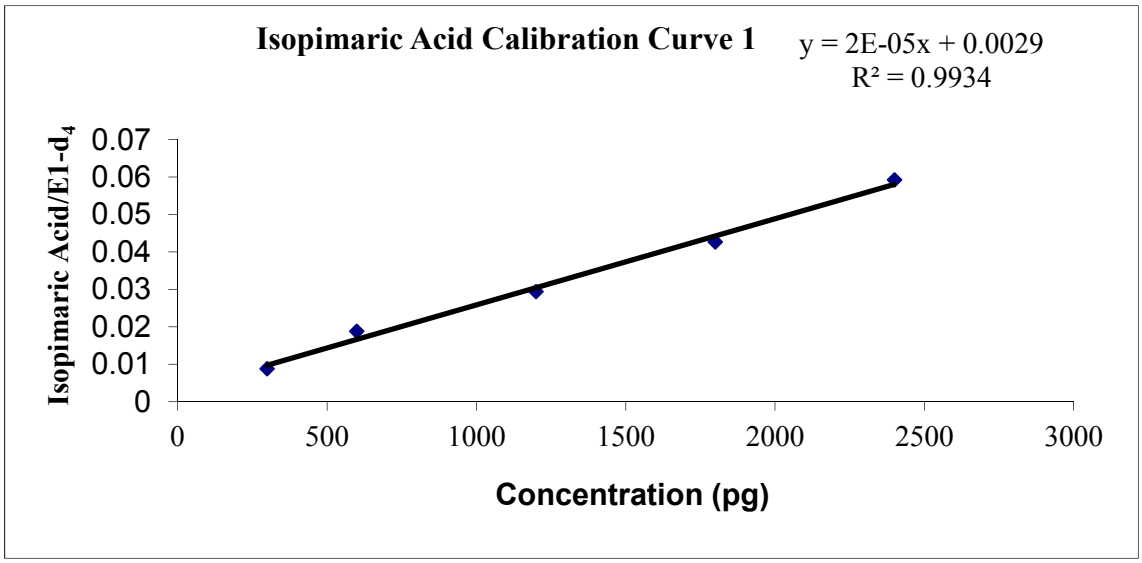
4B


Galaxolide Calibration Curve 3
y = 0.1081x - 0.0841
R² = 0.9992
Galaxolide/E1-d4
18
16
14
12
10
8
6
4
2
0
0
20
40
60
80
100
120
140
160
Concentration (ng)

