ANALYSIS OF STEROID HORMONES AS ENDOCRINE DISRUPTORS IN SEWAGE, SEAWATER AND MUSSELS USING GC-MS TECHNIQUES

GURUSANKAR SARAVANABHAVAN

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by

Gurusankar Saravanabhavan, M.Sc.

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Abstract

In recent years, there has been an increased concern over the appearance of endocrine disrupting chemicals (EDCs) in the aquatic environment due to their potential reproductive and early developmental toxicity to wildlife population. Municipal and industrial sewage effluents are considered as the major sources of EDCs. Among different classes of EDCs, natural and synthetic steroid estrogens have been identified as the most potent EDCs, as they can induce feminization in fish even at trace levels. Steroid hormones undergo extensive biodegradation during secondary treatment in municipal wastewater treatment processes. However, in Atlantic Canada only 50% of the population has municipal sewage treatment facilities. In many coastal towns and cities, including Halifax, Nova Scotia, and St. John's, Newfoundland & Labrador the raw sewage is directly discharged into harbours. Needless to say, sewage-related contaminants pose a serious threat to the marine ecosystem in these areas.

Mussels are widely used to assess environmental impact of pollutants (metals and organics) in the marine environment. Mussels living near sewage outfalls may be exposed to constant levels of steroid estrogens through their food and respiration. This study examines whether these organisms can be used as a good bio-indicator for steroidal pollution. In this work, the analysis of steroid estrogens (estrone, estradiol, ethynylestradiol, diethylstilbestrol and mestranol) and fecal biomarker coprostanol in raw sewage, seawater and mussels collected from St. John's and Halifax harbours was undertaken. Two analytical methods based on GC- (ion trap) MS/MS were developed for mussel tissue and sewage analysis. A major factor in the success of

method development was the removal of interferences of tissue matrix. Performance characteristics of these methods were evaluated using careful recovery experiments. Percentage recovery of analytes measured by spiking analyte standards in mussel and distilled water were >60% and >80% respectively. Reproducibility of the analytical methods calculated based on relative standard deviation values ranged from 7.7% to 13.3% for the analysis of mussel tissue and 3.0% to 6.8% for sewage effluents.

This study reveals the presence of steroid estrogens estrone (E_1) and estradiol (E_2) and of high levels of coprostanol in seawater samples collected from both harbours indicating extensive fecal contamination and significant steroidal pollution. In addition, estrone (E_1) was measured in mussel samples obtained from some of the harbour locations. Concentration of coprostanol in mussels was used as a qualitative indicator of relative sewage contamination among the sampling sites. Further research should be undertaken to sample a larger number of mussel sites over a longer period to determine whether these organisms are reliable bio-indicators of steroidal pollution.

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My family takes a special place in this section. Their support and encouragement made me very proud of myself today. I am very thankful to my wife Roma for her love and affection. Without her sacrifices this thesis would have been an impossible task for me.

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LIST OF ABBREVIATIONS

APCI	Atmospheric pressure chemical ionization
BHA	Butyl hydroxyanisole
BHT	Butylated hydroxytoluene
BPA	Bisphenol-A
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
CI	Chemical ionization
CID	Collision induced dissociation
DDE	Dichlorodiphenyl dichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DES	Diethylstilbestrol
DNA	Deoxyribonucleic acid
E_1	Estrone
E_2	17β- estradiol
E_3	Estriol
EDC	Endocrine disrupting chemical
EE_2	17α- ethynylestradiol
EI	Electron impact ionization
EPA	Environmental protection agency
ERE	Estrogen response element
ESI	Electrospray ionization
FDA	Food and drug administration
GC- (ion trap) MS/MS	Gas chromatography- (ion trap) mass spectrometry/mass spectrometry
GC-MS	Gas chromatography-mass spectrometry
HPLC	High performance liquid chromatography
LC	Liquid chromatography
LOD	Limit of detection
MSD	Mass selective detector
NP	Nonylphenol
NP1EO	Nonylphenol monoethoxylate
NP2EO	Nonylphenol diethoxylate
NPEO	Nonylphenol polyethoxylates
OP	Octylphenol
PAH	Polycyclic aromatic hydrocarbon
PFPA	Pentafluoro propionic anhydride
POP	Persistent organic pollutant
PVC	Polyvinyl chloride
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RSD	Relative standard deviation
SIM	Selected ion monitoring
S/N	Signal to noise ratio

SPE	Solid phase extraction
SPME	Solid phase microextraction
STP	Sewage treatment plant
TBAH	Tetra butyl ammonium hydroxide
TBT	Tributyltin
TIC	Total ion chromatogram
TLC	Thin layer chromatography
TMCS	Trimethyl chlorosilane
TMS	Trimethyl silyl derivatives

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Dedicated

To

My Family

INTRODUCTION

1.1 Emerging contaminants in the aquatic environment

Water is essential for life on Earth. Although 80% of the earth's surface is covered with water, only one percent of this volume is usable for human consumption. Unfortunately, various man-made chemicals, emerging from different sources, pollute the aquatic system. With the advent of sophisticated analytical techniques, in recent times, many 'new' micro-contaminants have been detected in environmental matrices (Desbrow et al., 1998; Ternes, 1998; Fatoki and Noma, 2002; Matthiessen and Law, 2002). Contamination in aquatic environment is primarily due to the discharge of domestic and industrial effluents into water bodies. Industrialization, population growth, and urbanization put an ever-increasing demand for clean water. This demand leads to recycling and reuse of water. In many places, freshwater needs have been partly fulfilled by introducing municipal and industrial wastewater effluents after adequate treatment into drinking water aquifers and surface water. During dry seasons, it is not difficult to realize that the wastewater effluents constitute a major proportion of water flowing in rivers and other water bodies. Secondly, dumping of municipal sewage into oceans is considered as one of the cheapest way to dispose of sewage. By this method, problems associated with land disposal methods are often overcome. In addition, the organic matter present in the sewage acts as a good food source for the growth of flora and fauna in the sea. Hence, many cities near large marine or inland coastal areas traditionally dispose of their municipal sewage directly into the sea with or without any treatment.

Apart from organic matter, sewage contains many toxic chemicals often at concentrations causing detrimental effects on aquatic organisms. However, only a few priority pollutants such as pesticides and polyaromatic hydrocarbons (PAH) and other well-studied chemicals like disinfectant by-products have been monitored regularly in drinking water and surface waters. Recently, chemicals that interfere with the normal functioning of the natural hormones have attracted the attention of the scientific community (Lee and Peart, 2002). These chemicals are collectively known as endocrine disrupting chemicals (EDCs).

1.2. Endocrine disruption hypothesis

It has been known for quite some time that exposure to certain environmental contaminants can cause reproductive and developmental disorders in humans and wildlife populations. Chick edema disease in herring gulls (Gilbertson, 1983), reproductive impairment in wood ducks (White and Hoffman, 1995), developmental abnormalities in alligators (Guillette et al., 1994) are linked to exposure to persistent organic pollutants (POP) such as polychlorinated biphenyls (PCBs), and dioxin and furans. Likewise, exposure to organochlorine pesticides resulted in a decline in bald eagle populations. Low embryonic survival in bald eagles have been linked with the bioaccumulation of dichlorodiphenyltrichloroethane (DDT), its metabolite dichlordiphenyl dichloroethylene (DDE), chlordane and dieldrin in their eggs (Faber and Hickey, 1973; Bowerman et al., 1995). Although many such pollutants have been implicated with reproductive abnormalities in wildlife, no concrete mechanism was established to explain the biological action of these chemicals on the organisms.

A common thread between all these incidents emerged in the 1990s. Ability of certain environmental chemicals to interact with the endocrine system of an intact organism has been implicated in all the observed field evidence (Colborn et al., 1993). Endocrine disruption phenomenon is not limited to wildlife populations. Humans have an endocrine system that is very similar to many other vertebrate animals. Hence, endocrine disruption is very likely to affect humans in a similar way as in wildlife populations (Colborn et al., 1993; Phillips and Harrison, 1999). In the book "*Our Stolen Future*", Colborn et al. (1996), narrates the reproductive effects of many of the environmental contaminants in wildlife and their implications for humans.

1.3 Endocrine disrupting chemicals and pharmaceuticals

The endocrine system comprises a group of glands that secrete chemical signaling agents called hormones. Many natural and synthetic chemicals released in the environment are found to interfere in the normal functioning of the hormones and hence are called endocrine disruptors. Environmental Protection Agency (EPA, US) defines an endocrine disrupting chemical (EDC) as "any exogenous chemical that can affect the synthesis, secretion, transport, binding, action or elimination of the natural hormones that are responsible for the maintenance of homeostasis, reproduction, development and behavior" (EPA report, 1997). As evidence for reproductive disorders in aquatic and wildlife populations has accumulated over the years, chemicals that are capable of disrupting natural sex hormones have been studied thoroughly. Contaminants that disrupt the functions of the female sex hormone 17β-estradiol (E_2) are commonly referred to as 'environmental estrogens'. Myriad

chemicals, structurally dissimilar to natural estrogens have been found to elicit estrogenic response in fish and other aquatic organisms. Table 1.1 shows some important classes of estrogenic chemicals, their primary use, and the probable source of entry into the aquatic system. Some of these newer chemical classes are discussed briefly here.

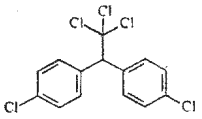
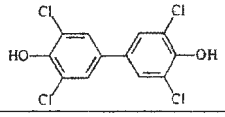
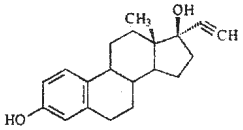
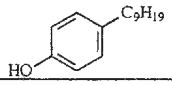
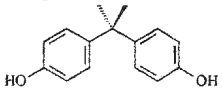
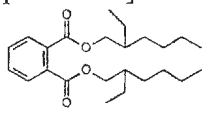
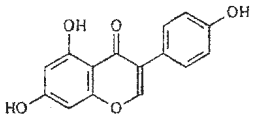
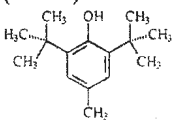
(1). Synthetic hormones are a class of pharmaceuticals designed purposely to interact with the human endocrine system. They have been used to cure many medical disorders resulting from hormone imbalance such as post-menopausal symptoms, and osteoporosis. Synthetic hormones like diethylstilbestrol (DES) are widely used as growth promoters in cattle. Synthetic estrogens such as 17α -ethynylestradiol (EE_2) combined with progestin are used as active ingredients in contraceptive pills (Goldzieher and Rice-Wray, 1966). About 88 kg of these estrogens are used annually in the US alone in the manufacture of oral contraceptive pills. Compared to natural estrogens, synthetic hormones degrade slowly in the environment. The presence of these drugs in sewage has been reported as early as 1980 (Tabak et al., 1981). Arcand-Hoy et al. (1998) estimated the expected introduction concentration (EIC) of EE_2, E_2 (see Figure 1.4 for structures) in wastewater treatment facilities based on their usage in various pharmaceutical products. EIC for EE_2 and E_2 are calculated to be 2.16 ng/L and 14.2 ng/L respectively. Moreover, estrogenic potency of EE_2 is higher than that of E_2 (Fang et al., 2001), and hence its occurrence in the aquatic environment is of great concern.

(2). Phytoestrogens are a group of compounds of natural origin that raised concern due to their ability to mimic human estrogens. Plants produce them as a defense

mechanism against ultraviolet radiation. Humans and herbivores are exposed to phytoestrogens primarily through their diet. However, studies showed that when phytoestrogens are consumed as a bulk portion of the meal, they could affect the fertility in animals (Phytoestrogens, 2001). Two major classes of phytoestrogens widely studied are lignans and isoflavones. Some of the commonly encountered phytoestrogens, such as genistein, daidzein, and entrodiol, are found in fruits (dates, cherries, apple) vegetables (beans, carrots potatoes), seasonings (garlic) and grains such as soyabeans (Phytoestrogens, 2001). Phytoestrogens are readily metabolized and excreted by the body. Unlike synthetic hormones, these compounds degrade quickly in the environment. Interestingly, phytoestrogens are shown to have beneficial effects on human health. Many of these compounds are now being considered for treatment in different types of cancers. Therefore, the overall benefits and health risk of these compounds are still a matter of debate (Barrett, 1996).

(3). Often referred as xenoestrogens, industrial chemicals form the largest group of estrogenic chemicals. It is interesting to note that the estrogenic activity of some of these chemicals was discovered accidentally when they fouled estrogen bioassays in laboratories (Soto et al., 1991; Krishnan et al., 1993). Subsequently, many bioassay techniques have been standardized to test the estrogenic activity of industrial chemicals. Some of the chemicals that are frequently encountered in the aquatic environment are discussed briefly below.

Table 1.1. Types of estrogenic chemicals, their uses and the entry route in aquatic system

Chemical class	Representative chemical	Primary use	Major sources
Pesticides	DDT 	Agriculture, domestic use	Agricultural run-offs, domestic sewage effluents
PCBs/Dioxins	3,3',5,5' –Tetrachloro- 4,4'-biphenyldiol 	Heat transfer fluids	Industrial effluents
Synthetic hormones	Ethynylestradiol 	Contraceptive pills, hormone replacement therapy	Domestic sewage effluents
Alkyl phenols	Nonylphenol 	Industrial surfactants and detergents	Industrial sewage effluents
Bisphenols	Bisphenol-A 	Production of polycarbonate polymers, dental sealant	Domestic as well as industrial sewage effluents
Phthalate esters	DEHP [bis(2-ethylhexyl) phthalates] 	Plasticisers	Industrial effluents
Phytoestrogens	Genistein 	Occurs naturally in plants	Domestic sewage effluents
Organometallics	Tributyltin compounds $Sn(C_4H_9)_3^+$	Anti-foulants, wood preservatives	Direct contamination of seawater
Other phenolic compounds	Butylated hydroxytoluene (BHT) 	Anti-oxidants	Domestic sewage effluents

(a). Alkylphenol polyethoxylates (APEs) are one of the most widely detected industrial contaminants in the aquatic environment (Sole et al., 2000; Bennett and Metcalfe, 2000; Ferguson et al., 2001). These compounds are widely used in domestic and industrial cleaning detergents. More than 500,000 tons of APEs are produced annually worldwide. Nonylphenol polyethoxylates (NPEO) represents 80% of this total volume. NPEO consists of nonylphenol attached to varying numbers of ethylene oxide (between 1 to 100) units. Upon biodegradation, NPEOs form free nonylphenol (NP), mono- and di- ethoxylates (NP1EO, NP2EO) and their corresponding carboxylic acids (Ahel et al. 1994a, 1994b). These compounds are detected in low μg/L concentrations in industrial effluents (Bennett and Metcalfe, 2000; Ferguson et al., 2001). Moreover, these metabolites with short ethoxylate side chains are found to be highly lipophilic. Bioaccumulation of NP1EO, NP2EO and NP has been reported in various aquatic organisms (Staples et al., 1998). Although, these compounds are very weak estrogens, their ubiquitous presence in the environment makes them an important class of estrogenic chemicals.

(b). Bisphenol A (BPA) is another industrial contaminant often encountered in sewage. About 650 million tons of BPA have been used annually in Europe alone. It is a monomer for the production of polycarbonate plastics. Polycarbonates are used for the production of variety of household articles such as polycarbonate milk bottles, tableware, etc. It is also used in the liners of canned food to prevent the direct contact between the metal and preserved food. BPA based sealants are used in some dental resins formulations (Bisphenol A, June 2000). Recent studies have shown that BPA could leach from some of these products (Gwynne, L., 2000). They are primarily

detected in industrial effluents at concentrations ranging from 0.1 μg/L to 4.09 μg/L (Christiansen et al. 2002). Bisphenol A was found to undergo biodegradation quickly by the action of bacteria in river water with a half-life of about 2-3 days (Kang and Kondo, 2002).

(c). Phthalate esters are used as additives in cosmetics, lubricants (European Council for Plasticisers and Intermediates, 2001) and are the most widely used softeners in toys made from plastics such as polyvinyl chloride (PVC). Phthalates are not tightly bound to the plastic but are present in the mobile component of the plastic matrix. Therefore, these compounds slowly leach from the plastics due to volatilization, surface contact, and mechanical pressure from children toys. Stringer et al. (2000) analyzed phthalates used in soft toys and teethers made from PVC manufactured in different countries. They found that nine phthalate esters commonly used in plastic manufacturing constitute about 10-40% of the toys by weight. Phthalates are known for their anti-androgenic properties and hence suppress the activities of the male sex hormone testosterone (Gray et al., 1999; Raloff, 2000); however, some phthalate esters such as benzylbutyl phthalates are found to be estrogenic (Sonnenschein and Soto, 1998). Although they are not highly persistent in the environment, due to their widespread use, they are often detected in the aquatic environment.

(d). Phenolic anti-oxidants such as butylated hydroxytoluene (BHT) and butyl hydroxyanisole (BHA) are used to prolong the shelf life of foodstuffs. They are mainly added as preservatives to fat rich food products such as butter, cereals, and meat. Estrogenic activity of these compounds has been identified using the E-SCREEN assay (Soto et al., 1995). BHA is widely used as an antioxidant to control

oxidation of short chain fatty acids in coconut and palm oils. The maximum allowed level of BHA in various foodstuffs permitted by the food and drug administration (FDA) varies from 50 ppm in cereals to 1000 ppm in active yeast (Sonnenschein and Soto, 1998).

(e). Tributyltin (TBT) is a biocide used as an antifouling agent in paints applied to ship hulls to avoid marine organisms from attaching to the hull. It is also used in wood treatment and preservation, and as anti-fungal agents in textiles, and breweries. TBT is highly toxic to many invertebrates such as snails. Female species are the most affected on exposure to TBT and develop male characteristics (Maguire, 2002). Exposure to TBT results in a variety of developmental abnormalities in aquatic organisms such as mussels, clams, and oysters (Depledge and Billinghurst, 1999). Many countries, including Canada restrict the use of tributyltin, to some degree.

1.4. Endocrine disruption in the aquatic environment

In the past decade, there has been an increased concern over the appearance of endocrine disrupting chemicals in the aquatic environment and their biological effects on aquatic organisms. Increasing incidence of developmental and reproductive abnormalities in fish and other aquatic vertebrates has been reported worldwide. (Guillette and Crain, 2000; Kime, 1998). Recently, Oberdorster and Cheek (2000) reviewed the endocrine disruption observed in marine and estuarine organisms with particular emphasis on invertebrates.

Jobling et al. (1998) reported widespread occurrence of intersexuality (simultaneous presence of both male and female gonadal characteristics) in fish (roach, *Rutilus*

rutilus) throughout the United Kingdom. Particularly, fishes captured downstream of sewage treatment plants (STPs) were affected tremendously. Moreover, the number of male fishes with normal testes growth was found to be very less indicating that the 'feminization' of male fishes are the root cause of intersexuality. Exposure of roach fish to treated sewage effluent in laboratory studies resulted in feminization, confirming the presence of estrogenic chemicals in sewage effluents (Rodgers-Gray et al., 2001). Similar sexual disruption has been observed in other species of fishes as well (Kime, 1998).

Exposure to estrogenic chemicals can potentially lead to hormone imbalance in aquatic organisms. Folmer et al. (1996) reported alterations in normal sex hormone levels encountered in the fish captured near STP outfalls. In female fish, estrogens induce synthesis of egg yolk protein vitellogenin. Vitellogenin appears as a redundant protein in males. Therefore, an increased level of vitellogenin in male fish is widely considered as an indication of exposure to estrogenic chemicals (Folmar et al., 1996; Jobling et al., 1998). When caged fishes were exposed to STP effluents, higher plasma concentration of vitellogenin was observed in different species of male fishes (Rodgers-Gray et al., 2000; Harries et al., 1997).

Different laboratory investigations have been reported to supplement these field evidences. When roach fishes were exposed to the natural sex hormone 17β- estradiol (E_2) at concentrations between 1 and 10 ng/L, higher plasma concentrations of vitellogenin were observed (Routledge et al., 1998). Similar vitellogenin induction was seen in fishes exposed to suspected estrogen mimics as well. Exposure to alkylphenols and bisphenol A increased plasma vitellogenin levels and inhibited

testicular growth in different species of fish (Jobling et al., 1998; Tabata et al., 2001). Synthetic hormone 17α-ethynylestradiol (EE_2) induced vitellogenin synthesis in male fish at concentration as low as 1 ng/L (Purdom et al., 1994).

Information on endocrine disruption in aquatic invertebrates is very limited. The endocrine system of invertebrates is more complex involving different types of hormones. Among them, ecdysteroids and terpenoids are the major classes of steroid hormones occurring in crustaceans, molluscs, annelids and nematodes. Their functional role in invertebrates is similar to that of vertebrate-type sex hormones in vertebrates. Ecdysteroids are responsible for the growth, differentiation, reproduction and molting, and synthesis of vitellogenin while terpenoids regulate male differentiation and embryogenesis (LeBlanc, 2000). In addition, vertebrate-type sex steroids (estrogens and androgens) have been identified in crustaceans (Reis-Henriques et al., 1990). In laboratory experiments, administration of vertebrate sex steroids has been found to alter the endocrine system of certain invertebrates, implying their role in the development of gonads, eggs and secondary sex characteristics. For example, injection of estradiol in starfish (*Sclerasterias mollis*) caused an increase in the size of the oocytes and ovarian protein levels (Barker and Xu, 1993). Likewise, the administration of testosterone to female crabs has stimulated the conversion of ovaries to testes (Sarojini, 1993). Mussels contain vitellogenin- like egg yolk proteins that are inducible by female sex hormones. Blaise et al. (1999) reported the induction of this protein by the vertebrate sex hormone estradiol (E_2).