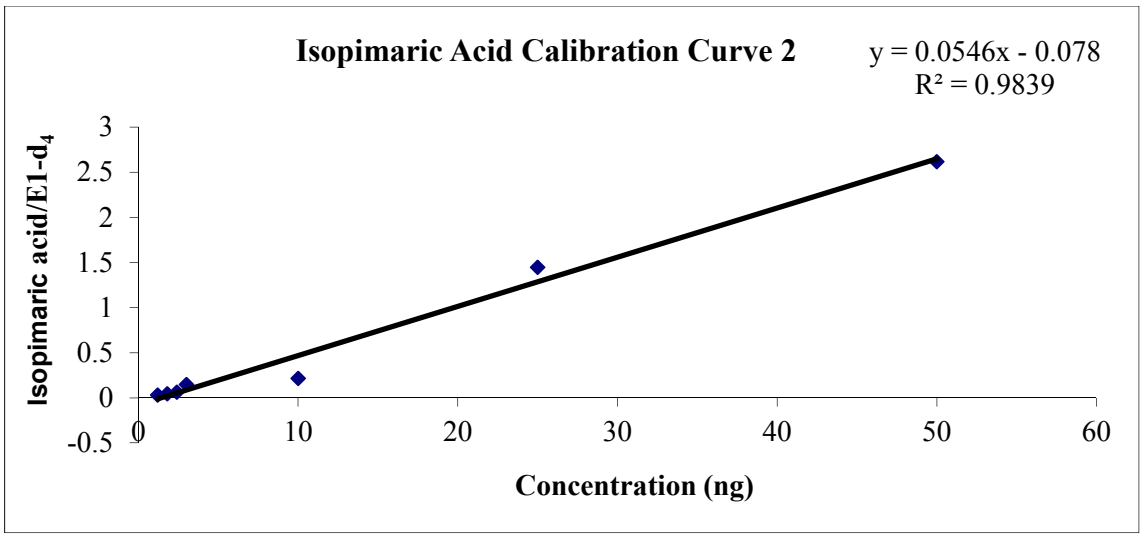
4C


N-Butylbenzenesulfonamide Calibration Curve
y = 0.0014x + 0.0135
R² = 0.9971
NBBSA/E1-d4
1
0.8
0.6
0.4
0.2
0
-100
0
100
200
300
400
500
600
700
NBBSA Concentration (ng)

5

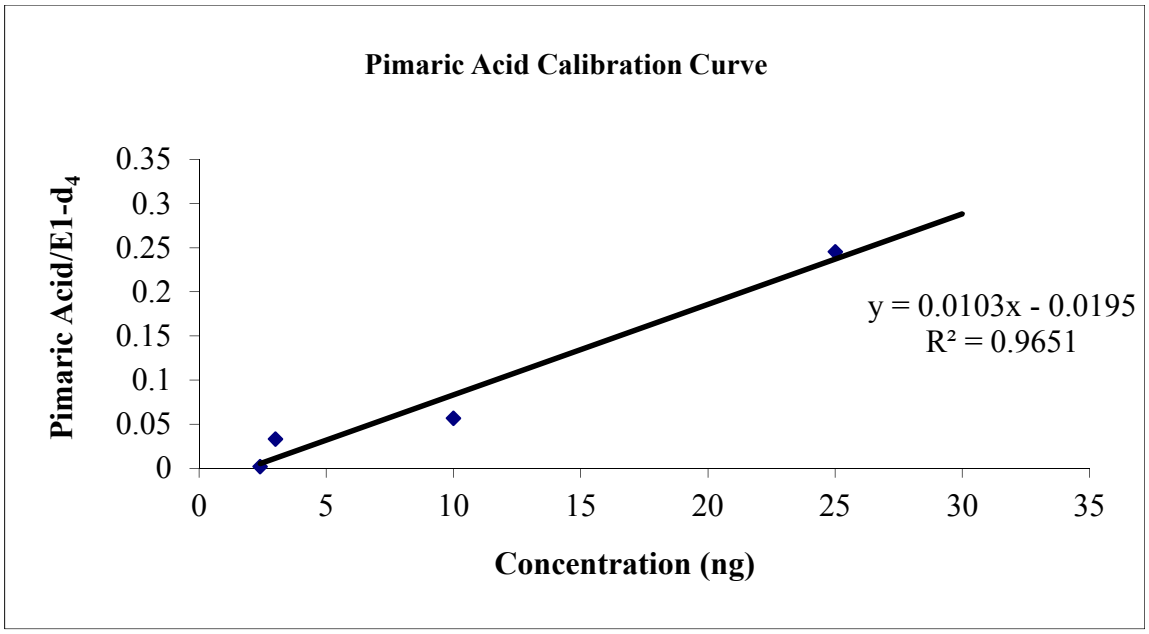

Isopimaric Acid Calibration Curve 1
y = 2E-05x + 0.0029
R² = 0.9934
Isopimaric Acid/E1-d4
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0
500
1000
1500
2000
2500
3000
Concentration (pg)

6A

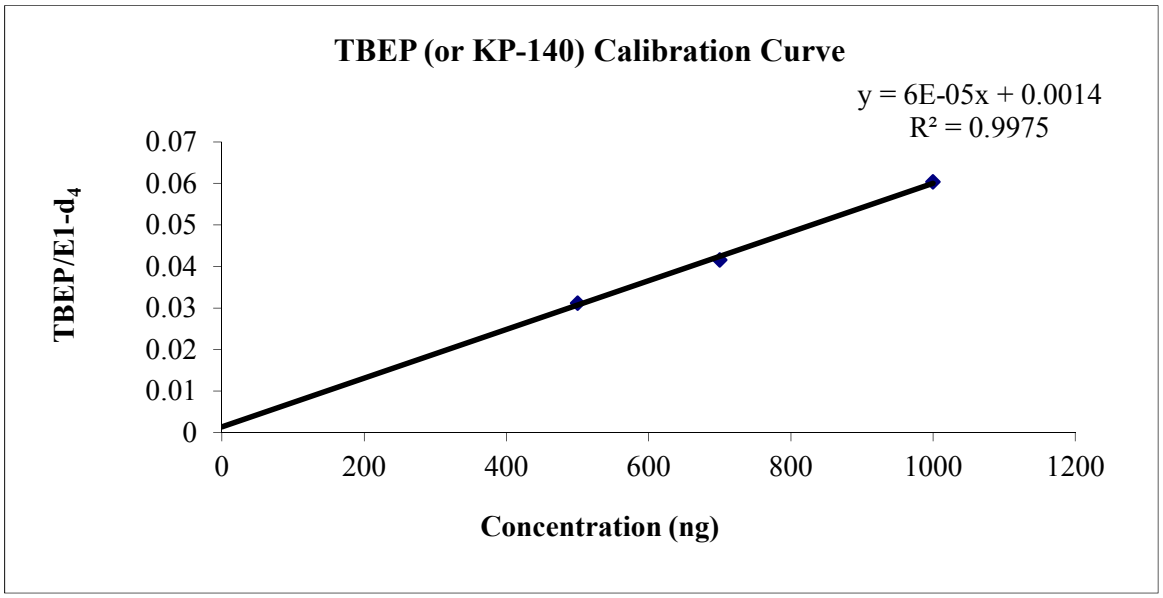

Isopimaric Acid Calibration Curve 2
y = 0.0546x - 0.078
R² = 0.9839
Isopimaric acid/E1-d4
3
2.5
2
1.5
1
0.5
0
-0.5
0
10
20
30
40
50
60
Concentration (ng)

6B

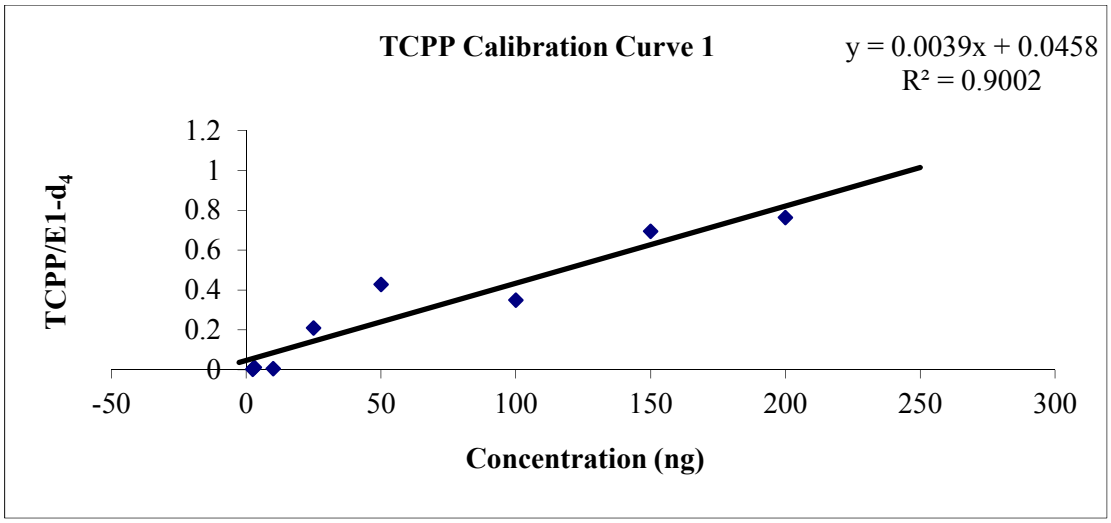
7


Pimaric Acid Calibration Curve
Pimaric Acid/E1-d4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
20
25
30
35
Concentration (ng)
y = 0.0103x - 0.0195
R² = 0.9651