Endocrine disruption in different species of invertebrates has been observed both in field and laboratory investigations. Depledge and Billinghurst (1999) presented a

concise account on endocrine disruption in marine invertebrates. Exposure of freshwater mussels to sewage effluents resulted in an increase in vitellogenin levels in their hemolymph and the gonads (Gagne et al., 2001). Similar increase in vitellogenin levels was seen in a downstream site during *in-situ* exposure of caged mussels to municipal effluents (Gagne et al., 2001). Exposure of barnacles (*Elminius modestus*) to nonylphenol (NP) in the laboratory resulted in the disruption of timing of larval development (Billinghurst et al., 2001).

Invertebrates exposed to certain endocrine disrupting chemicals are found to develop a condition known as imposex. Imposex is defined as the imposition of morphological characteristics of one sex onto another. Imposex can interfere in the ability to release eggs in females and suppress oogenesis (LeBlanc, 2000). Field evidence confirms that exposure of female snails to TBT compounds result in the development of full-fledged penis (Matthiessen and Gibbs, 1998). At concentration as low as 0.5 ng/L, TBT caused the development of penis in female dogwhelk (*Nucella lapillus*), which blocked the oviducts and prevented reproduction (Maguire, 2002).

1.5. Endocrine disruption

1.5.1. Hormone action

Hormones play a vital role in the regulation of various biochemical activities in humans and animals. Major functional domains of hormones are (i) Reproduction (ii) Growth and development (iii) Maintenance of internal environment and (iv) Production, utilization and storage of energy (Wilson et al., 1998; Bayliss, 2002). Hormones formed from cholesterol are called steroid hormones. Adrenal and gonadal

hormones fall in this category. During the biosynthesis of sex hormones, cholesterol is first converted into pregnenolone that serves as a precursor for the synthesis of sex hormones. In gonads, pregnenolone is converted to predominant male hormone testosterone through a series of enzymatic reactions. In females, testosterone is subsequently converted to the female sex hormone 17β-estradiol (E_2). Hormone synthesis is highly regulated by various feedback mechanisms (Wilson et al., 1998).

Sex hormones bind to plasma binding proteins such as sex hormone binding globulins in blood and are transported to different target tissues. At the target cells, the hormones cross the cell membrane and bind to the specific receptors to form receptor–steroid complex. The receptor-steroid complex binds to chromatin (DNA and associated proteins) in the cell nucleus, stimulating the synthesis of specific RNAs and proteins. Although hormone receptors show a high affinity towards respective steroids, they do not display absolute specificity. Hence, different ligands with similar structure to that of steroids can bind to the same receptor.

1.5.2. Mechanism of endocrine disruption

An endocrine disrupting chemical (EDC) can interfere in the functioning of the hormone system in a variety of ways. Moreover, as the hormone action is a multi-stage process, some chemicals can attack the hormonal system at more than one stage. Based on the known mechanism of endogenous hormone action, an endocrine disruptor can be visualized to elicit a biological response by one or more of the following ways (WWF report, Canada):

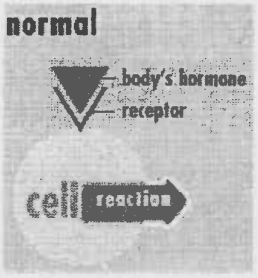
(1). Alteration in the production of endogenous hormones: EDCs can alter the steroidogenesis (synthesis of steroid hormones) by altering the availability of cholesterol to begin steroidogenesis or by altering the activities of the enzymes involved in the process. In addition, EDCs are known to interfere in the feedback mechanisms regulating the hormone synthesis. This would cause the secretion of hormones at the wrong time or disturb the time span of hormone production (Crain et al., 2000).

(2). Alteration in the bioavailability of the hormones: Bioavailability of endogenous hormones at target cells depends on many factors including plasma and tissue concentration, sequestration by binding proteins, and hepatic metabolism. For example, the concentration of free estrogens in the bloodstream as well as inside the target cell is governed by the concentration of steroid-binding proteins. The concentration of steroid-binding proteins is in turn controlled by a feedback mechanism and dependant on the concentration of free estrogens. Therefore, EDCs that mimic natural estrogens could give a false signal for the production of an excess of steroid-binding proteins thereby affecting the concentration of endogenous estrogens at the target sites.

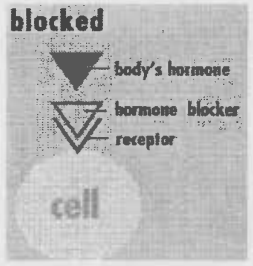
(3). Mimicry of endogenous hormones: It is widely accepted that the first step in the action of a hormone disruptor is it's binding to the receptors at the target sites. However, some chemicals are known to elicit biological response in an independent pathway without binding to the receptors (Zacharewski, 1997). Binding of EDCs to the receptors can affect the action of endogenous hormones in different ways as shown in Figure 1.1. Estrogenic chemicals can bind to any of the three types of

estrogen receptors, α, β_1, β_2 or to the two different estrogen response elements in DNA of the target cells (Purchase and Randall, 1998). The binding then causes alteration in mRNA transcription and gives a corresponding change in protein synthesis. The magnitude of cellular response produced by EDCs depends on many factors and could be stronger or weaker than that of natural hormones. Alternatively, EDCs can just block the receptor site thereby inhibiting the binding of natural hormone. This will result in the suppression of hormone response in the organism.

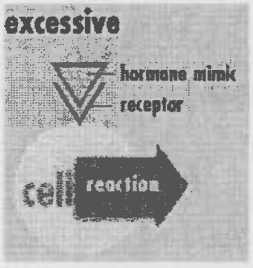
Different quantitative structure-activity relationship models (QSAR models) have been developed (Tong et al., 1997) to understand the binding of various estrogenic chemicals to estrogen receptors. In Figure 1.2, the relative binding affinity of some of these chemicals with respect to estradiol is shown. The binding affinity of a given pollutant to the estrogen receptor is found to depend on two main factors such as (i) Presence of phenolic group with hydrogen bonding ability (similar to the phenolic group in 17β-estradiol (E_2), Figure 1.2); and (ii) A hydrogen bonding donor group, like hydroxyl group of E_2, at 17^{th} carbon, and the precise distance between these two hydrogen bonding groups (Fang et al., 2001). In addition to these requirements, the steric hydrophobic centers at 7^{th} and 11^{th} carbon and general hydrophobicity are also some of the other parameters that influence the binding affinity.



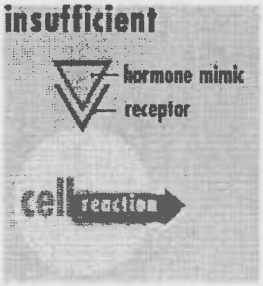
Normal hormone activates the receptor at the appropriate level



Hormone blocker interferes with the signal from the body hormones



Hormone disrupters give a signal stronger than the body's hormone (and at the wrong time), or --



Hormone disrupter gives a signal weaker than normal, also at the wrong time.

Figure 1.1. Action of endocrine disruptors.
An endocrine disruptor can mimic the natural hormones in different ways. They can act either as hormone agonist or antagonist as shown. (Source: Reproduced with permission from http://www.som.tulane.edu/ecme/eehome/)

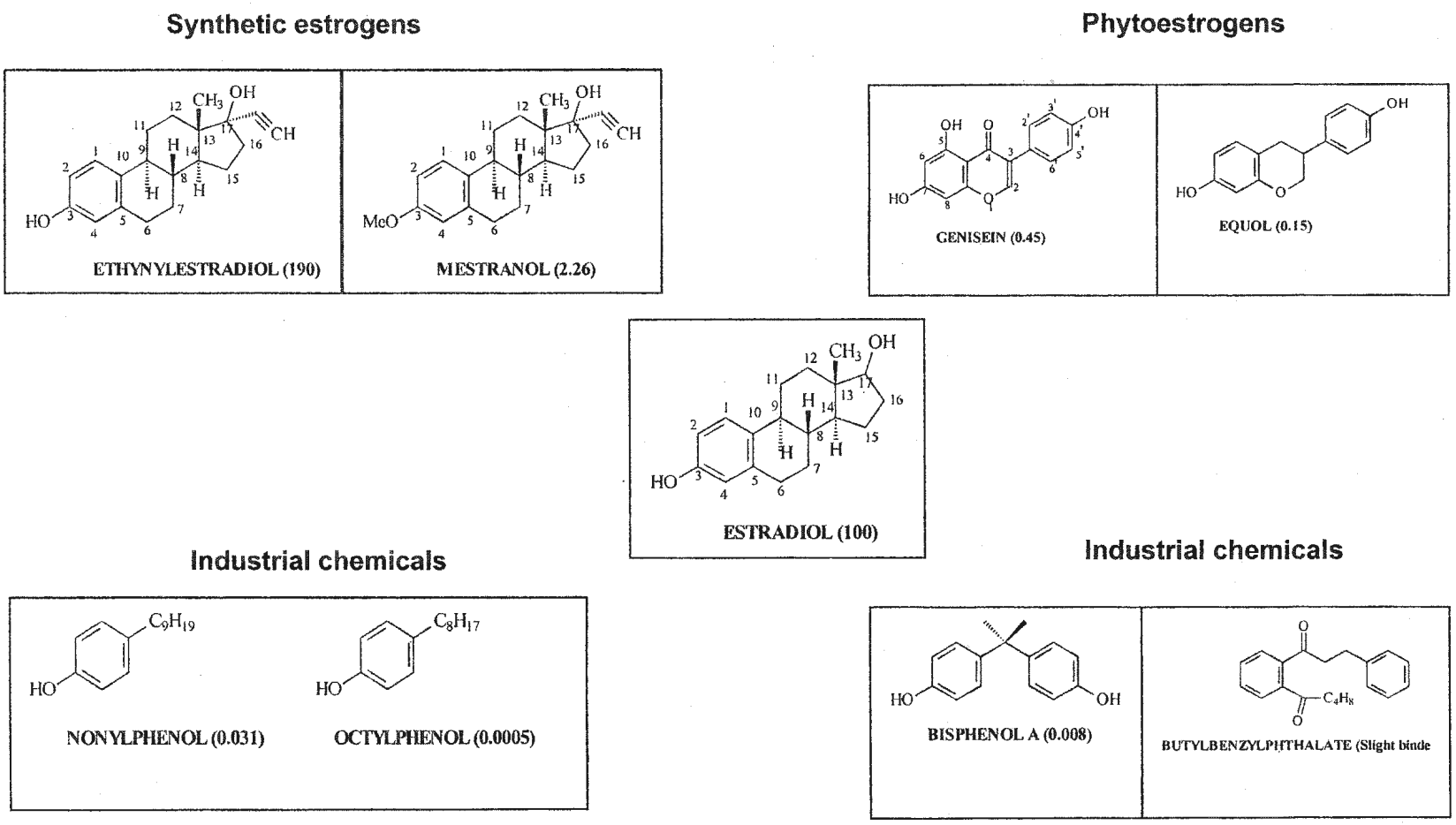


Figure 1.2. Structural similarities between different types of environmental estrogens and the natural estrogen estradiol. The estrogenic potency measured using estrogen receptor (ER) competitive binding assay is indicated in the parentheses (Fang et al., 2001).

Exposure to EDCs can give rise to both reversible and irreversible health effects in an intact organism. Generally, reversible endocrine disrupting effects are observed when a fully developed organism is exposed to EDCs for a shorter duration. These effects will disappear once the organism is removed from the exposure scenario (Sonnenschein and Soto, 1998). In contrast, exposure to EDCs during early developmental stages (for example, exposure in fetus) leads to permanent damages in the organism. The effect of synthetic estrogen, diethylstilbestrol (DES) in humans is one of the many examples to show such effect in the human populations. The first undisputed evidence of endocrine disruption by DES was reported in 1971. DES was given to about 5 million pregnant women across the world in the 1950s to avoid spontaneous abortion during pregnancy. When they attained puberty, the children of these 'DES mothers' developed a multitude of health effects due to their exposure to DES when they were in their mother's womb. The daughters of these women, when they grew up, developed clear cell adenocarcinoma (CCA), a rare form of cervical cancer (Herbst et al., 1971). The sons of these "DES mothers" suffered from several permanent changes in their genital tract (National Research Council, 2000).

1.5.3. Screening for estrogenic chemicals

About 87,000 compounds are listed for initial sorting in the EPA's endocrine disruptor screening program. This list includes 900 pesticides, 75,000 industrial chemicals, and 8,500 cosmetics and food additives. To evaluate the endocrine disruption of such a large number of chemicals, a multi-tier screening approach has been devised (EPA, 2002). Many *in-vivo* and *in-vitro* bioassay techniques have been

developed for the screening of chemicals. For initial sorting, in-vitro bioassays are preferred over *in-vivo* bioassays as the former method is rapid, simple, and inexpensive (Zacharewski, 1997). However, *in-vitro* techniques do not take the pharmacokinetics of the chemicals into consideration and therefore cannot duplicate the *in-vivo* conditions. Therefore, *in-vitro* assays should be used as complementary and not as a substitute for *in-vivo* testing procedures (Jobling, 1998).

Different types of *in-vitro* bioassays have been developed based on the known mechanism of action of estrogens on estrogen responsive cells. Among them, assays based on the binding to estrogen receptor (receptor binding assays), proliferative effects of estrogens on breast cells (cell proliferation assays commonly known as E-SCREEN assay), expression of proteins such as vitellogenin (protein expression/enzyme activity assays), expression of the estrogen response element (ERE) – regulated reporter gene assays are widely used. The methodologies, merits and weaknesses of each bioassay are reviewed by Zacharewski, (1997).

Often, estrogenicity of the suspected chemicals is evaluated using different bioassays utilizing different endpoints. Therefore, interpretation of the data from bioassays, to predict the estrogenic activity of the chemicals in humans and other species is extremely problematic. To overcome this difficulty, Calabrese et al., (1997), proposed a relative ranking scheme by taking into account (a) The type of endpoints used in the bioassay; (b) Ability to induce estrogenic activity in *in-vivo* testing; (c) Estrogenic activity in whole animal models; and (d) Estrogenic potency measured using ER binding affinity. Figure 1.3 shows the estrogenic potency of different chemicals calculated based on this scheme in fish, human and birds. In the real world scenario,

Summary of Relative Ranking Calculations for Selected Agents

Agent	Number of bioassays	Stages represented[a]	Percentage estradiol equivalent based on phylogenetic relationship to		
			Human	Fish	Bird
DES	7	1–4	86.2	86.6	84.3
2',4',6'-trichloro-4-hydroxybiphenyl	3	1, 4, 5	62.6	59.9	59.9
Methoxychlor	3	1, 5	52.7	49.6	49.6
β-Sitosterol	2	3, 4	51.5	51.5	47.4
Kepone	6	1, 3–5	24.0	22.3	24.8
Octylphenol	6	1–4	19.5	18.7	16.7
Coumestrol	5	2–5	18.8	16.9	15.3
Nonylphenol	8	1–5	17.3	16.5	14.5
Bisphenol-A	6	1–4	15.5	9.4	9.4
Endosulfan	5	1–4	13.3	6.1	6.1
NP2EO	7	1–4	12.9	12.2	10.5
Toxaphene	4	1–4	12.9	5.9	5.9
BBP	2	1, 2	9.8	9.8	6.8
Genistein	4	1–3	10.6	8.4	6.4
NP1EC	7	1–4	9.6	8.8	7.1
BHA	2	1, 2	6.1	6.1	2.1

Note. DES, diethylstilbestrol; *o,p'*-DDT, *o,p'*-dichlorodiphenyl trichloroethane; NP2EO, 4-nonylphenoldiethoxylate; BBP, butylbenzyl phthalate; NP1EC, 4-nonyl-phenoxycarboxylic acid; BHA, butylated hydroxyanisole.

[a] Refers to the five stages identified in the process of endocrine disruption.

Figure 1.3. Relative ranking of estrogenic chemicals (Source: Reproduced with permission from Calabrese et al., 1997)

however, several other environmental factors such as fate and dispersion of the chemicals, their bioaccumulation potential in organisms and their possible biomagnification in the food chain are considered before prioritizing pollutants for regulatory activities.

1.6. Fate of steroid hormones in municipal sewage treatment plants

Estrogens are the primary female sex hormones secreted by all vertebrates including humans. Both men and women secrete estrogens, however, men do it to a lesser extent compared to women. The amount of estrogens secreted by men and women changes during different stages of the human life cycle. Table 1.2 shows the average urinary excretion rates for men and women at different stages (Johnson et al., 2000). Hepatic metabolism of natural estrogens occurs via 16α -hydroxylation and 2-hydroxylation pathways. For example, 16α -hydroxylation of estradiol produces estriol (E_3). Estrogens undergo conjugation reaction predominantly with glucuronic acid and to a lesser extent with sulphonic acid before their excretion. The synthetic estrogen 17α -ethynylestradiol does not undergo hepatic metabolism, but undergoes conjugation reactions before excretion (Christiansen et al., 2002). The majority of estrogens are excreted in urine while some of them are excreted in feces. Estrogen conjugates are cleaved by β-glucuronidase enzyme produced by E.coli bacteria in the gut and hence excreted in free form in the feces. In the conjugated form, estrogens lose their biological activity.

Numerous studies have reported the presence of free estrogens in STP influents, effluents and receiving waters (Lee and Peart, 1998; Snyder et al., 1999; Sole et al.,

2000). Moreover, no significant amount of conjugated estrogens was detected in STP effluents (Belfroid et al., 1999). As mentioned, β-glucuronidase enzyme produced by E. coli bacteria cleaves estrogen conjugates (glucuronides) and liberate free estrogens. As these bacteria are abundant in sewage, it is hypothesized that estrogen de-conjugation will occur in sewers and STP thus releasing biologically active estrogens into effluents and surface waters. Baronti et al. (2000) measured the concentration of natural steroids for 5 months in six activated sludge sewage treatment plants (ASSTP) in Rome. The authors found that the concentration of the steroids were much lower in the effluents compared to that of influents indicating efficient biodegradation. Recently, many studies on the biodegradation of free and conjugated estrogens have been reported in the literature (Panter et al., 1999; Ternes et al., 1999b; Layton et al., 2000). Ternes et al. (1999b) found that estrogen conjugates are transformed quickly to free estrogens in activated sludge. During biodegradation, estradiol (E_2) is first oxidized to estrone (E_1), which then undergoes mineralization (Jurgens et al., 2002). Furthermore, the synthetic estrogen 17α -ethynylestradiol (EE_2) is found to undergo biodegradation in activated sludge slowly compared to estradiol (Ternes et al., 1999b). Layton et al., (2000) studied the rate of mineralization of steroidal hormones by biosolids present in water treatment plants and found that 17α -ethynylestradiol undergoes mineralization 25 to 75 times slower than 17β-estradiol.

Few studies have investigated the rate of removal of steroid estrogens in sewage treatment plant facilities. Wastewater treatment with activated sludge was found to be efficient in removing steroid hormones (Baronti et al., 2000). Ternes et al. (1999a) found that trickle filter based secondary treatment was less effective than activated

sludge treatment process for estrogen removal. As expected, high levels of estrogens were detected in STP effluents that underwent only primary treatment (Desbrow et al., 1998). Removal of organic contaminants in STPs is found to depend on many factors such as sorption, chemical degradation, biodegradation and volatilization (Rogers, 1996). Using fate & distribution models, Mastrup et al. (2003) predicted that more than 60% of EE_2 entering into the sewage treatment plant (STP) would be found in the effluents. Based on the concentrations of estrogens in influents and effluents in six STPs using activated sludge treatment, Baronti et al. (2000) estimated estrogen removal rates at 95 ± 6%, 87 ± 9% and 85 ± 14% for E_3, E_2, and EE_2 respectively. Nasu et al. (2001) studied the concentration of many endocrine disrupting chemicals in the influents and effluents of 27 sewage treatment plants in Japan. They found that estradiol (E_2) was removed less effectively with a removal rate of 64% - 69% in STPs. Ternes et al. (1999a) compared the estrogen removal rates in STPs in Brazil and Germany. They found that the estrogen removal rates in German STPs were lower (about 65%) than that measured in Brazil (above 78%). Low temperatures during sampling periods are found to be the contributing factors to the low removal rates at German STPs.

1.7. Mussels as bioindicators of environmental estrogens

Many aquatic organisms living in the marine environment are constantly exposed to man-made chemicals. In particular, marine invertebrates concentrate contaminants

Table 1.2. Average urinary excretion of estrogens by humans. Estrogens are excreted predominantly as their glucuronide conjugates in urine (Source: Taken from Johnson et al., 2000).

Estrogen	Urinary excretion per day (μg)			
	Men	Menstruating women	Pregnant women	Menopausal women
Estrone (E_1)	3.9	8	600	4.0
Estradiol (E_2)	1.6	3.5	259	2.3
Estriol (E_3)	1.5	4.8	6000	1.0

both adsorbed onto the particles and present freely in water column due to their limited capability to metabolize chemicals. Among them, mussels are one of the widely used bio-indicators to assess environmental contaminants in the aquatic environment (Gulf of Maine Environmental Monitoring Program, 1995). This is because,:

(i). Mussels respire water and feed on organic particles suspended in the water column. Therefore, they are exposed to water-soluble and particle bound contaminants present in the aquatic environment;

(ii). They are sedentary creatures and hence the variations in contaminants concentrations due to the mobility of species would be negligible;

(iii). They are a commercially important food source and therefore information on accumulation of chemicals in their tissue is essential for public health safety;

(iv). They are present in abundance and can be easily collected from the shore. Bioaccumulation of various environmental toxins such as PAHs (Marvin et al., 2000: Hellou et al., 2000), metals (Szefer et al., 1997; Regoli and Orlando, 1992), and organochlorine compounds (Ramesh et al., 1990) has been investigated in mussels during various 'mussel watch' programs worldwide. Accumulation of various endocrine disrupting chemicals in mussel tissue have been studied. Wahlberg et al. (1990) studied the uptake of NP, NP1EO, and NP2EO by blue mussel (*Mytilus edulis*) near the wastewater outlet of a surfactant manufacturing company in Sweden. They have detected NP, NP1EO, and NP2EO in mussel tissue at concentrations 0.2 μg/g -0.4 μg/g, 0.15 μg/g -0.25 μg/g, 0.05 μg/g -0.15 μg/g respectively. Bennett and

Metcalfe, (2000) reported similar levels of NP (0.12-0.46 μg/g, wet weight), NP1EO (<LOD-0.12 μg/g, wet weight) and NP2EO (<LOD-0.14 μg/g, wet weight) in freshwater mussels (*Elliptio complanata*) found near STPs in Hamilton harbor. Ferrara et al. (2001) studied the accumulation of octylphenol (OP) and nonylphenol (NP) compounds in seafood in the Adriatic Sea, Italy and found that OP and NP occurs in mussels at levels ranging from 4.4 to 4.9 ng/g (wet weight) and 254 to 265 ng/g (wet weight) respectively.

Mussels are found to bioaccumulate the fecal biomarker coprostanol (5β-cholestan-3β-ol) and hence widely used as bioindicator for the evaluation of impact of sewage discharges in aquatic environment (Sherwin et al., 1993; Cathum and Sabik, 2001; Hellou et al., 2003). Therefore, in this study, mussels (*Mytilus edulis*) were chosen as bioindicators of the presence of sewage-related anthropogenic steroids.

1.8. Present methods of analysis of steroid estrogens

1.8.1. Extraction and cleanup of steroids from tissue samples

Most of the research on the analysis of estrogens has been done on tissue samples from meat and meat products. This reflects the fact that natural and synthetic estrogens are used as growth promoters in cattle and many regulatory organizations control the levels of estrogen residue in meat (Daeseleire et al., 1992). Due to the complexity of the tissue matrix, a series of analytical steps are generally involved in the extraction and analysis of steroids. Different solvents such as acetonitrile, methanol, *tert* -butyl methyl ether, and chloroform/methanol have been used for the extraction of estrogens (Covey et al., 1988; Lagana and Marino, 1991; Hartmann and

Steinhart, 1997; Fritsche et al., 1999). A recent study by Cathum and Sabik (2001) reported microwave-assisted extraction of estrogens using 50% acetone in hexane from mussel tissue.

Several reference methods require an enzymatic hydrolysis step using β-glucuronidase to hydrolyze estrogen conjugates after preliminary extraction (Hartmann and Steinhart, 1997). Different enzyme suspensions such as Helix pomatia have been used to de-conjugate estrogen glucuronides (Covey et al., 1985; Daeseleire, et al., 1998). As only a small portion of the estrogen is found in the conjugated form in tissues, many researchers have questioned the importance of the enzymatic hydrolysis step (Busico et al., 1992; Hartmann and Steinhart, 1997). Moreover, the glucuronide conjugates have been shown to undergo de-conjugation reaction in wastewater and receiving water, and hence exposure of mussels to these compounds would be minimal.

A variety of cleanup procedures have been employed for the subsequent isolation of estrogens from tissue extracts. Stan and Abraham (1980) developed a cleanup method based on liquid-liquid extraction (LLE) of tissue extract between acetonitrile and hexane followed by a silica column cleanup for the analysis of anabolic steroids in meat. Currently, solid phase extraction (SPE) methods are widely employed for cleanup of tissue extracts. Many of the steroid estrogens have a phenolic group in their chemical structure and hence, in some studies, anion exchange resin cartridges were used for selective isolation of estrogens (Covey et al., 1985; Covey et al., 1988). After initial SPE cleanup, Daeseleire et al. (1998) used a combination of SPE and HPLC fractionation for the isolation of estrogens.

Different column chromatographic methods for the purification of estrogens were also developed. Selective isolation of anabolic androgenic and estrogenic compounds from tissue extract was carried out using immunoaffinity column chromatography (Van Ginkel et al., 1989; Bagnati et al., 1990). Cathum and Sabik (2001) purified steroid estrogens in mussel tissue using a silica column cleanup procedure after derivatization with pentafluorobenzyl bromide. Reis-Henriques et al. (1990) used ion-exchange columns (Dowex AG1 X2) for the separation of estrogens from lipids and other neutral steroids in mussel tissue.

1.8.2. Extraction of steroids from water

Methods currently available for the extraction of estrogenic compounds from sewage and STP effluents are recently reviewed by Lopez de Alda and Barcelo (2001a). Extraction of estrogens from water and wastewater samples was predominantly carried out by SPE methodologies. A few studies employed liquid-liquid extraction techniques for the isolation of estrogens from different kinds of wastewater (Tabak et al., 1981; Cathum and Sabik, 2001; Mol et al., 2000). Okeyo et al., (1998) developed a solid phase microextraction (SPME) method for the extraction and analysis of steroid estrogens from water and biological fluids.

Different SPE adsorbents have been investigated for their suitability to extract estrogenic chemicals. Reverse phase materials (C_8, C_{18}) both in cartridge (Desbrow et al., 1998; Lee and Peart, 1998; Lopez de Alda and Barcelo, 2000; Sole et al., 2000) and disc (Snyder et al., 1999; Mol et al., 2000) formats were widely used in sewage and surface water analysis. Other adsorbents such as Graphitized carbon (Baronti et

al., 2000; Lagana et al., 2000) and polymeric adsorbents like polystyrene divinylbenzene disks (SDB-XC) were also used for the extraction of estrogens. The extraction efficiencies of all three types of materials for model estrogens are usually high (above 80%). However, in a recent study, Kuch and Ballschmiter (2000) reported that sampling with amberlite XAD –2 polymeric material led to poor recovery (<40%) for the surrogate standard (cholesterol acetate) in field samples. In this study, good recovery (94%) was achieved using C_{18} adsorbent materials.

In order to avoid clogging, wastewater samples were usually filtered prior to SPE. It was found that the steroid estrogens are not bound to glass fiber materials generally used for filtration (Desbrow et al., 1998). However, as a precautionary measure, some investigators preferred to wash the filter bed with methanol and added the washing to the effluent sample before SPE (Baronti et al., 2000; Lagana et al., 2000).

The volume of water sample processed differs widely and depends on factors like type of water sample (STP influent, effluent, or surface water) and the method detection limit. Usually, less than 500 mL of raw sewage or 1 L of surface water or STP effluent is analyzed for estrogens. However, in certain cases large volume sampling becomes necessary to achieve lower detection limits (Kuch and Ballschmiter, 2000).

1.8.3. Instrumental methods for steroid analysis

A wide variety of instrumental techniques have been used for the analysis of steroid estrogens. Methods based on thin layer chromatography (TLC) were developed in the early 1980s (Tabak et al., 1981) for the analysis of synthetic steroids in STP effluents.

Today, techniques based on modern chromatographic methods such as high pressure liquid chromatography (HPLC) and gas chromatography (GC) are quite common for the analysis of estrogens in environmental samples.

Some of the advantages of HPLC methods such as direct analysis of polar analytes without prior derivatization and the concurrent reduction in overall sample preparation time are very useful. Moreover, HPLC based methods are very easy to automate. Lopez de Alda and Barcelo (2001b) reported a method for on-line detection of estrogens in water samples.

Reverse phase HPLC columns (C_{18}) are generally used for the separation of natural and synthetic steroids. HPLC methods using fluorescence detector (Snyder et al., 1999), and UV diode array detector (Lopez de Alda and Barcelo 2000) are reported for the analysis of estrogenic chemicals in wastewater. Detection limits of these methods are in the order of 50-100 ng/L and therefore useful for the detection of steroid estrogens in samples where the concentration is generally high. HPLC coupled with mass spectrometric detector (MS) has been used to achieve lower detection limits required for the analysis of steroids in surface water samples. Analytical methods based on both atmospheric pressure chemical ionization – mass spectrometry (APCI) and electrospray ionization – mass spectrometry (ESI) have been reported. Sole et al., (2000) compared the efficiency of both these ionization techniques for the analysis of a group of estrogens and progestogens. They found that ESI in negative ion mode is sensitive for estrogens while ESI and APCI (both in positive ion mode) are suitable for progestogens.

Gas chromatography-mass spectrometry (GC-MS) is the most commonly used instrumental method for the analysis of steroids. GC analysis of some estrogens without derivatization is possible, but often leads to poor peak shapes. Therefore, a derivatization step is generally included in trace analysis (Mol et al, 2000). Estrogens are derivatized using different reagents such as bis- (trimethylsilyl)trifluoroacetamide (Okeyo and Snow, 1998; Saravanabhavan et al., 2002;), pentafluropropionic anhydride (Lee and Peart, 1998), heptafluorobutyric anhydride (Huang and Sedlak, 2001), and acetic anhydride (Larsson et al., 1999). GC columns with low polarity phases (DB-5 or its equivalent) are found to be satisfactory for baseline separation for the most common analytes. With GC-MS methods, detection limit between 2 and 5 ng/L for estrogens in wastewater was reported (Lee and Peart, 1998; Cathum and Sabik, 2001). However, to avoid the interference of matrix materials, analytes were purified using elaborate cleanup procedures before GC analysis.

More advanced detection systems such as MS/MS coupled to HPLC or GC have been used to achieve very low detection limits. The utility of GC- (ion trap) MS/MS for the trace analysis of steroids in STP effluents and surface waters have been demonstrated (Belfroid et al., 1999; Ternes et al., 1999a). Baronti et al. (2000) reported a sensitive analytical technique based on LC-MS-MS for the detection of steroid estrogen at concentrations between 8-30 pg/L in surface water samples. Lagana et al. (2000) compared the sensitivity of HPLC-MS-MS and GC-MS methods and found that MS/MS detection (detection limit ~ 0.5 ng/L) was at least one order of magnitude more sensitive for estrogens than a simple MS detection (detection limit between 5-10 ng/L). Croley et al. (2000) compared the sensitivities of LC-MS-MS and GC-MS-MS

methods for the analysis of steroids in serum samples. Both methods are found to be very suitable for trace analysis and 17β- estradiol (E_2) can be quantified in serum samples at levels as low as 10 pg/μL and 5 pg/μL in GC-MS-MS and LC-MS-MS respectively.

1.9. Research objectives

The level of wastewater treatment (primary, secondary or tertiary) varies greatly among provinces in Canada. Almost all municipalities in Ontario have wastewater treatment facilities to treat sewage. On the other hand, in the Atlantic region only 50% of total population have sewage treatment facilities and in the remaining places the sewage is directly disposed into the ocean. It is sad to note that even in major cities in Atlantic Provinces such as St. John's, NL and Halifax, NS only very few wastewater treatment plants exist to treat raw sewage. In St. John's alone, about 33.2 billion liters of raw sewage generated annually is dumped into the ocean (Bonner and Wristen, 1999). Needless to say, sewage related contaminants are causing great concern to the marine ecosystem.

Natural and synthetic steroids are somewhat lipophilic (with $\log K_{ow} \sim 4$) and may therefore partition into lipid tissues of organisms in the environment (Lai et al., 2000). Some of the chemical properties of selected steroids are given in Table 1.3. Mussels living near sewage outfalls in St. John's and Halifax harbours are likely exposed to steroid hormones through their food and respiration. Therefore, it is of interest to examine whether these compounds can be detected in mussels. Such a study will

require a robust analytical method because of the complex nature of mussel tissue and the trace levels of analytes. The objectives of this investigation were,

(1). To develop a sensitive analytical method based on GC-MS techniques for the analysis of natural and synthetic steroid standards spiked in mussels. The target analytes investigated included natural estrogens: i.e. estrone (E_1), 17β- estradiol (E_2), estriol (E_3); and synthetic estrogens, i.e. 17α -ethynylestradiol (EE_2), mestranol, and diethylstilbestrol (DES). Estrone 3-methyl ether was used as internal standard (IS) for quantifying analytes in real samples. Figure 1.4 shows the chemical structure of all the target analytes.

(2). The second objective of this work was to implement the analytical method for the analysis of target compounds in mussel samples collected from Halifax and St. John's harbours.

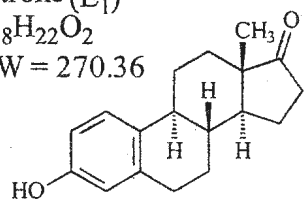
(3). The third and final objective of this investigation was to isolate and measure estrogenic compounds in raw sewage and harbour seawater samples. Target analytes in sewage analysis included steroid estrogens and coprostanol, the intestinal degradation product of cholesterol in humans and a widely used biomarker for fecal contamination. This information would be useful to correlate the presence of estrogens in the aquatic environment and any bioaccumulation of the estrogens in mussels.

Table 1.3. Chemical properties of natural and synthetic steroids analyzed in this study

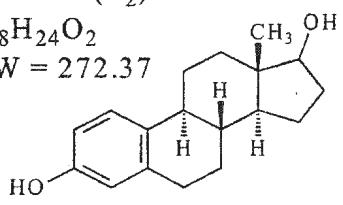
Estrogen	Molecular weight	Vapor pressure	Water solubility (mg/L)	Log K_{OW}
Estradiol	272.39	3E-08	13	4.01
Estrone	270.37	3E-08	13	3.13
Estriol	288.39	9E-13	13	2.60
Ethynylestradiol	296.40	6E-09	4.8	3.67
Mestranol	310.42	1E-07	0.3	4.10

Lipophilicity of organic contaminants is measured in terms of octanol-water partitioning coefficient ($LogK_{ow}$). As shown in the table, steroid estrogens are moderately lipophilic (Lai et al., 2000).

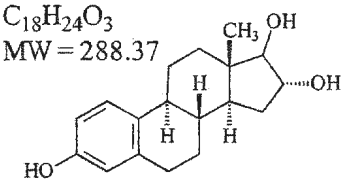
Estrone (E_1)
$C_{18}H_{22}O_2$
MW = 270.36



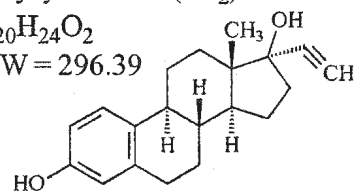
Estradiol (E_2)
$C_{18}H_{24}O_2$
MW = 272.37



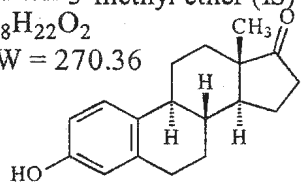
Estriol (E_3)
$C_{18}H_{24}O_3$
MW = 288.37



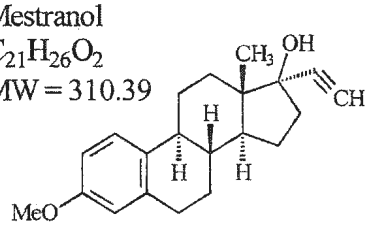
Ethynylestradiol (EE_2)
$C_{20}H_{24}O_2$
MW = 296.39



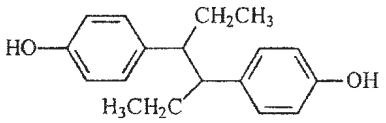
Estrone 3-methyl ether (IS)
$C_{18}H_{22}O_2$
MW = 270.36



Mestranol
$C_{21}H_{26}O_2$
MW = 310.39



Diethylstilbestrol
$C_{18}H_{20}O_2$
MW = 268.34



Coprostanol
$C_{27}H_{48}O$
MW = 388.65

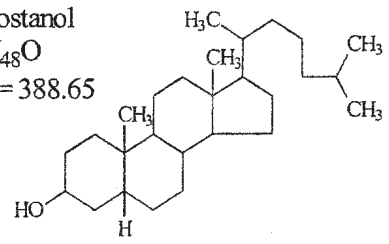


Figure 1.4. Structure of target analytes investigated in this study

MATERIALS AND METHODS

2.1. Materials and standards

Steroid standards, estrone (E_1), estradiol (E_2), estriol (E_3), ethynylestradiol (EE_2) and diethylstilbestrol (DES) were obtained from Sigma Chemical Co., US. The 3-methyl ethers of E_1, E_2, EE_2, E_3, diethylstilbestrol dimethyl ether, and coprostanol standards were procured from Sterloids Inc., US. All standards had a purity of >98% and used as such without further purification. Trace analysis grade solvents (Optima, Fisher Chemicals, US) were used throughout this study. Nanopure water (distilled water followed by Nanopure II system, Barnstead-Thermolyne, US) was used in the extraction protocol as well as for the general rinsing of glassware. The glassware was cleaned first with a mild laboratory detergent and was thoroughly rinsed with tap water. This was followed by rinsing with nanopure water. All glassware, excluding volumetric glassware, was baked at 400°C for six hours. To avoid contamination between samples, disposable vials were used for final sample preparation for GC –MS analysis.

Stock solutions of steroid standards were prepared in methanol at 1 mg/mL concentration. A working solution was prepared by mixing together individual steroid (E_1, E_2, E_3, EE_2, DES and coprostanol) stock solutions followed by dilution with methanol to get a concentration of 25 μg/mL. Standard mixture of 3-methyl derivative of steroids was prepared in the same manner for extractive alkylation methodology. All standard working solutions were stored at 4°C. All the solutions were brought to room temperature before use. Evaporation of solvent during

dispensing of aliquots was kept to a minimum. This was confirmed by recording the weights of the stock solution before and after sample preparation.

2.2. Sampling

2.2.1. Sewage and seawater samples

About three L of water samples were collected on August 15, 2002 from two harbour sites; one at Northwest Arm (H-2) and one near Downtown (H-1) in Halifax, NS. Water sampling locations are depicted in Figure 2.1. Water samples were collected in borosilicate bottles and transported to the lab. The samples were preserved from biodegradation by adding 5 mL of concentrated H_2SO_4/L of water sample. The samples were shipped to the St. John's lab in a cooler where they were stored in the dark at 4°C and processed within five days.

Water and sewage sampling locations in and near St. John's harbour, NL is shown in Figure 2.2. Raw sewage samples from Beck's Cove (X-1), Southside (X-2) and in the Goulds township (not shown in the figure) pumping stations in St. John's (population: 115,000) were collected on November 4, 2002. Southside and Beck's Cove pumping stations pump untreated sewage water into the St. John's harbour. The Southside pumping station has the biggest pumping capacity and pumps about 9 million gallons of raw sewage daily. At Beck's Cove pumping station, the wastewater is from a sewer line that mixes the surface run off (storm sewer) and the raw domestic sewage. The Goulds pumping station is located on the outskirts of St. John's and serves a much smaller community of people (population: 4,000), processing about 700,000 gallons of raw sewage daily.

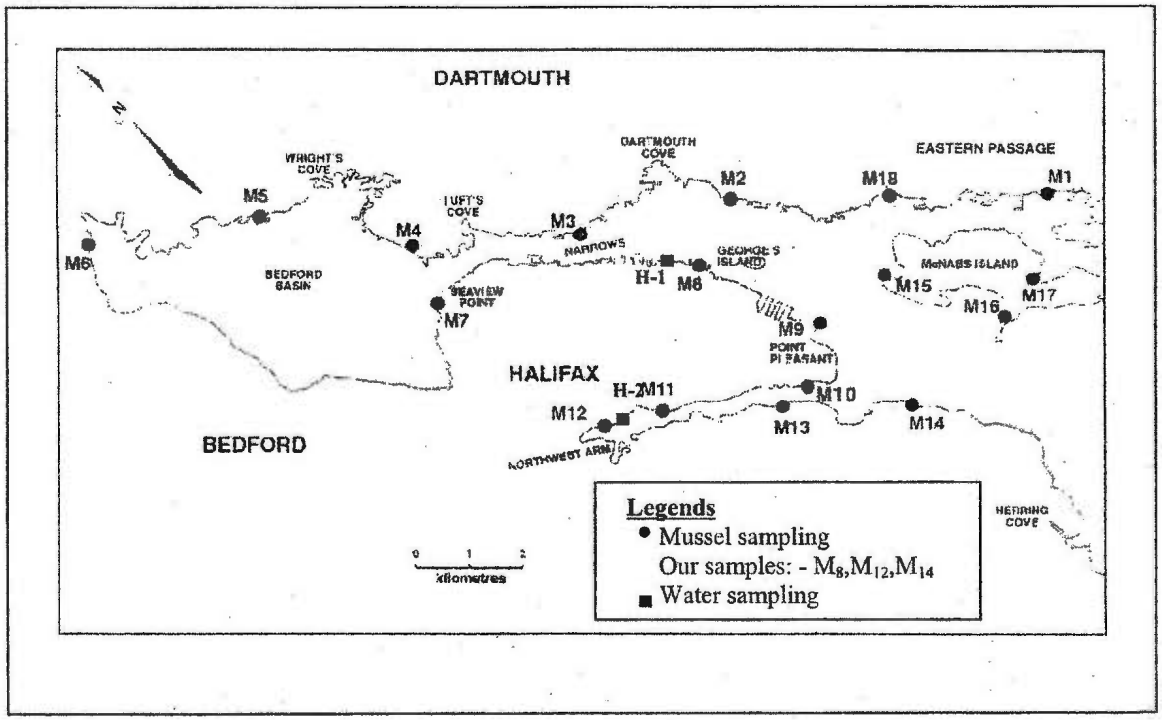


Figure 2.1. Sampling locations in Halifax harbour, NS.