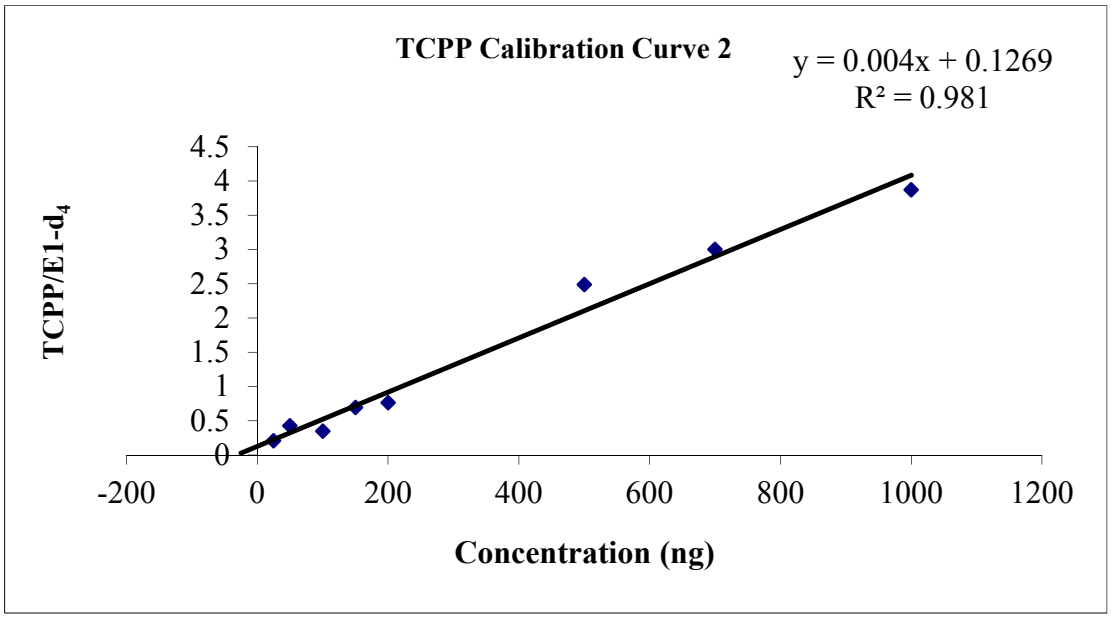
8


TBEP (or KP-140) Calibration Curve
TBEP/E1-d4
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0
200
400
600
800
1000
1200
Concentration (ng)
y = 6E-05x + 0.0014
R² = 0.9975