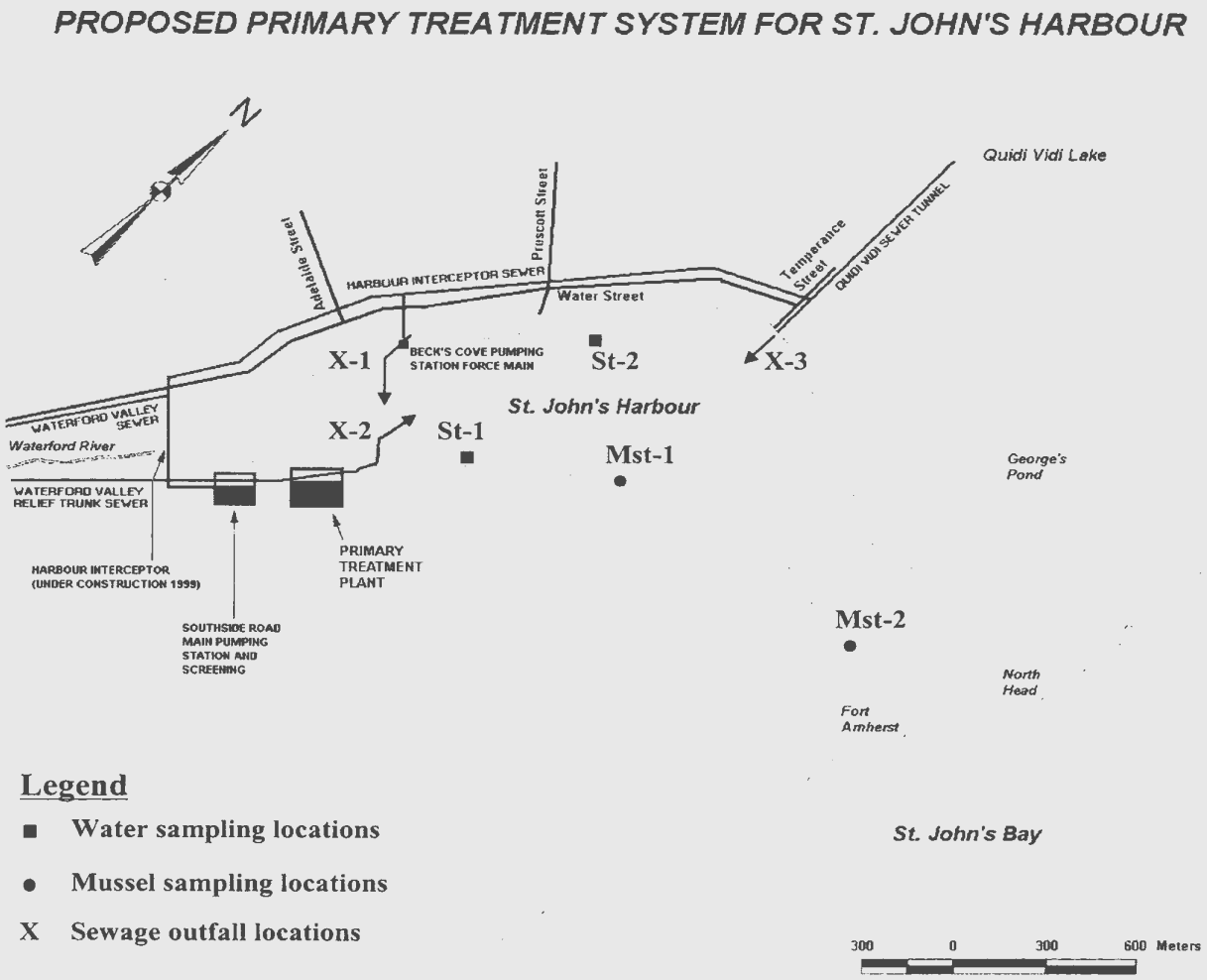


Figure 2.2. Sampling locations in St. John's harbour, NL.
Courtesy: St. John's Harbour ACAP Inc.: - http://www.thezone.net/stjacap/atfa4.html

Seawater samples from St. John's harbour were collected from two sites on November 8, 2002. Site 1 (St-1) is situated about 50 yards from the point where sewage from Southside pumping station mixes with seawater. Site 2 (St-2) is situated at about 200 yards from the point where the sewage from Beak's cove pumping station mixes into the sea.

2.2.2. Mussel samples

Mussels (*Mytilus Edulis*) of restricted size range (length between 4 – 5 cm) were collected around Halifax harbour. This sampling is a part of Harbour study conducted by Dr. Jocelyne Hellou at Bedford Institute of Oceanography, Department of Fisheries and Oceans, Halifax, NS. Mussel sampling sites around the harbour are shown in Figure 2.1. All samples were collected in February 2001. In the present study, mussel samples collected from Sites M_8, M_{12} and M_{14} were analyzed for steroid estrogens.

In St. John's harbour, mussels were collected during May and November 2002. Mussels (length between 2-3 cm) were collected from Site MST-1 and Site MST-2 as shown in Figure 2.2. Site MST-1 is located near the harbour water sampling Site St-1 and represents the most contaminated site. Mussels could not be found in any other on-shore locations in the harbour. During the second sampling period in November 2002, no mussels were found at this site. Site MST-2 is located just outside the harbour and represents a low contamination site.

After collection, all mussel samples were brought to the lab and maintained in separate tanks with running seawater for 3 hours to remove particulate matters from

their gut. Then the mussels were dissected and the soft tissues were pooled together. The samples were stored frozen until their analysis.

2.2.3. Control samples

Live mussels obtained from a local seafood shop were used in the recovery studies. They were treated in the same way as the field samples (Section 2.2.2). These mussels were grown at Newfoundland sites free of any contamination from human sewage and therefore served as control sample.

2.3. Sample preparation

2.3.1. Overview of analytical protocols

The steps involved in the mussel tissue analysis are shown in Figure 2.3. Briefly, after homogenizing the mussel tissue, the analytes were extracted using methanol and water. After centrifugation, the supernatant was decanted in a clean test tube. This aqueous methanolic extract was partitioned with hexane. The hexane layer, which contained non-polar lipids, was discarded. This was followed by rotary evaporation to remove most of the methanol. The aqueous extract was then subjected to solid phase extraction (SPE) protocol. The analytes were eluted from the SPE cartridge using ethyl acetate. Cleanup of SPE extract was done either by silica column or by extractive alkylation. After cleanup, the extract was derivatized for GC-MS analysis.

The schematic for water analysis protocol is shown in Figure 2.4. A sewage or seawater sample was first filtered through glass fiber filter (GF/C) of 1.2 μm pore size to remove the suspended solids and debris. After adjusting the pH to 4.5, 1L of

ANALYSIS OF STEROID ESTROGENS IN MUSSEL TISSUE

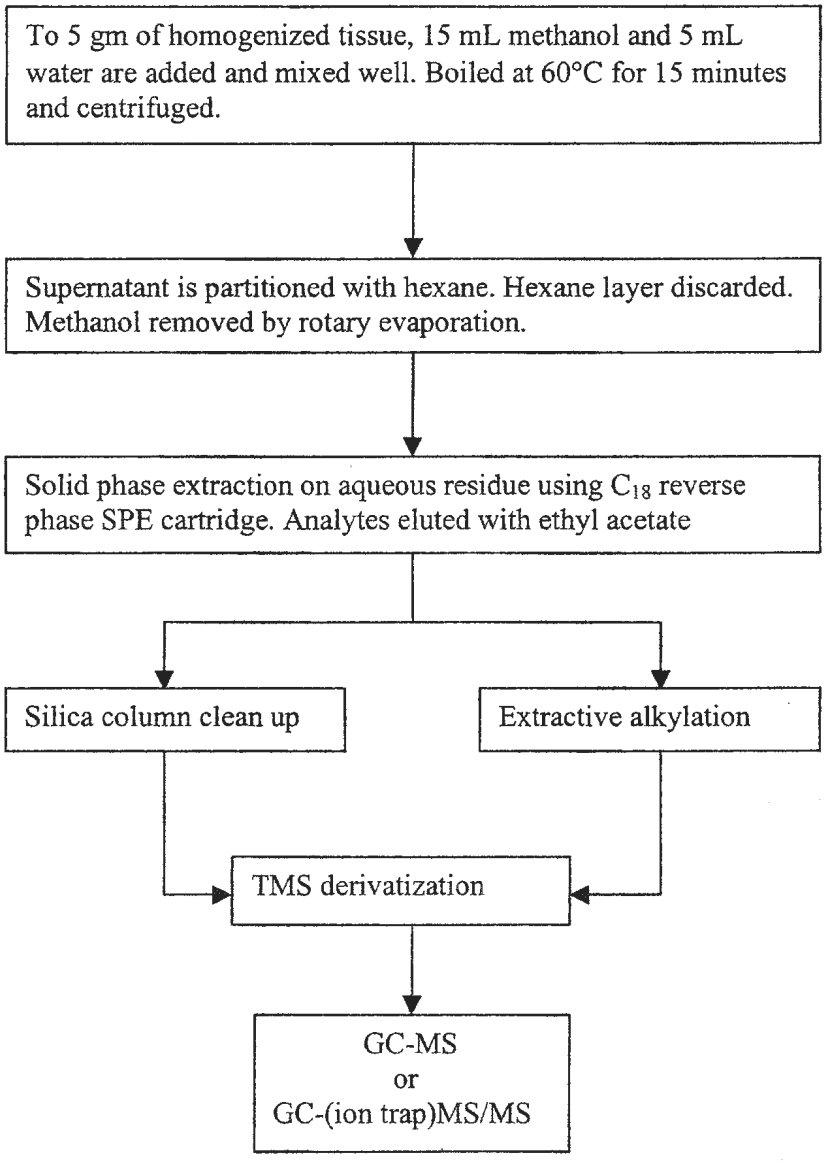


FIGURE 2.3. Schematic of mussel analysis

SEWAGE AND SEAWATER ANALYSIS

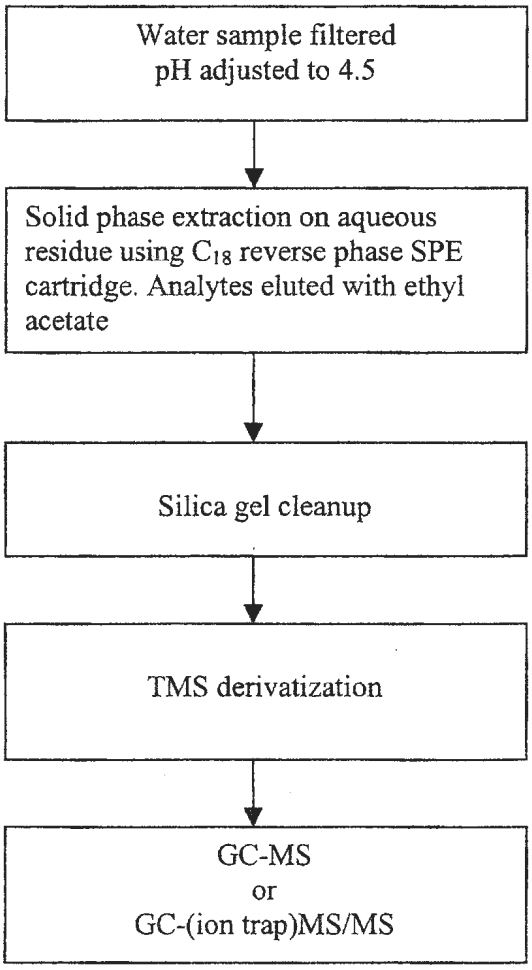


Figure 2.4. Schematic of water analysis

filtered seawater or 250 mL of filtered raw sewage was loaded on a C_{18} reverse phase SPE cartridge at a flow rate of about 5 mL/min. The analytes were eluted from the SPE cartridge using ethyl acetate. The SPE extract was then subjected to silica column cleanup and finally derivatized for GC-MS analysis.

2.3.2. Homogenization of mussel tissue

Mussels were dissected by opening their shell using a sharp knife. The soft tissue together with the fluid was collected in a beaker. The tissue sample was homogenized using a rod homogenizer (Polytron, Brinkmann Instruments, Canada). The homogenized tissue was stored at -10°C until analyzed.

2.3.3. Extraction of analytes

To 5 grams of homogenized mussel tissue in a beaker, 15 mL methanol and 5 mL water was added and mixed well by stirring for 5 minutes at room temperature. The beaker was then immersed for 15 minutes in a hot water bath at 60°C in order to precipitate the proteins and assist in analyte dissolution. The sample was cooled and then centrifuged at *2500 g* for 10 minutes. The aqueous/methanolic extract was decanted.

2.3.4. Lipid removal

Much of the non-polar lipids present in the aqueous/methanolic extract were removed by shaking with 10 mL hexane in a separating funnel. In order to improve the phase separation, the mixture was centrifuged for 2 minutes in a tabletop centrifuge. The

hexane layer was discarded. The aqueous/methanolic fraction was transferred to a round bottom flask and most of the methanol was removed by rotary vacuum evaporation at 40°C. The aqueous extract was diluted to 50 mL with nanopure water and subjected to solid phase extraction.

2.3.5. Solid Phase Extraction (SPE)

A C_{18} reverse phase cartridge (ENVI-18, 500 mg, Supelco, USA) was used for SPE cleanup procedures. The cartridge was conditioned with 4 mL of methanol followed by 4 mL of water, as per the manufacturer's instruction. The cartridge was fitted on a vacuum manifold (Supelco, USA) connected to a water aspirator. The aqueous extract (about 50 mL) was loaded on the cartridge at two mL/min. After loading, the cartridge was washed with 4 mL of 25% aqueous methanol followed by drying at full vacuum for 2 minutes. The cartridge was further dried under vacuum over KOH for 30 minutes to ensure that most of the moisture was removed from SPE cartridge. Then 5 mL of ethyl acetate was added to the cartridge and left to equilibrate for 5 min. Finally the analytes were eluted by applying a slight vacuum.

2.3.6. Extractive alkylation

Based on the method of Mckillop et al. (1974) for the synthesis of phenyl ethers, a protocol was developed for the extractive alkylation of phenolic steroids. The ethyl acetate extract (after SPE) was evaporated to dryness under a gentle stream of N_2 and the residue re-dissolved in 5 mL of 1N NaOH. The extract was partitioned with 3 mL of 15% methylene chloride in hexane. Then 0.3 mL of 1M tetra butyl ammonium

hydroxide (TBAH, Sigma Aldrich, US) was added followed by 5 mL of 0.2M dimethyl sulfate (Sigma Aldrich, US) in methylene chloride. The contents were stirred vigorously for 24 hours at room temperature. The methylene chloride layer was separated using a small separatory funnel. The aqueous layer was re-extracted twice with 5 mL methylene chloride and combined with the original sample. The organic sample was then washed with 5 mL of 0.2 M aqueous ammonia, followed by 5 mL of 1N NaOH and finally with 5 mL of saturated NaCl solution. The methylene chloride was concentrated to a residue by gentle evaporation under N_2. To remove trace levels of water, the residue was dissolved in 2 mL of methylene chloride and passed through a small column filled with anhydrous sodium sulfate just before derivatization and GC-MS analysis.

2.3.7. Silica column cleanup

Silica gel (grade 923, 100-200 mesh, Aldrich Chemical Co., US) was activated at 130 °C for 12 hours. Three percent-deactivated silica was prepared by adding the required amount of water to the silica and stored in a tightly sealed glass bottle. A silica column was made as follows: 1 gm of silica was mixed with 3 mL methylene chloride and the slurry was loaded into a glass column fitted with a sintered filter. The column was first washed with 6 mL of methylene chloride, followed by 3 mL of 32% ethyl acetate in hexane and finally with 3 mL of 8% ethyl acetate in cyclohexane.

The mussel extract (after SPE) was evaporated to dryness under a flow of N_2; the residue was re-dissolved in 500 µl of 8% ethyl acetate in cyclohexane and, applied onto the silica column. The sample vial was rinsed with two aliquots of solvent and

added onto the column. The analytes were eluted in three different fractions using the following solvent combination.

(a). Fraction 1: The first fraction was collected by eluting with 6 mL of 8% ethyl acetate in cyclohexane by gravity. This fraction contains 30% of mestranol.

(b). Fraction 2: The column was then eluted with 15 mL of 12 % ethyl acetate in cyclohexane. This fraction consists of DES, E_1, and the remaining mestranol. About 40% of EE_2 was also eluted in this fraction.

(c). Fraction 3: The remaining portion of E_2 and EE_2 were eluted from the column using 10mL of 32% ethyl acetate in hexane.

All fractions were collected separately and subjected to derivatization to study the effect of matrix. After optimization studies (see section 3.1.4.), fraction 1 and 2 could be combined before GC-MS analysis. Fraction 3 had a strong yellow color indicating the presence of high background materials (matrix). Therefore, this fraction was analyzed separately.

In water and sewage analysis, the SPE extract was evaporated to dryness and the residue dissolved in minimal quantity of 32% ethyl acetate in cyclohexane. It was loaded on a 3% deactivated silica column prepared as described above. The column was eluted with 10 mL of 32% ethyl acetate in cyclohexane and no further fraction was required. The extract was then subjected to derivatization.

2.3.8. Derivatization of phenolic and hydroxyl functional groups

In order to improve the GC behavior of the analytes, the free phenolic and hydroxyl groups of the analytes were derivatized using either N,O-

bis(trimethylsilyl)trifluoroacetamide (BSTFA) or pentafluoropropionic anhydride (PFPA) reagent.

The free phenolic and alcoholic groups of the analytes were converted into their corresponding trimethylsilyl ethers using Sylon BFT ampoules (Supelco, USA). Each ampoule consists of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS). The sample previously evaporated to dryness under gentle stream of N_2 was dissolved in 250 µl of dry pyridine. To this, one BFT ampoule (0.1 mL) was added and the content mixed well. The reaction proceeded at 60°C for 1 hour on a heating block. The derivatized sample was evaporated to dryness under N_2. The residue was dissolved in 200 µl hexane before GC-MS analysis.

Based on the protocol described by Lee and Peart (1998), fluoroacyl derivatives were prepared using PFPA reagent (Supelco, USA). Briefly, the analytes were dissolved in 100 µl ethyl acetate and 50 µl of PFPA reagent was added. After thorough mixing, the content was allowed to stand at room temperature for 30 min. Then 3 mL of 2% aqueous K_2CO_3 solution was added. Analytes were extracted by partitioning with 3 mL of diethyl ether. Diethyl ether extraction was repeated twice and all the extracts were combined together. The volume of the sample was reduced to about 2 mL by evaporation under N_2. The sample was then dried by passing through a small column filled with 1 g of anhydrous sodium sulfate. The extract was evaporated to dryness and the residue dissolved in 200 µl of hexane before GC-MS analysis.

2.4 Instrumental methods

2.4.1. GC-MS method

GC-MS work (used extensively for method development and optimization) was carried out using a HP 5890 series GC coupled with 5971 mass selective detector (MSD). The GC and MS operating conditions are given in Table 2.1. In chemical ionization (CI) studies, ammonia (NH_3) was used as CI gas. The CI gas pressure was optimized using HP chemstation software. All target analytes were successfully separated and baseline resolved as shown in the Figure 2.5. Except for DES, all the analytes formed only one derivative with BSTFA and PFPA reagent. The absence of any underivatized analytes in the total ion chromatogram (TIC) indicated that the derivatization reactions were quantitative. DES is known to undergo isomerization during TMS derivatization (Bagnati et al., 1990). Cis- and trans- isomers of the product were well separated under the developed chromatographic conditions.

Analytes were positively identified in a sample by matching (a) their retention time (± 0.2 min) with that of standards; and (b) by comparing the relative ion abundance of three characteristic ions for each analytes. The characteristic ions used for quantification of analytes by selected ion monitoring (SIM) technique are listed Table 2.2.

2.4.2. GC- (ion trap) MS/MS method

A Varian GC- (ion-trap)-MS system (Saturn 3, Varian Inc., US) was used primarily for the analysis of real environmental samples. Chromatographic and mass spectrometric conditions used in this study are summarized in Table 2.1. Various

Table 2.1. Chromatographic and mass spectrometric conditions used in steroid analysis

Parameter	Details	
	GC-MS (HP) system	GC- (ion trap) MS/MS (Varian) system
Column	DB-5; 28 meters; 0.25 μm film thickness	DB-5; 30 meters; 0.5 μm film thickness
Carrier gas	Helium, 12 psi	Helium, 25 psi
Injector temperature	No temperature programming. Held constant at 250°C	50°C - 100°C/min – 300°C (32.5 minutes)
Sample injection	1 μl; splitless mode (2 min)	1 μl; on-column injection
Oven temperature programming	80°C(2 min.) – 30°C/min. – 242°C (2 min) - 1°C/min – 246°C – 15°C/min – 280°C (3min)	50°C (3.5 min.) - 20°C/min - 240°C - 2°C/min. - 290°C (15 min).
MS conditions	Electron energy: 70 eV; SIM scan rate: 9 cycles/sec. Interface temperature: 280°C; Ion Source temperature: 180°C Electron multiplier voltage: 200 V above autotune value	Electron energy: 70 eV; Mass range: 50 to 650 amu; Interface temperature: 280°C; Ion source temperature: 225°C Electron multiplier voltage: 200 V above autotune value

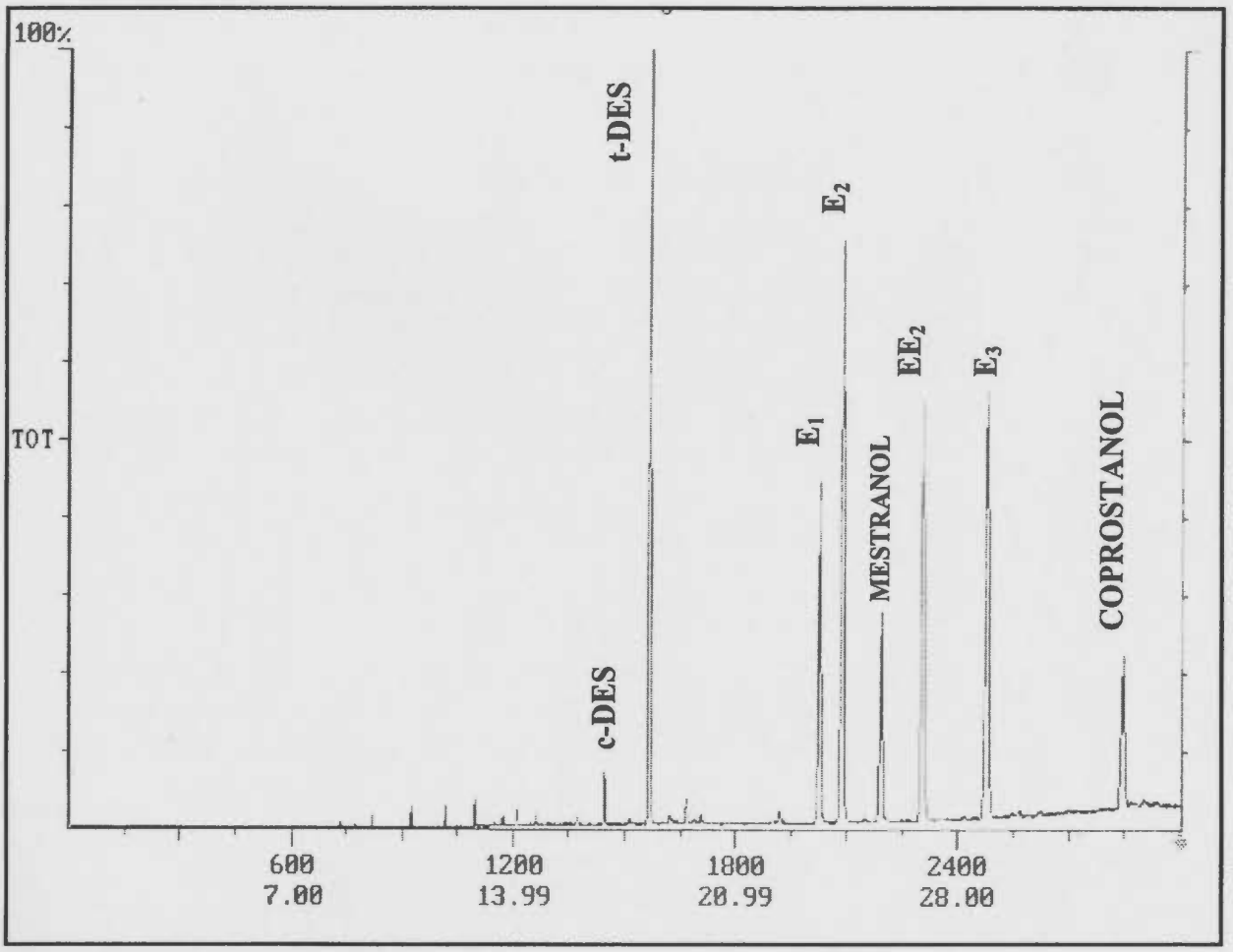


Figure 2.5. Selected ion chromatogram of estrogen standards. 50 ng of pure standards was derivatized using BSTFA and chromatographed. GC-MS operating conditions are given in Table 2.1.

Legends: c-DES: cis-DIETHYL STILBESTROL; t-DES: trans-DIETHYL STILBESTROL; E_1: ESTRONE; E2: ESTRADIOL; EE_2: ETHINYL ESTRADIOL; E3: ESTRIOL

Table 2.2. List of characteristic ions of TMS derivatives of some of steroid analytes used in the quantification by GC- (SIM)-MS

Analyte*	Characteristic ions (m/z)	
	Electron impact ionization	Chemical ionization
DES	217, 383, 412	267, 281, 296
E_1	257, 342, 218	160, 199, 284
E_2	232, 285, 416	173, 227, 358
EE_2	287, 425, 440	227, 367, 382
E_3	311, 345, 504	253, 287, 446

* Analyte abbreviations as shown in Figure 2.5.

Table 2.3. Optimized GC- (ion trap) MS/MS conditions for the analysis of TMS derivatives of target analytes CID voltages, excitation storage level, the mass of parent ion and the three most abundant daughter ions for analytes are given.

Analyte*	Parent ion (m/z)	CID voltage (Resonant excitation, V)	Excitation storage level (m/z)	Most abundant daughter ions (m/z)
DES	412	1.55	183	217, 383, 397
E_1	342	0.95	151	244, 257, 325
E_2	416	1.35	184	285, 298, 326
MESTRANOL	367	1.50	162	173, 193, 324
EE_2	425	1.50	188	193, 231, 407
E_3	386	1.10	171	296, 357, 371
COPROSTANOL	370	0.65	164	341, 355, 370

* Analyte abbreviations as shown in Figure 2.5.

steps in an MS/MS experiment are described in detail by Chapman (1993). Two important parameters are collision induced dissociation (CID) voltage and excitation storage level. CID voltage determines the kinetic energy of the parent ion during the CID process. Higher energy collisions would result in more fragmentation and might result in the absence of parent ion in the daughter ion spectra. On the other hand, low energy collisions would lead to low fragmentation and higher abundance of parent ion. Therefore, the CID voltage is optimized and leads to a parent ion abundance of about 5-10% in the daughter ion scan. Excitation storage level determines the lowest daughter ion mass that will be stable inside the ion–trap before the daughter ion scan. These parameters were optimized for each analyte using the tool kit program of the Varian GC-MS software. The daughter ion spectrum for the target analytes are shown in Appendix A and the optimized MS/MS conditions are given in Table 2.3. The daughter ion spectra and the retention times of pure standards were used for the qualitative identification of analytes in a given sample. During quantification, peak areas were calculated based on the extracted ion chromatogram of analytes obtained using the most abundant daughter ions. A list of daughter ions used for analyte quantification by this technique is given in Table 2.3.

2.4.3. Quantification of estrogens

Estrogens are quantified using the internal standard technique as described by Debbrecht (1995). 3-methyl estrone, at 10 ng/μL is used as internal standard. Mixture of estrogen standards at 1, 5, 10, and 50 ng/μL concentrations were prepared and chromatographed together with the internal standard. Calibration curves for each

analyte was developed by plotting the ion abundance ratio in the ordinate and weight ratio in the abscissa and shown in Appendix B. The slope of the calibration curve gives the response factor for the analyte.

Calculation for unknown:

$$\text{Ion abundance ratio} = \frac{\text{Ion abundance of analyte } (A_C)}{\text{Ion abundance of internal standard } (A_{IS})}$$

$$\text{Weight ratio} = \frac{\text{Weight of Analyte injected } (W_C)}{\text{Weight of internal standard injected } (W_{IS})}$$

Response factor (R) = Slope of ion abundance ratio vs. weight ratio

Hence, Weight ratio = Ion abundance ratio/Response factor

(Or)

$$\frac{W_C}{W_{IS}} = \frac{A_C}{A_{IS}} \times \frac{1}{R}$$

Therefore weight of the analyte in unknown,

$$W_C = \frac{A_C}{A_{IS}} \times \frac{1}{R} \times W_{IS}$$

RESULTS AND DISCUSSION

3.1. Introduction to method development

Detection of trace levels of steroid estrogens from tissue samples is a challenging analytical task. Tissue analysis is generally complicated by the presence of a variety of biological molecules like proteins, carbohydrates and lipids. Therefore, the biochemical composition of the given tissue type would dictate the choice of cleanup procedures. In mussels, the percentage composition of proteins, carbohydrates and lipids are estimated as 58%, 22%, and 7% respectively (Dare and Edwards, 1975; Okumus and Stirling, 1998). Teshima and Kanazawa (1973) reported that cholesterol and its derivatives, 2,2-dehydrocholestrol, and 2,4-methylenecholesterol, are the major steroidal classes in mussels. However, very little is known about the occurrence of different types of estrogenic steroids in mussels. Reis-Henriques et al. (1990) identified the natural estrogens E_1, E_2 and E_3 in mussel tissue. Both sterols and natural estrogens share the basic perhydrocyclopentanophenanthrene chemical structure. Hence, the presence of different types of sterols, at high concentrations, complicates the analysis of estrogens present in trace levels (Cathum and Sabik, 2001).

The key to success in trace analysis of steroid estrogens in mussel tissues lies in the efficient removal of the large amount of extractable matrix of the mussels. As the chemical nature of the background materials varies widely, tissue analysis generally involves a multi-step protocol. In many instances, this approach results in poor recoveries for the target compounds due to the loss of analytes during various steps. To achieve the best recoveries, the analytical protocol was optimized for each step,

one at a time (i.e. extraction, cleanup, derivatization and analysis). Finally, the recovery of analytes was studied over the entire procedure and the results are presented in this chapter.

3.1.1. Extraction of analytes from tissue

Choice of a solvent or solvent system for extraction is based on its ability to dissolve steroids at trace levels (ng/g) quantitatively from tissue matrix. In the present study, methanol was chosen as the extraction solvent because (i) target analytes are freely soluble in methanol; (ii) water constitute about 80% by weight of fresh mussel tissue, and hence, a solvent that is miscible with water would enhance the dissolution of the analytes from tissues; and (iii) methanol helps in protein precipitation, and this helps avoid a separate protein removal step in the analysis.

Addition of 15 mL of methanol and 5 mL water was found to be sufficient to extract the target analytes quantitatively from 5 g of wet mussel tissue. For complete protein removal, the mixture (tissue + methanol) was heated to 60°C in a water bath as suggested by Hartmann and Steinhart (1997). Although methanol is a good extraction solvent for steroids, it extracted many other lipid components and some carbohydrates from mussel tissue. Preliminary GC analysis showed that a high matrix interfered with TMS derivatives particularly at the GC injection step. Much work was involved to remove the background matrix before the final instrumental analysis step.

Before any extensive cleanup was undertaken, non-polar lipids were removed by partitioning with hexane. To ensure no loss of analytes, their partitioning behavior between aqueous/methanol extract and hexane was studied. For this, 100 ng mixture

of target analytes was spiked in 20 mL of 3:1 methanol: water (by volume) and partitioned with a 10 mL portion of hexane. The hexane fraction was separated, passed through a small column of anhydrous sodium sulfate (to remove moisture) and analyzed for the presence of analytes using GC-MS after derivatization with BSTFA. None of the steroid hormones were found in the hexane fraction. However most of the coprostanol (>90%) and other sterols such as cholesterol were partitioned in hexane fraction. As coprostanol was not a target analyte in mussel tissue analysis, the hexane fraction was discarded.

3.1.2. Optimization of solid phase extraction (SPE)

The SPE method was developed based on guidelines worked out by Thurman and Mills (1998). During optimization experiments, target analytes were spiked in nanopure water and passed through the SPE cartridge (C18, ENVI-18, Supelco) at a flow rate of about 2 mL/min. The following parameters were optimized to achieve better analyte recovery.

(a). Choice of eluting solvent: Many solvents, either alone or in combination with other solvents were used to elute estrogens from C_{18} reverse phase SPE cartridge (Lopez de Alda and Barcelo, 2001). Although methanol and acetone would be good solvents for eluting these analytes, due to their high elution strengths, these solvents would elute more matrix materials. Hence, ethyl acetate was chosen for desorption of steroids from the SPE cartridge. Five mL of ethyl acetate was found to be sufficient to elute all analytes quantitatively.

(b). Washing: Washing the SPE cartridge with a suitable solvent before the actual elution of analytes helps to remove some of the background materials. To optimize the composition of wash solvent, three separate experiments were carried out. In each case 4 mL of 10%, 25%, and 35% methanol (aqueous) was used as wash solvent. The washings were collected in separate vials and analyzed for the presence of analytes using GC-MS. The results indicated that about 10% of estriol (E_3) was eluted during washing with 35% methanol; while none of the analytes were found in 10% and 25% wash compositions. Therefore, 25% aqueous methanol was chosen as the wash solvent.

(c). Drying: Drying the SPE cartridge before eluting the analytes was found to be beneficial. As ethyl acetate has very low miscibility with water, the cartridge was dried thoroughly to remove moisture so that the interaction of bound analytes with ethyl acetate could be improved.

(d). Finally, recovery of analytes using this SPE procedure was ascertained by analyzing 50 mL of water spiked with 100 ng of analytes. After the washing and drying steps, the analytes were eluted using 5 mL of ethyl acetate. As a precautionary measure, ethyl acetate extract was passed through a small anhydrous sodium sulfate column before derivatization with BSTFA and then analyzed using GC-MS. Percentage recoveries of some of the target analytes are shown in the Table 3.1.

Recoveries of all the analytes except E_3 were above 90% and the repeatability was acceptable. The recovery of E_3 was acceptable, but lower, ranging between 70 to 85%. Although the recovery of the analytes from water was excellent, attempts to obtain good reproducible recoveries of analytes spiked in mussel matrix proved futile.

Table 3.1. Optimization of solid phase extraction procedure. Recovery of analytes from nanopure water

Analyte	Percentage Recovery		Average recovery (%)	Relative Standard Deviation* (%)
	Experiment 1	Experiment 2		
DES	94	101	98	5.0
E_1	92	99	96	5.1
E_2	93	94	94	0.7
EE_2	101	95	98	4.3
E_3	70	85	78	13.6

Where DES : Diethylstilbestrol; E_1 : Estrone; E_2 : 17-β estradiol; EE_2 : Ethynyl estradiol; E_3 : Estriol. 100 ng of analytes were spiked in 50 mL of nanopure water.

*Relative standard deviation (RSD) = $\frac{\text{Standard deviation}}{\text{Mean}} \times 100$

Poor percentage recoveries often with high variability between trials were observed when mussel extracts spiked with analytes was isolated by SPE. The total ion chromatogram (TIC) of mussel extract after SPE cleanup is shown in Figure 3.1. As can be seen, the TIC is characterized by number of wide peaks with high ion abundance. Therefore, steroid analytes even if they were present, would be buried in the background thereby hindering their detection. Recovery rates for analytes measured by GC-(SIM)-MS were generally low (< 50%) as shown in Table 3.2. Hence, the development of suitable cleanup procedures after SPE extraction was undertaken. To this end, two different cleanup procedures, one, based on extractive alkylation (section 3.1.3) and the other, silica column fractionation (section 3.1.4) were investigated.

3.1.3. Cleanup using extractive alkylation

All steroid estrogens (except mestranol) selected for this study contain one or more phenolic hydroxyl group. This property is explored for their selective isolation from matrix materials by extractive alkylation. Extractive alkylation procedures have been used extensively in synthetic organic chemistry (Weber and Gokel, 1977). However, its use as an extraction technique in analytical chemistry is not extensively explored. Lisi et al. (1991) used this technique for the analysis of diuretics in human urine. As well, Ervik and Gustavii (1974) developed a method for the analysis of chlorthalidone in plasma samples in nanogram levels.

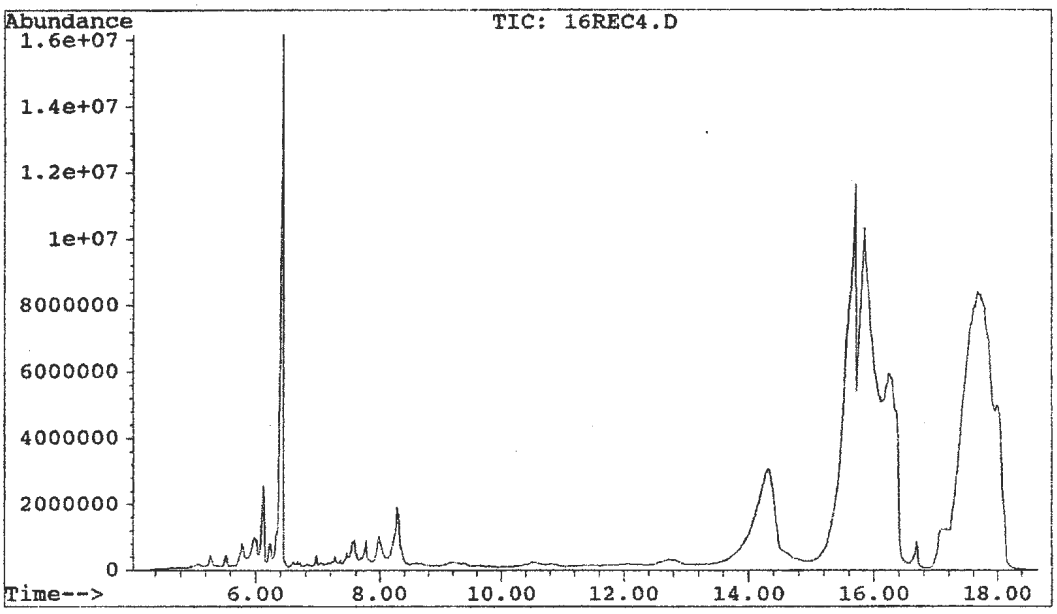


Figure 3.1. Total ion chromatogram of analytes spiked in mussel extract after SPE cleanup. The chromatographic conditions are given in Table 2.1.

Table 3.2. Recovery of target analytes spiked in mussel tissue extract following SPE procedure

Analyte*	Percentage Recovery (n=1)
DES	25
E_1	44
E_2	44
EE_2	75
E_3	30

* Analyte abbreviation as in Table 3.1. Percentage recovery obtained when 100 ng of analyte mixture was spiked into mussel extract (5 g wet mussel) is shown.

In one study, the synthesis of phenol ethers using an extractive alkylation technique was examined by Mckillop et al., (1974). Based on this work, a protocol was developed for the analysis of phenolic steroids from mussel tissue extract. The phenolic hydroxyl group of the analytes was methylated using a two-phase extractive alkylation technique. The basic reactions involved in this technique are depicted in Figure 3.2. Briefly, the analytes are added to a two-phase system consisting of an aqueous phase and an organic phase (methylene chloride). Strong alkaline conditions in the aqueous phase ionizes the phenolic hydroxyl group of the target analytes to phenoxide ions. These ions are then transported to the organic phase in the form of ion-pair by a phase transfer catalyst (Weber and Gokel, 1977). Once in the organic phase, the phenoxide ion reacts with methylating agent to form methyl ethers of the target analytes.

During the extractive methylation procedure, the synthetic estrogen 17α-ethynylestradiol (EE_2) is converted into mestranol. Therefore mestranol, if already present in the tissue, could affect the quantification of EE_2. Hence it was necessary to isolate mestranol before extractive alkylation. This was achieved by partitioning analytes in aqueous NaOH with 3 mL of 15% methylene chloride in hexane.

To study the time required for the quantitative conversion of steroid estrogens into corresponding methyl ethers, experiments were performed by spiking 1 μg of analyte standards in distilled water. The alkylation reaction was terminated at different time intervals and the methyl ethers quantified using GC-MS. A 12 hour reaction time was found to be necessary for the conversion (>85%) of steroid estrogens into their methyl ethers.

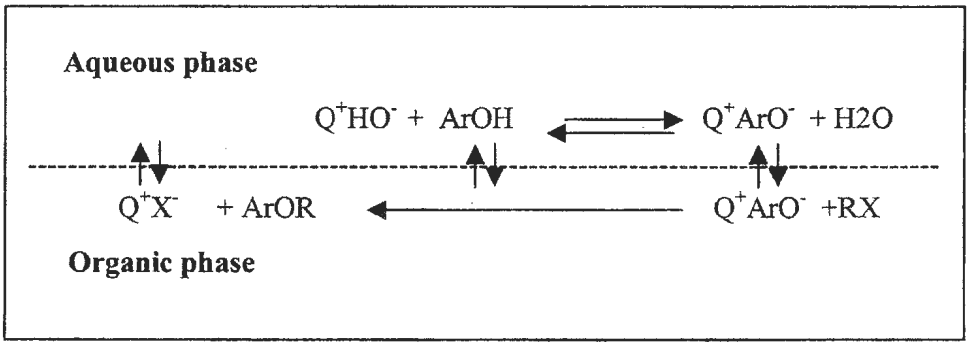


Figure 3.2. Fundamental reactions in extractive alkylation procedure (Mckillop et al., 1974) .