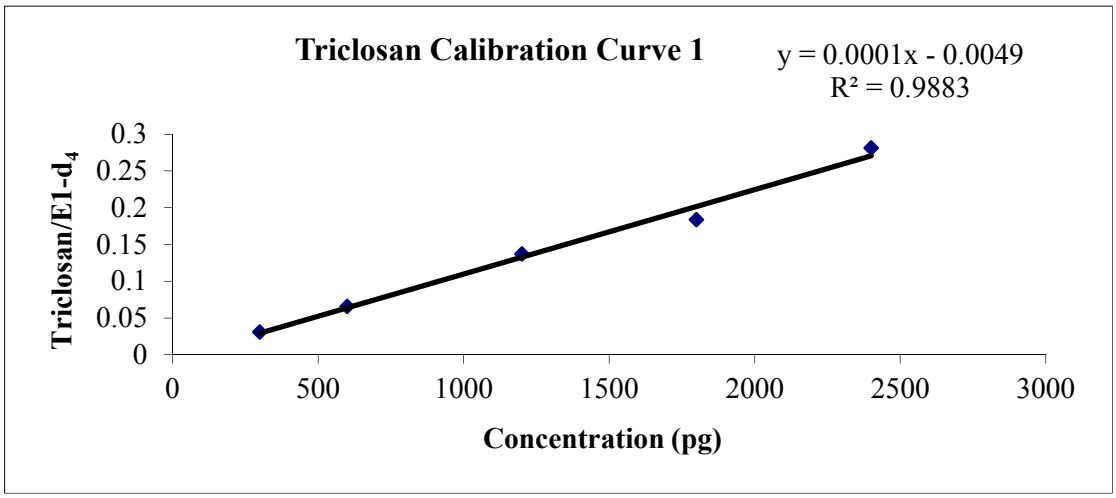
9A

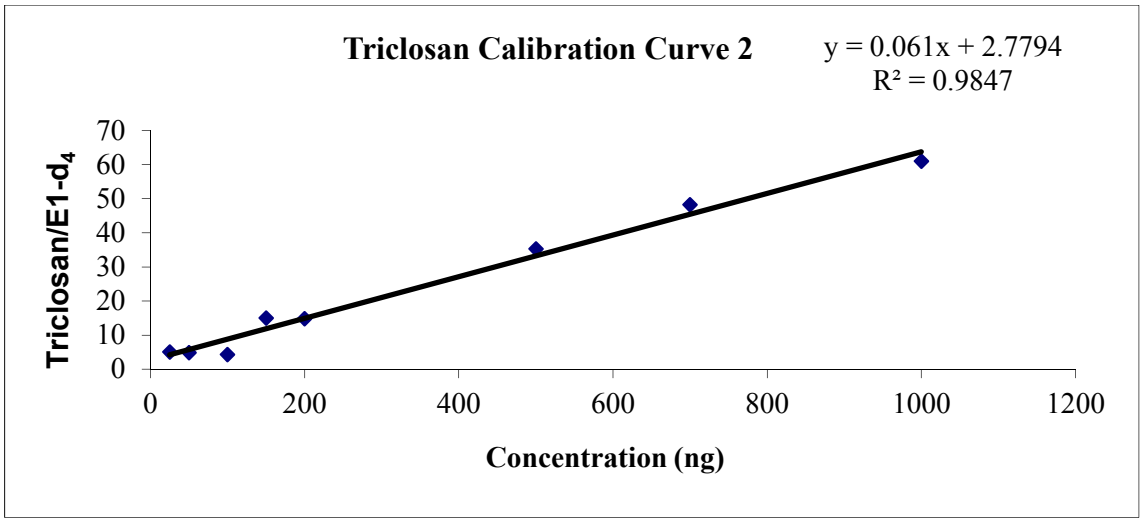

TCPP Calibration Curve 1
y = 0.0039x + 0.0458
R² = 0.9002
TCPP/E1-d4
1.2
1
0.8
0.6
0.4
0.2
0
-50
0
50
100
150
200
250
300
Concentration (ng)

9B


TCPP Calibration Curve 2
y = 0.004x + 0.1269
R² = 0.981
TCPP/E1-d4
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
-200
0
200
400
600
800
1000
1200
Concentration (ng)

10A


Triclosan Calibration Curve 1
y = 0.0001x - 0.0049
R² = 0.9883
Triclosan/E1-d4
0.3
0.25
0.2
0.15
0.1
0.05
0
0
500
1000
1500
2000
2500
3000
Concentration (pg)


Triclosan Calibration Curve 2
y = 0.061x + 2.7794
R² = 0.9847
Triclosan/E1-d4
70
60
50
40
30
20
10
0
0
200
400
600
800
1000
1200
Concentration (ng)

10B

Appendix F: The amount of AA in each fraction of effluent and influent samples analysed.

Fraction	AA of Effluent Fraction(ngFeq/L)	AA of Influent Fraction(ngFeq/L)
27	4100	2200
31	3100	500
32	5400	2000
33	8400	7700
34	4900	7800
35	3900	7600
36	3000	<LOD
37	2800	2500
38	2900	8600
40	2300	3900
45	6600	2400
46	4800	4100
58	2700	4300
59	1500	7200
60	1400	3700