ArOH: aryl alcohol; Q^+HO^-: phase transfer catalyst; RX: alkylating reagent; ArOR : phenol ether

Table 3.3. Optimization of extractive alkylation cleanup. Mean recovery of analytes from nanopure water

Analyte*	Average recovery (n=2) (%)	RSD (%)
DES	92	8
E_1	90	12
E_2	106	9
EE_2	98	5
E_3	88	6.5

*Analyte abbreviations as in Table 3.1. 100 ng of analytes spiked in nanopure water was extracted using SPE followed by extractive alkylation cleanup.

Duplicate recovery experiments performed by spiking analytes at 100 ng level in distilled water gave very promising results as shown in Table 3.3. However, recovery of analytes spiked into mussel matrix proved to be more difficult. A series of experiments were performed where lower and lower amounts of analytes were spiked into mussel extract followed by SPE and extractive alkylation. It was found that analytes could not be detected below the 400 ng (per gram of wet mussel tissue) spike level with good reproducibility.

3.1.4. Silica column cleanup

In the second sample cleanup approach, removal of background materials using a silica column cleanup was attempted. The analytes were eluted using ethyl acetate/cyclohexane solvent system. To ascertain the right percentage of ethyl acetate, a series of experiments were done using estradiol as the model compound. Attempts to elute E_3 from column have failed because, due to its high polarity, it was bound to the column very strongly and required elution with high polar solvents (100% ethyl acetate). This elution solvent eluted considerable background materials leading to an ineffective silica column cleanup. Therefore, the recovery of E_3 was sacrificed to get better recoveries for rest of the analytes.

By trial and error, it was found that 10 mL of 32% ethyl acetate in cyclohexane was sufficient to elute all target analytes (recovery >95%). This cleanup protocol was then applied to tissue analysis. A mussel extract (after SPE) was spiked with target analytes and evaporated to dryness under the flow of N_2. The residue was dissolved in a minimal quantity of 32% ethyl acetate in cyclohexane and loaded onto the silica

column. The analytes were eluted with 10 mL of 32% ethyl acetate in cyclohexane. The eluent was analyzed using GC-(ion trap)MS/MS. In spite of the cleanup, the percentage recovery of analytes from mussel extract was not very encouraging. To understand if the poor analyte recoveries are due to the complications in GC derivatization step, an alternative derivatization technique was investigated.

Preparation of TMS ethers of steroid estrogens has been traditionally used in trace analysis. Their ease of formation and good volatility improves the chromatographic behavior of steroids. However, TMS derivatization reactions are easily fouled by the presence of background materials in real sample analysis. Mol et al. (2000) reported that TMS derivatization of steroid estrogens are strongly affected by matrix components. Poor recoveries observed in this study might be due to such interferences. A recent study (Lee and Peart, 1998) compared TMS derivatization of steroid estrogens with fluoroacyl derivatization techniques. It was found that PFPA derivatives of estrogens are easily formed and stable for weeks at –20°C. Therefore, as an alternative to TMS derivatization, suitability of PFPA derivatives of target analytes in mussel analysis was studied. However, the PFPA derivatives did not improve the recovery of analytes significantly. Moreover, one of the synthetic estrogens, EE_2, formed two distinct derivatives with PFPA reagent. Clearly, low recovery of TMS and PFPA derivatives is due to the interference of background materials in GC derivatization step. To examine this problem more carefully, a detailed fractionation of analytes on silica was carried out.

In order to evaluate the elution behavior of mussel matrix on silica, a study was conducted using thin layer chromatographic plates coated with 0.2 mm of silica gel

(TLC plates, Polygram Sil G/UV254, Macherey-Nagel, Germany). A small amount of the mussel extract (after SPE) was developed in 50% ethyl acetate in cyclohexane. The matrix materials have separated into six bands, ranging in R_f values from 0.09 to 0.85. Analyte standards were also analyzed in the same fashion in order to ascertain their R_f values relative to the background matrix. Figure 3.3 depict the position of various matrix bands and target analytes on a TLC plate developed using 50% ethyl acetate/cyclohexane system.

With this preliminary information, the mussel matrix was further investigated by fractionation on silica. After the mussel extract (after SPE) was loaded on the silica column, fractions were collected with solvents of increasing polarity (4%, 8%, 12%, 16%, 32%, and 50% ethyl acetate in cyclohexane) and analyzed using TLC. Based on these results, 8%, 12% and 32% ethyl acetate in cyclohexane were chosen for the fractionation of the analytes by silica column.

Cleanup of an analyte-spiked SPE extract was repeated using silica column fractionation procedure. The elution behavior of steroid standards is given Table 3.4. Recovery of analytes and their reproducibility were ascertained by spiking 100 ng of target analytes in mussel matrix. The silica column fractions were analyzed separately and the analyte concentrations were combined. Except for E_2, good overall recoveries were obtained for all target analytes as shown in Table 3.5.

In order to shorten the workup, the silica column fractions were combined before GC analysis and the recoveries of analytes were compared. The results of one such

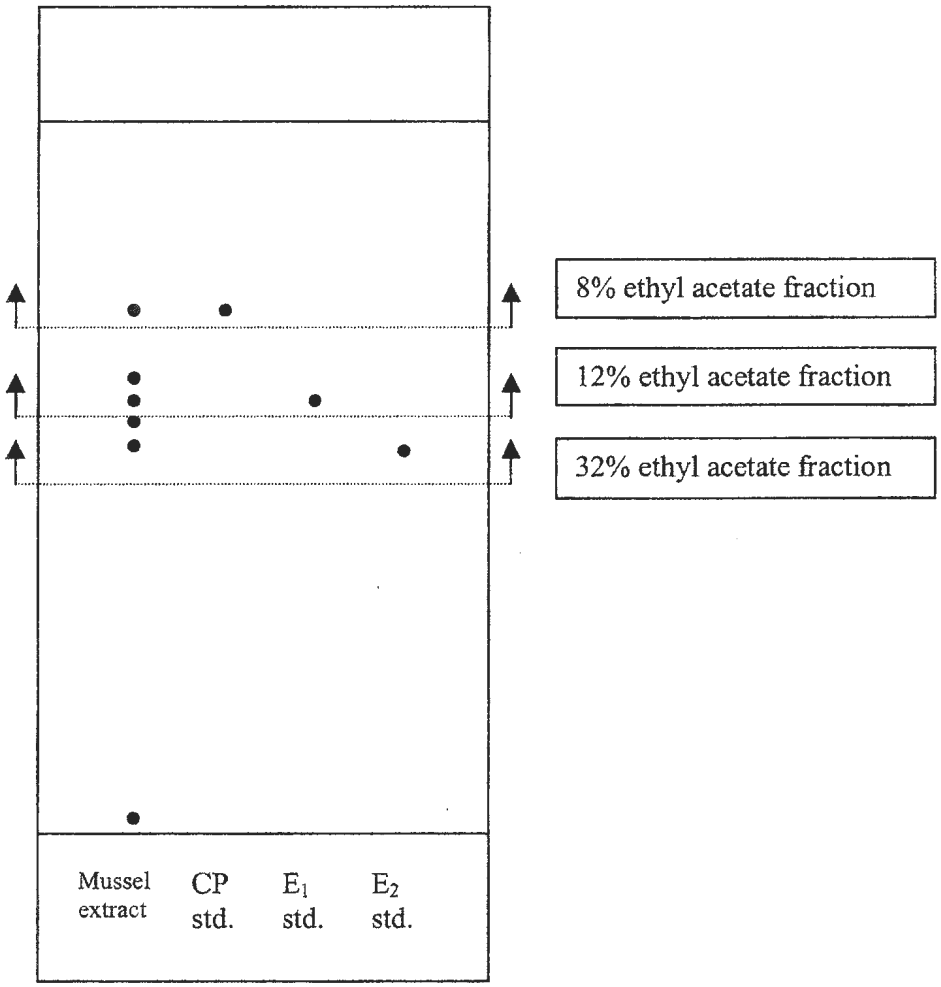


Figure 3.3. Elution behaviors of mussel extract and target analytes on a TLC plate. The plate was developed on a TLC plate with 50% ethyl acetate in cyclohexane. Dotted lines represent the elution of analytes during fractionation on a silica column.

Table 3.4. Elution behavior of analytes on silica. Fractionation of 100 ng of analytes spiked in mussel matrix

Analyte*	Elution of analytes		
	8% ethyl acetate in cyclohexane (Fraction 1)	12% ethyl acetate in cyclohexane (Fraction 2)	32% ethyl acetate in cyclohexane (Fraction 3)
DES	---	✓	---
E_1	---	✓	---
E_2	---	----	✓
Mestranol	✓	✓	---
EE_2	---	✓	✓

*Analyte abbreviations are as given in Table 3.1.

Note: E_3 was not included. See section 3.1.4 for details.

Table 3.5. Recovery of target analytes spiked in mussel tissue extract (after SPE) following silica column cleanup

Analyte*	Percentage recovery		Average recovery (%)	Relative standard deviation (%)
	Experiment 1	Experiment 2		
DES	102	97	99	3.5
E_1	94	90	92	3.1
E_2	61	68	65	7.6
Mestranol	84	93	89	7.2
EE_2	104	95	99	6.4

*Analyte abbreviations as in Table 3.1. Mussel extract (after SPE), obtained from 5 g of mussel tissue (wet wt.), was spiked with 100 ng of analyte mixture and fractionated on silica column. Each of the three individual silica column fractions was analyzed separately and the analyte concentration combined. RSD values are calculated as shown in Table 3.1.

Table 3.6. Optimization of silica column cleanup. Percentage recovery of analytes spiked in mussel matrix when silica column fractions were combined.

Analyte*	Overall recovery of analyte (%)		
	Fractions analyzed individually (from Table 3.5)	Fractions 1 &2 were combined (n = 1)	All fractions were combined (n = 1)
DES	99	90	93
E_1	92	87	32
E_2	65	56	58
Mestranol	89	73	66
EE_2	99	84	91

* Analyte abbreviations are as shown in Table 3.1.

analysis is shown in Table 3.6. The recoveries of DES, E_2 and EE_2 are largely unaffected when all the three fractions were combined together. However, the recovery of E_1 was drastically reduced to 30%. Acceptable recovery for E_1 was obtained only when fractions 1 & 2 were combined together before analysis and fraction 3 analyzed separately. It is believed that the background matrix present in fraction 3 interfered in the GC analysis of E_1. Hence, all further analyte analysis involved the analysis of fraction 1&2 together and fraction 3 separately.

3.1.5. Mussel matrix

The primary objective of this study's method development was the selective isolation and analysis of target steroids at trace levels, amid a complex matrix. It is therefore of interest to measure the mass of matrix material after various stages in the cleanup procedure as depicted in Figure 2.3. After each cleanup step, a portion of the sample was evaporated to dryness and weighed. The results are presented in Table 3.7. As can be noted, after SPE cleanup, 15% of the mussel matrix still remained. The silica column fractionation lowered this to 4%.

3.2. Choice of instrumental method

The suitability of electron impact (EI) and chemical ionization (CI) in GC/MS analysis of steroid analytes in mussel tissues was studied. Both the analytes and the matrix compounds fragmented extensively in EI technique leading to high background signal in the chromatogram. Figure 3.4 (a) shows the mass chromatogram of TMS-derivatized mussel matrix recorded under SIM mode. The chromatogram is

Table 3.7. Amount of mussel matrix at different stages of sample workup

Step	Dry weight/5 g of wet mussel tissue	Percent dry weight/gram of wet mussel tissue
Homogenized tissue (5 g wet weight)	0.89 ± 0.02 g	18%
After extraction and protein removal	0.5046 g	10%
After hexane extraction	0.3330 g	6.6%
After solid Phase Extraction	0.135 ± 0.02 g	2.7%
After silica column fractionation	0.0359 ± 0.007 g	0.7%

5 g of the homogenized tissue (St. John's harbour mussels) was processed using the developed analytical method. At each step, a portion of the sample was evaporated to dryness at 50°C and weighed to constant weight.

characterized by a complex array of matrix compounds of various abundances. For comparison, the elution pattern of analyte standards recorded under identical conditions is shown in Figure 3.4 (b). As can be seen, the spurious peaks from the background materials co-elute with analytes resulting in improper identification and quantification.

In Figure 3.5, the selected ion chromatogram of TMS-derivatized mussel matrix obtained under CI condition is shown. Qualitative comparison of chromatograms recorded under EI (Figure 3.4a) and CI (Figure 3.5) conditions indicated less background noise in CI conditions. In CI, the gaseous analytes are ionized by the ions of reagent gas (ammonia) instead of energetic electrons. The energy transfer during this ion-molecule interaction is far less than to that of electron impact (Chapman, 1993). Therefore, the analytes undergo less fragmentation thereby producing primarily molecular ions. Likewise, the background materials would also undergo less fragmentation and hence less background noise in the chromatogram.

Excellent analyte recoveries were achieved when mussel tissue spiked with of target analytes at 400 ng/g was analyzed under CI conditions (Saravanabhavan, 2002). However, target analytes could not be detected at trace levels (<100 ng/g) in the presence of the tissue matrix. This can be attributed to the lower sensitivity of CI for the target analytes. In Table 3.8, typical instrumental responses of target analytes in CI, and EI mode are given. In each case, the peak area was measured by summing the ion abundance of three characteristic ions of the analytes (Table 2.2). Although the background in CI is lower than EI, instrumental sensitivity in CI is much less. Hence, the suitability of GC-(ion trap)MS/MS for the steroid analysis was investigated.

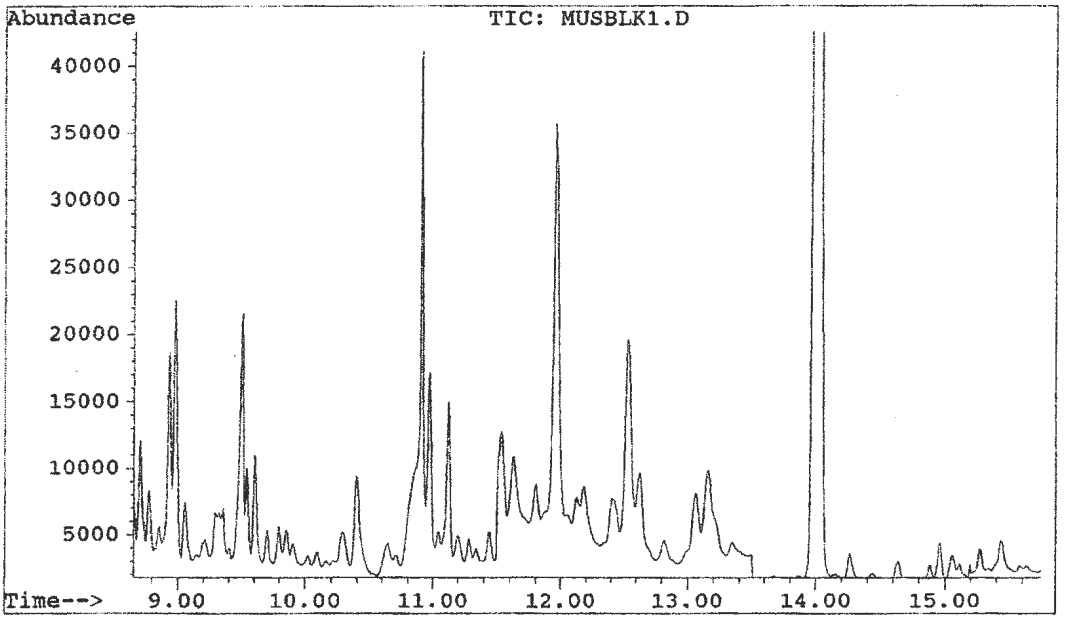


Figure 3.4a. Selected ion chromatogram of TMS- derivatized mussel matrix acquired in GC-(EI) MS. The chromatographic conditions are given in Table 2.1.

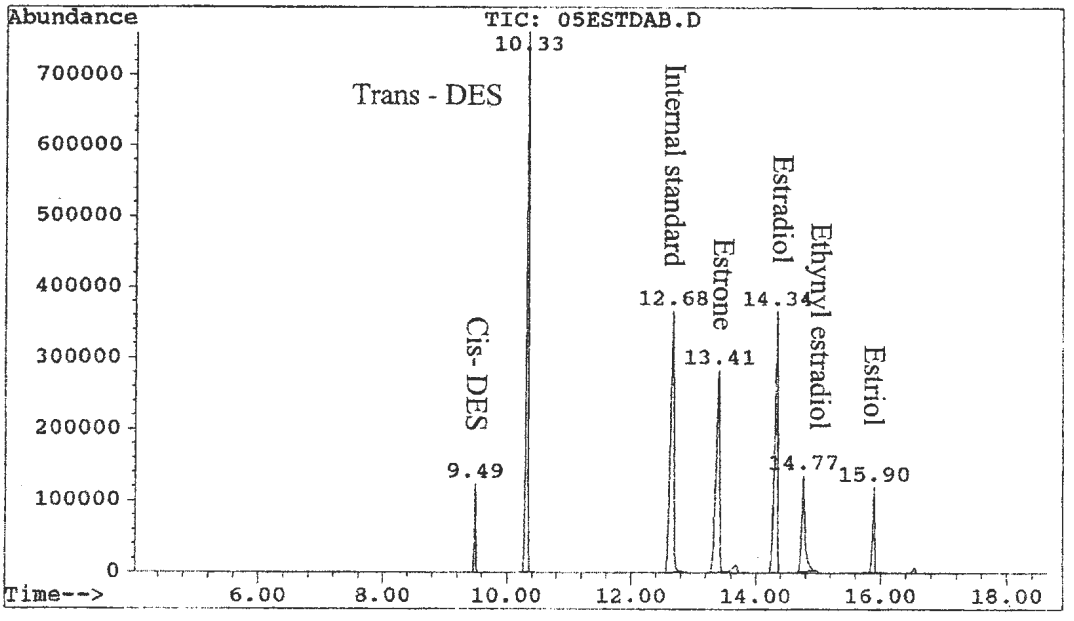


Figure 3.4b. Selected ion chromatogram of TMS-derivatized analyte standards obtained in GC-(EI) MS. The chromatographic conditions are given in Table 2.1.

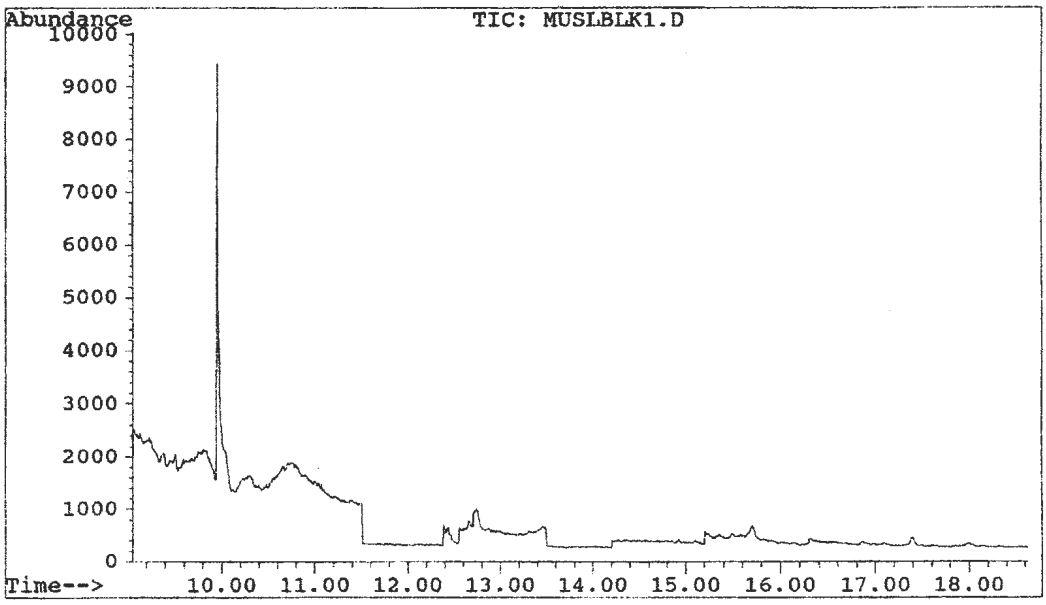


Figure 3.5. Selected ion chromatogram of mussel matrix acquired using GC-(CI)MS. Chromatographic conditions are given in Table 2.1.

Table 3.8. Comparison of instrumental responses of steroid estrogens in GC-MS in EI and CI modes.

Analyte	Retention time (min.)	Peak area of analytes	
		Electron Impact ionization	Chemical Ionization (NH_3)
DES	9.01	3255368	588714
E_1	12.26	3918482	652067
E_2	12.96	4212050	418551
EE_2	14.56	1421861	246217
E_3	15.61	2217534	273917

In each case, 5 ng of analytes were injected. The GC-MS was operated in SIM mode Operating conditions are shown in Table 2.2.

GC-(ion trap)MS/MS has been widely used in the organic trace analysis for complex matrices such as sewage, tissues, blood and serum (Chapman, 1993). However, only few investigators have used this instrumental method for the analysis of steroid estrogens in environmental samples. Ternes et al., (1999a) developed a sensitive method based on GC-MS-MS for the determination of estrogens in raw sewage and surface water where a limit of detection (LOD) of 1 ng/L and 0.5 ng/L for natural estrogens from sewage and surface water respectively were obtained. In a similar investigation, Belfroid et al., (1999) have developed an improved analytical method for estrogen analysis based on MS/MS detection. LOD for steroidal analytes were better, ranging from 0.1 ng/L to 0.3 ng/L for surface water and from 0.1 ng/L to 2.4 ng/L for sewage effluent.

The isolation of a parent ion of the target analytes from ions of matrix materials and its subsequent fragmentation to produce a daughter ion spectrum improves the selectivity of MS/MS detection. For quantitative work, additional selectivity can be achieved by extracting a few characteristic ions from the daughter ion spectrum. Table 2.3 lists the optimized GC- (ion trap) MS-MS conditions used in this study. In Figure 3.6, a portion of a MS/MS chromatogram obtained during the analysis of the target analytes in tissue matrix is depicted. Using this technique, detection of analytes at levels as low as 50 pg/μL (S/N = 10) was achieved. Therefore, GC-MS-MS technique was chosen for the quantification of analytes in environmental samples.

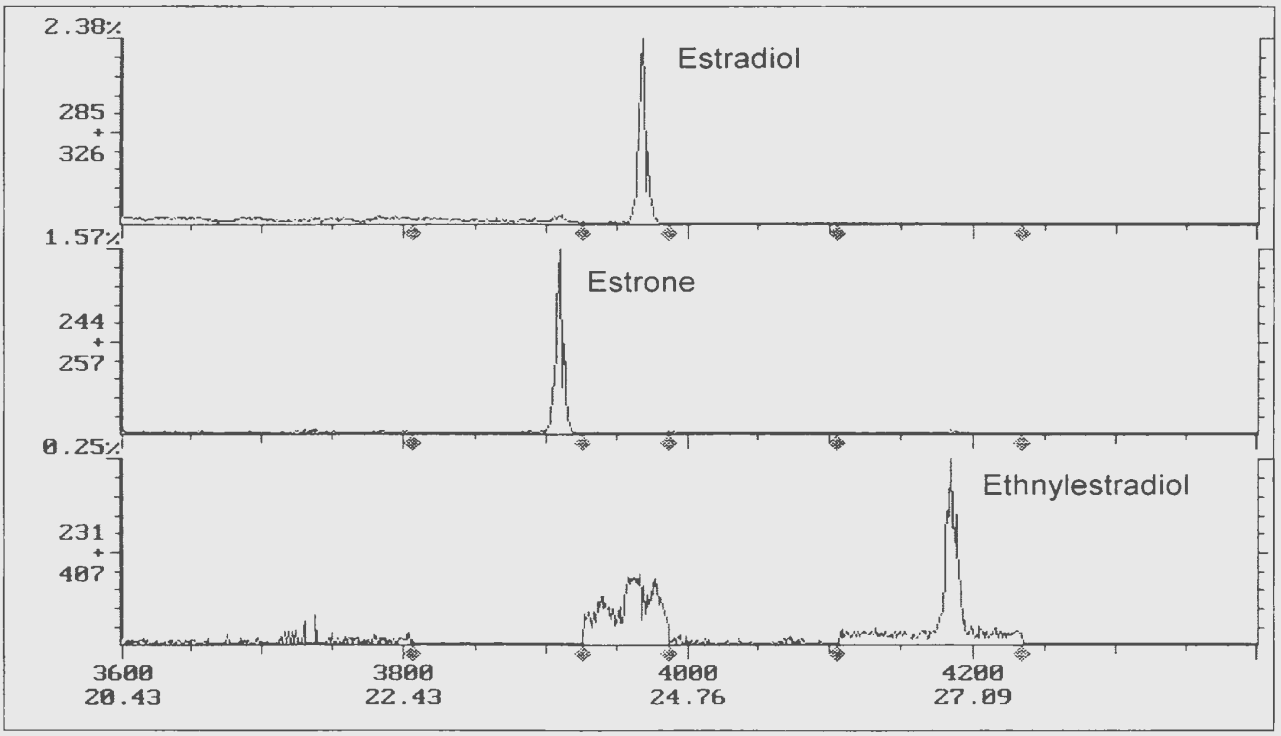


Figure 3.6. Extracted ion chromatogram of target analytes in mussel matrix acquired using GC-(ion trap)MS/MS. The experimental conditions are given in Table 2.1 and 2.3.

3.3. Overall method performance

Quantitative performance characteristics of the analytical methods are evaluated using percentage recovery, precision and limit of detection.

3.3.1. Analysis of spiked mussel tissue

Recovery studies were performed using mussel tissue samples procured from a local seafood shop. Absence of target analytes in these samples (prior to spiking) was ascertained by a blank mussel analysis. Recovery studies were performed using homogenized tissue samples spiked with target analytes at 20 ng/g (wet weight). Analytical protocol described in Figure 2.3 was followed for the analysis of 5 g of the wet tissue sample. During GC-(ion trap)MS/MS analysis, duplicate injections were made for each sample to determine peak areas. After each injection, the GC column was baked out at 290° C for 15 minutes, to remove any residue from the column and to minimize carryover. Often a solvent blank was run to check for carryover.

The percentage recoveries for each analyte over the entire sample workup (Figure 2.3.) were determined. Table 3.9 shows the results of percentage recoveries, precision, and limit of detection for target analytes. The recoveries of all the target analytes were above 60% except for mestranol. The recovery of mestranol was poor (44%) for some unknown reason. Reproducibility of the method based on relative standard deviation (for triplicate analyses) ranged from 7.7% to 13.3%.

Table 3.9. Recovery of target analytes spiked in mussel tissue using mussel analysis protocol

Analyte	Limit of detection	Percentage recovery			Average recovery (%)	RSD (%)
		Trial 1	Trial 2	Trial 3		
DES	0.1 ng/g	79	72	84	78	7.7
E_1	0.1 ng/g	88	76	97	87	12.1
E_2	0.3 ng/g	64	56	73	64	13.3
Mestranol	0.5 ng/g	41	41	51	44	13.2
EE_2	1.0 ng/g	79	71	84	78	8.5

In each trial, 5 g of (wet weight) tissue spiked with 100 ng of analytes were extracted using the developed method (Figure 2.3.) and analyzed by GC-MS/MS.

LOD values (S/N = 3) were calculated based on the wet weight of the mussel tissue. RSD values are calculated as shown in Table 3.1.

Limit of detection (LOD) of the analytical method was estimated and was dependent on the sensitivity of the instrument, background ions generated by the tissue matrix, and the amount of tissue sample used in the analysis. A signal to noise ratio (S/N) of three was used to report LOD. LOD values for the target analytes ranged from 0.1 ng/g (DES and E_1) to 1 ng/g (EE_2), (Table 3.9). Hence, the developed method was found to be sufficient to detect steroid estrogens at low to sub-ppb levels (ng/g) from tissue matrix.

3.3.2. Analysis of spiked water

Different approaches have been reported for performing recovery studies of steroids in water and wastewater. Some investigators preferred to match the matrix components (sewage effluent, surface water) in the recovery studies while others simply used distilled water. Lagana et al. (2000) spiked analytes in aliquots of composite sewage effluent either before or after the extraction procedure. The difference in the concentration between the two samples is then used to estimate recovery. Baronti et al. (2000) spiked the analytes in the sample matrix in which the analyte concentration were predetermined (blank value). In this case, the recoveries were calculated after appropriate blank subtraction. Alternatively, tap water (Desbrow et al., 1998), ground water (Snyder et al., 1999) and distilled water (Sole et al., 2000; Lopez de Alda and Barcelo, 2000; Kuch and Ballschmiter, 2000; Cathum and Sabik, 2001) matrices were used as a substitute for sewage matrix in recovery studies. Lee and Peart (1998) performed recovery experiments in distilled water, sewage water

and sewage water previously extracted. The percentage recovery values did not vary significantly between these three experiments.

In this work, the recovery studies were performed using nanopure water. For this, 1 L of water was spiked with 100 ng of analyte standards. The water was then mixed well using a stirrer for about 30 minutes at room temperature. The analytes were extracted using the workup protocol illustrated in Figure 2.4. Table 3.10 shows the percentage recoveries of analytes, the precision and the LOD of the analytical method. The recoveries of analytes were above 80% in all cases. The reproducibility measured in terms of RSD values was found to vary from 3.0 % to 6.8 %.

3.4. Analysis of real environmental samples

3.4.1. Sewage and seawater samples

The concentration of steroid estrogens in seawater depends on many factors, including the sewage discharge rate, dilution factor, and the distance of the sampling location from the sewage outfall. In the present study, seawater samples from two locations (H-1 and H-2) in Halifax harbour (Figure 2.1.) were analyzed using the developed analytical method. Two of the target analytes, estrone (E1) and coprostanol were identified in all the analyzed samples. In Figure 3.7a, a portion of GC-(ion trap)MS/MS mass chromatogram of a seawater analysis is shown. For comparison, the elution of estrone standard under identical chromatographic condition is given (Figure 3.7b). Close retention time matching observed in these analyses together with mass spectral matching confirmed the presence of estrone. The concentrations of target analytes from Site H-1 and H-2 are tabulated in Table 3.11. In both sites, both

Table 3.10. Recovery of target analytes from nanopure water

Analyte	Limit of detection (ng/L)	Percentage recovery (%)			Average recovery (%)	RSD (%)
		Trial 1	Trial 2	Trial 3		
DES	0.5	94	101	97	97	3.6
E_1	0.5	93	99	102	98	4.7
E_2	0.5	89	86	98	91	6.8
Mestranol	1.2	85	90	83	86	4.2
EE_2	1.2	85	82	87	84	3.0
Coprostanol	2.5	88	89	82	86	4.4

100 ng of analyte mixture spiked in 1 L of nanopure water was extracted using the protocol developed for sewage analysis (Figure 2.4.) and analyzed using GC-MS/MS. RSD values are calculated as shown in Table 3.1. LOD values were measured at S/N = 3.

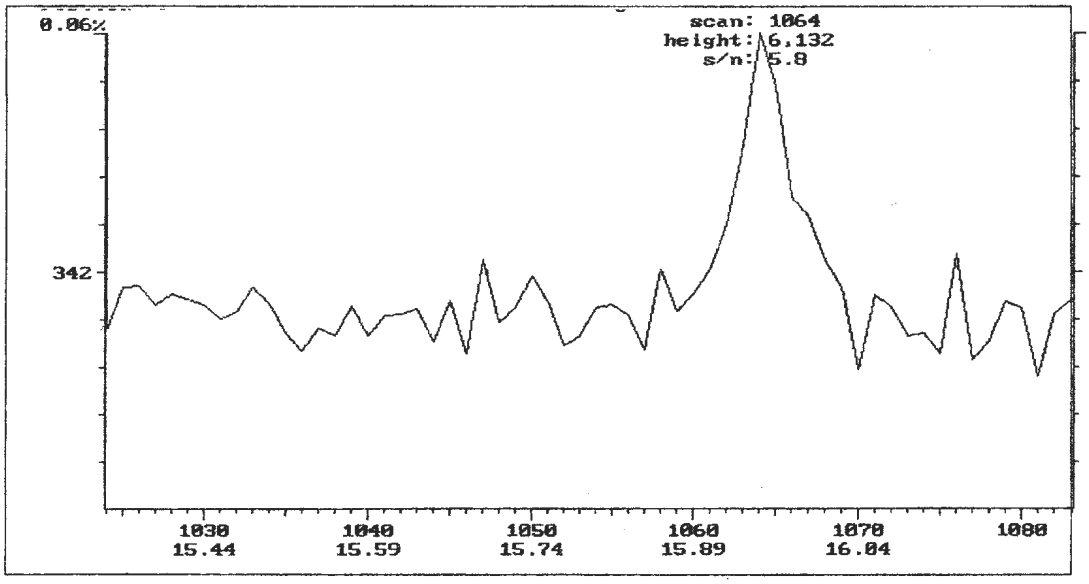


Figure 3.7a. Detection of estrone (E_1) in seawater sample from Halifax harbour. A portion of extracted ion GC-(ion trap)MS/MS mass chromatogram is shown. Retention time of E_1 from this analysis is compared to that of pure E_1 standard as shown below. Both mass chromatograms were taken under identical conditions. In this particular analysis, E_1 was detected at a signal to noise ratio of 6:1.

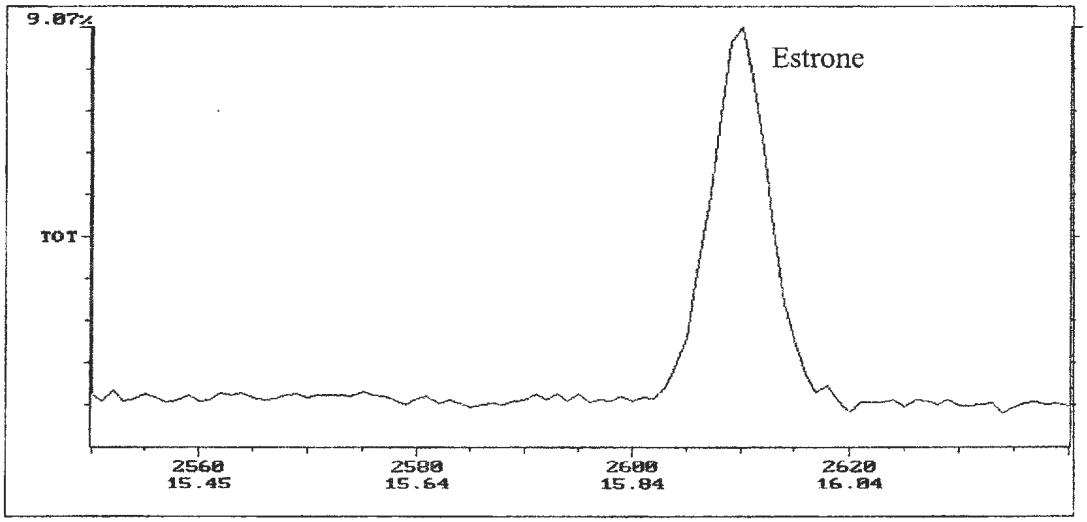


Figure 3.7b. GC-(ion trap)MS/MS mass chromatogram of estrone (E_1) standard.

Table 3.11. Analysis of seawater samples from Halifax harbour

Analyte	Site H-1 Downtown, Halifax (n = 2)	Site H-2 Northwest arm, Halifax Harbor (n = 3)
E_1	4.0 ± 1.1 ng/L	6.6 ± 0.9 ng/L
Coprostanol	1.3 ± 0.1 μg/L	29.2 ± 1.1 ng/L

the natural estrogen E_1 and the fecal biomarker coprostanol were detected. The concentration of coprostanol was higher at H-1 as expected because of the number of sewage outfalls near H-1.

It was of interest to see the levels of steroidal compounds in raw sewage before and after its discharge in the ocean. Therefore, samples of raw sewage were collected from three major sewage-pumping stations (X-1, X-2, X-3) in and around St. John's. As well, seawater from two locations (St-1 and St-2) in the harbour (Figure 2.2.) close to the point of sewage discharge was collected. Table 3.12 lists the concentration of target analytes found in these samples. In Figure 3.8, the GC-(ion trap)MS/MS mass chromatogram of sewage analysis is shown. E_1, E_2 and coprostanol were detected in all the sewage samples. Moreover, all these analytes were found in one of the seawater samples from St-1.

The concentration of E_1 and E_2 in sewage ranged from 5.7 to 20.2 ng/L & from 4.3 to 30.1 ng/L respectively. The concentration of coprostanol was three orders of magnitude higher than natural estrogens. In Table 3.13, the concentration of steroid estrogens reported by several investigators is presented. Baronti et al. (2000), reported 25 to 132 ng/L (E_1), 6.3 to 25 ng/L (E_2), and 0.40 to 13 ng/L (EE_2) in 6 STP influent samples in Rome. A Canadian study reported 14 to 109 ng/L (E_1), and 6 to 15 ng/L (E_2), in STP influents in various places across Canada (Lee and Peart, 1998). This study did not find EE_2 in any sewage effluent samples. The concentrations of steroids measured in the present study are in close agreement with the levels reported in the literature. Among the sewage - pumping stations, Southside station processes a higher volume of wastewater while Beck's Cove station receives wastewater from domestic

sewage as well as from surface run offs (combined sewer). Therefore as expected, the concentrations of steroids were higher at Southside station.

Steroids were also detected in St. John's harbour seawater samples at low ng/L levels. In St-1, natural estrogens E_1 and E_2 and coprostanol were detected at 1.5 ng/L, 1.8 ng/L and 4.9 μg/L, respectively. In St-2 only E_1 and coprostanol was detected at levels of 1.4 ng/L and 5.0 μg/L respectively. There was no significant difference in the concentrations of the steroids and coprostanol detected in these two locations, which might indicate efficient mixing of the sewage with seawater in St. John's harbour.

Table 3.12. Analysis of sewage and seawater samples from St. John's harbour

Analytes	Raw sewage sample (Duplicate analysis)			Seawater	
	Southside (X-1)	Goulds	Beck's cove (X-2)	Southside (St-1)	Beak's cove (St-2)
Estrone (E_1)	20.2 ± 3.1 ng/L	12.0 ± 2.7 ng/L	5.7 ± 0.9 ng/L	1.5 ng/L	1.4 ng/L
Estradiol (E_2)	30.1 ± 3.0 ng/L	10.9 ± 1.4 ng/L	4.3 ± 1.4 ng/L	1.8 ng/L	Not detected
Coprostanol (CP)	160.4 ± 3.8 µg/L	31.4 ± 4.9 µg/L	540.0 ± 60.0 ng/L	4.9 µg/L	5.0 µg/L

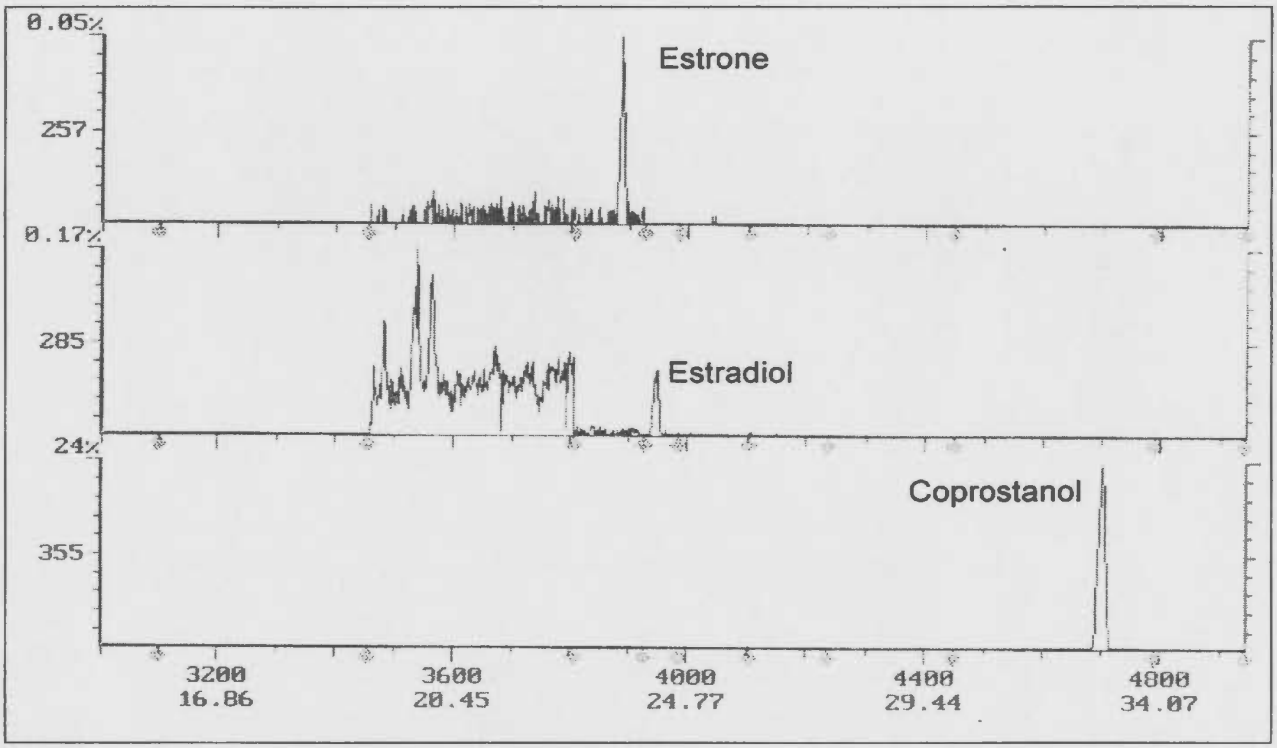


Figure 3.8. Analysis of sewage samples from St. John's harbour. Extracted ion GC-(ion trap)MS/MS mass chromatogram of a sewage sample. A list of daughter ions used for quantification is given in Table 2.2.

Table 3.13. Review of literature: Levels of target analytes in raw sewage, STP effluents and receiving water

Publication	Analyte studied	Sample type		
		Raw sewage/STP influent	Treated sewage/STP effluent	Receiving water
Cathum and Sabik (2001), Canada	E_1, E_2, and coprostanol (CP)	----	14.66µg/L (CP) < 2ng/L (E_1) < 2ng/L (E_2)	38 ng/L (E_2) 2 and 67 ng/L (CP) < 2 ng/L and 6 ng/L (E_1)
Sole et al. (2000), Denmark	E_1, E_2, E_3, DES, EE_2, mestranol	261 ng/L (E_3)	34 and 43 ng/L (DES)	-----
Kuch and Ballschmiter (2000), Germany	E_1, E_2, E_3, EE_2, mestranol	-----	3 to 13 ng/L (E_1) 1 to 13 ng/L (E_2) < 1 to 9 ng/L (E_3) < 1 to 5 ng/L (EE_2)	-----
Baronti et al. (2000), Italy	E_1, E_2, E_3, EE_2,	25 to 132 ng/L (E_1) 6.3 to 25 ng/L (E_2) 24 to 188 ng/L (E_3) 0.40 to 13 ng/L (EE_2)	3.3 to 82.1 ng/L (E_1) 0.44 to 3.5 ng/L (E_2) 0.44 to 18 ng/L (E_3) < DL to 1.7 ng/L (EE_2)	-----
Lee and Peart (1998), Canada	E_1, E_2, E_3, EE_2	14 to 109 ng/L (E_1) 6 to 15 ng/L (E_2) 53 to 250 ng/L (E_3)	5 to 19 ng/L (E_1) <5 ng/L (E_2) <10 to 34 ng/L (E_3)	-----
Belfroid et al. (1999), The Netherlands	E_1, E_2, EE_2	-----	< 0.6 to 1.1 ng/L (E_2) < 0.4 to 47 ng/L (E_1) <0.2 to 7.5 ng/L (EE_2)	<0.3 to 5.5 ng/L (E_2) <0.1 to 3.4 ng/L (E_1) <0.1 (EE_2)
Larsson et al. (1999), Sweden	E_1, E_2, EE_2	----	5.8 ng/L (E_1) 1.1 ng/L (E_2) 4.5 ng/L (EE_2)	-----
Desbrow et al. (1998), UK	E_1, E_2, EE_2	-----	1 to 80 ng/L (E_1) 1 to 50 ng/L (E_2) <DL to 7 ng/L (EE_2)	-----

3.4.2. Mussel samples

Very little information is available on the analysis of steroid hormone contaminants in mussels. In Table 3.14 the concentrations of sewage-related estrogens in mussels reported in the literature is summarized. Reis-Henriques et al. (1990) was the first to measure the natural levels of human estrogens in mussels (Mytilus edulis). E_1 and E_2 were found in low pg/g levels in both male and female mussels. Gross et al. (2000) studied the distribution of estrogen in different organelles of the mussels. They measured the concentration of E_2 in gonads, foot, mantle and internal fluid of a freshwater mussel species (*Elliptio buckleyi*) and found marginally higher concentrations of estrogens in gonads. Based on the radioimmunoassay (RIA) method, about 1.2 ng/g of total estrogens was measured in gonads out of which 0.8 ng/g was contributed by estradiol alone. Information on accumulation of coprostanol in mussels is also very limited. Sherwin et al. (1993) reported the concentration of coprostanol ranging from 80 to 620 ng/g (dry wt.) in mussels (*Mytilus galloprovincialis*) found in the Lagoon of Venice. Cathum and Sabik (2001), studied the accumulation of coprostanol and other steroids in mussels. Using GC-MS, they detected about 32 μg/g of coprostanol; however, the targeted steroid hormones were not detected. Recently Hellou et al. (2003) reported the concentration of coprostanol in mussels collected from various sites from Halifax harbour. This included mussel samples from site M_8, M_{12} and M_{14}, analyzed in this study.

In the present study, the concentrations of identified steroids measured in mussel samples collected from Halifax and St. John's harbour are tabulated in Table 3.15 and 3.16. Coprostanol was detected in all mussel samples indicating

Table 3.14. Review of literature: Concentration of target analytes in mussels

Publication	Analytes studied	Concentration of analytes (based on wet wt.)	Method of quantification
Reis-Henriques et al., (1990)	E1, E2, E3	E1 - 48 to 55 pg/g (Male) - 45 to 80 pg/g (Female) E2 – 21 to 38 pg/g (Male) - 23 to 53 pg/g (Female)	RadioImmuno Assay (RIA)
Gross et al., USGS report.	E2 and total estrogens	E2 - >0.8 ng/g (Gonad) - 0.5 ng/g (Foot) - 0.2 ng/g (Mantle) - 0.1 ng/g (Fluid)	RIA
Cathum and Sabik, (2001)	E1, E2, E3 and Coprostanol	E1, E2, E3 were not detected. Coprostanol: 32.25 μg/g	GC-MS
Sherwin et al., (1993)	Coprostanol	Coprostanol: 80 to 620 ng/g*	GC
Hellou et al., (2003)	Coprostanol	Coprostanol: 0.13 to 4.32 μg/g	GC-MS

* Based on dry weight.

Table 3.15. Analysis of mussel samples from Halifax harbour. All concentrations are based on dry weight of the mussels.

Analyte	Site M_8	Site M_{12}	Site M_{14}
E1	Not detected	0.3 ± 0.1 ng/g	Not detected
Coprostanol	34.7 ± 3.8 ng/g*	11.8 ± 1.4 ng/g*	3.4 ± 0.5 ng/g*

* These values represent the residual coprostanol in mussel extract after hexane extraction and do not reflect actual concentration. Values are given to show the relative sewage contamination among sampling sites.

Table 3.16. Analysis of mussel samples from St. John's harbour. All concentrations are based on dry weight of the mussels.

Analyte	MST-1 (Duplicate analysis)	MST-2 (Duplicate analysis)	
		August	November
E1	Not detected	Not detected	0.5 ± 0.1 ng/g
Coprostanol	10.2 ± 1.1 ng/g*	13.0 ± 2.4 ng/g*	43.1 ± 5.9 ng/g*

* These values represent the residual coprostanol in mussel extract after hexane extraction and do not reflect actual concentration. Values are given to show the relative sewage contamination among sampling sites.

prevalent fecal contamination at these sampling sites. As mentioned earlier, during mussel analysis most of the non-polar materials including coprostanol were removed by partitioning with hexane. Therefore the concentration of coprostanol reported indicates relative fecal contamination among sampling sites.

Out of five sampled sites, E_1 was identified at Site M_{12} (Halifax harbour) and at site MST-2 (St. John's harbour) at very low concentration. Figures 3.9a and 3.9b show a portion of the extracted ion chromatogram and the MS/MS library spectral match for coprostanol and estrone in harbour samples. MS/MS library for all the target analytes was generated by analyzing pure analyte standards. The presence of E_1 and coprostanol in these samples were confirmed by matching their retention times and MS/MS spectrum to that of pure standards.

It is interesting to note that one of the natural estrogens, E_1 was identified in mussels collected from harbour sampling sites. Future work should be undertaken to lower the detection limit of the analysis for E_1 and other steroids and to sample more mussel sites in order to ascertain the suitability of mussels as reliable bioindicators of steroidal pollution in coastal areas.

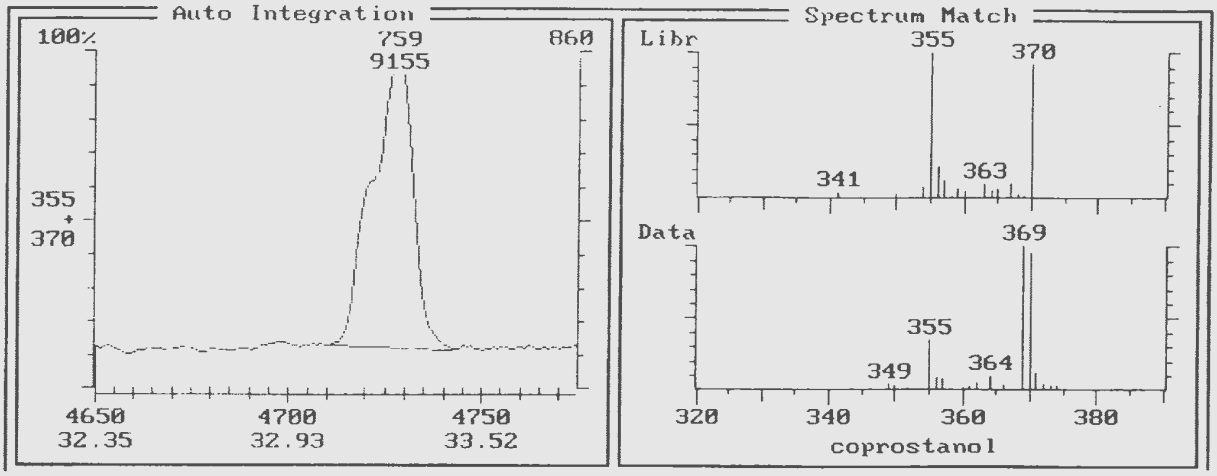


Figure 3.9a. Analysis of mussel samples using GC-(ion trap)MS/MS. Identification of coprostanol in a mussel sample from site MST-2 (St. John's harbour). A portion of extraction ion chromatogram and the daughter ion mass spectral match for coprostanol are shown.

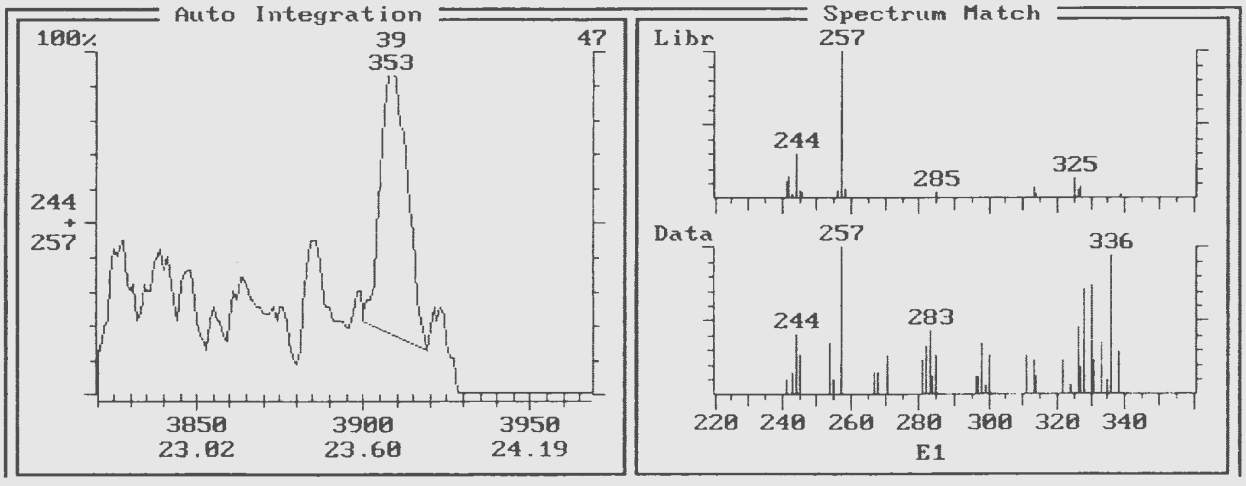


Figure 3.9b. Identification of estrone (E_1) in mussel samples. Extraction ion chromatogram of GC-(ion trap)MS/MS scan from Site 12 (Halifax harbour) and the daughter ion mass spectral match for E_1 are shown.

Conclusion

This research investigation focused on the possible presence of endocrine disrupting chemicals in seawater and mussel samples collected from St. John's, NL and Halifax, NS harbours. The major thrust of this study was to develop a sensitive and reliable method based on GC-(ion trap)MS/MS for the analysis of steroid estrogens in mussel tissue. As expected, the complexity of the tissue matrix posed serious problems for the trace analysis of steroids in mussels. A multi-step protocol was developed to remove interfering substances from the initial tissue extract. Analytes were extracted using methanol, which also helped to precipitate proteins. Neutral lipids were removed by partitioning with hexane. Coprostanol and non-target sterols such as cholesterol were removed in hexane fraction. GC derivatization reactions with PFPA and TMS reagents were found susceptible to matrix interference. Therefore, two different cleanup procedures were investigated to purify analytes before the derivatization step. The first, a novel cleanup procedure based on extractive alkylation was developed for this purpose. However, due to significant interferences from the tissue matrix this procedure was found to be unsuitable for trace analysis. The second, a silica column cleanup procedure was developed and found suitable.

Comparative study on different GC techniques indicated that GC-(ion trap)MS/MS would be the most reliable instrumental technique for the analysis of trace levels of steroid estrogens in mussels. Good reproducibility and satisfactory recovery (>60%)

of spiked analytes from complex tissue matrix demonstrates the robustness of this analytical approach. Although developed for mussel tissue analysis, this method can be a useful technique for the analysis of steroids from other types of tissues such as meat.

The scope of the developed analytical technique was demonstrated by analyzing mussel and harbour seawater samples from St. John's and Halifax harbours. Presence of fecal biomarker coprostanol in seawater samples indicates widespread sewage pollution. The natural estrogen, estrone, (E_1) was detected in low ng/L levels in all seawater samples while estradiol (E_2) was detected only at St-1 in St. John's Harbour. Due to their low capacity to metabolize foreign substances, mussels accumulate contaminants and could be susceptible to the presence of endocrine disrupting chemicals. In this study, coprostanol was used as a qualitative indicator for the exposure of mussels to raw sewage. A relatively higher level of coprostanol in mussels from Site-8 (Halifax harbor) is consistent with high sewage inputs in this area. Absence of mussels at on-shore locations in St. John's harbour at certain times of the year suggests that mussels are severely affected by sewage inputs. Natural estrogen estrone (E_1) was semi-quantitatively measured in mussel samples from one Halifax harbour site (Site-12) and one St. John's harbour site (MST-2; November).

The presence of steroid estrogens in harbour seawater as well as in mussels together provide good evidence for the exposure of these marine invertebrates to sewage-related contaminants. The results of this study are preliminary since only a few samples were taken for analysis. A more extensive seawater and mussel sampling

program must be undertaken to determine if mussels can be used as reliable bio-indicators of steroidal contamination in the marine environment.

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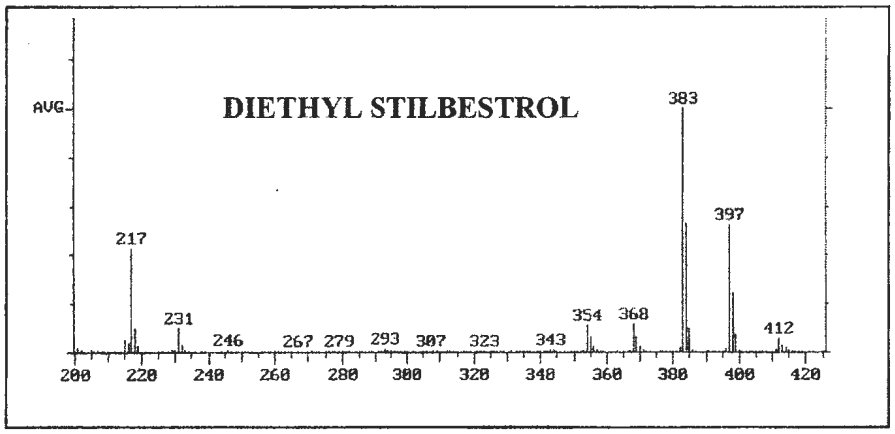
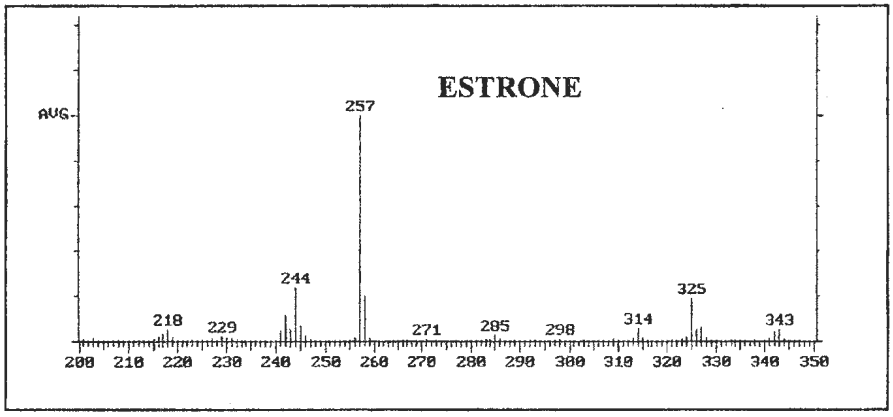
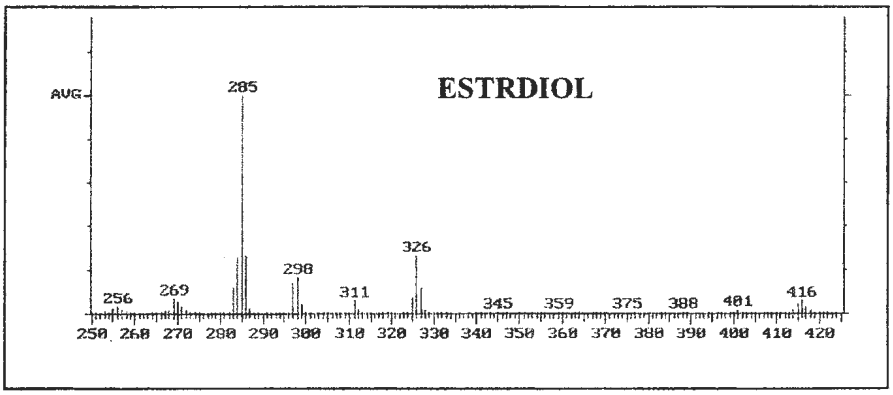
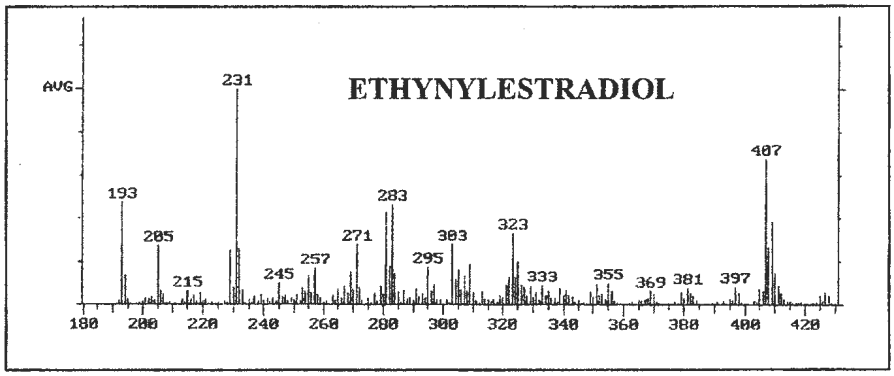
APPENDIX A

Daughter ion spectrum of analyte standards acquired in Varian GC-MS-MS instrument. The list of parent ions is shown in Table 2.3.








ETHYNYLESTRADIOL
AVG
231
193
205
215
245
257
271
283
295
303
323
333
355
369
381
397
407
180
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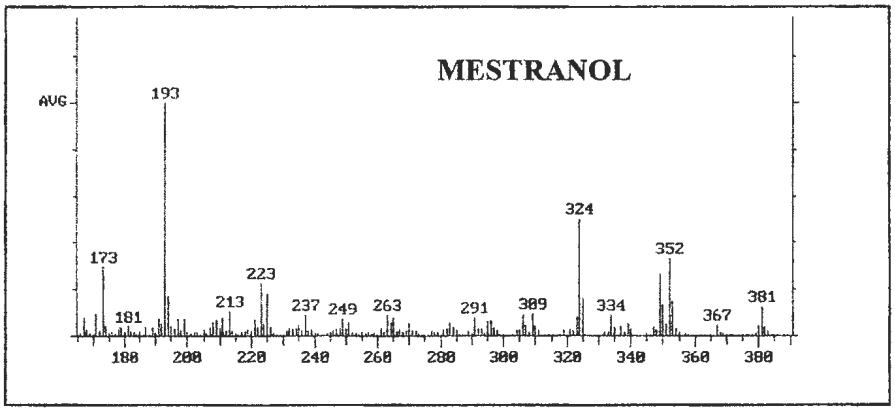
240
260
280
300
320
340
360
380
400
420


MESTRANOL
AVG
193
173
181
213
223
237
249
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220
240
260
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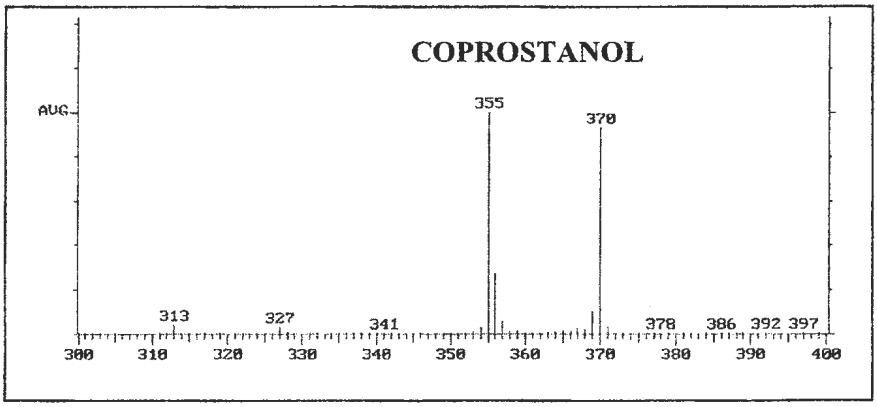
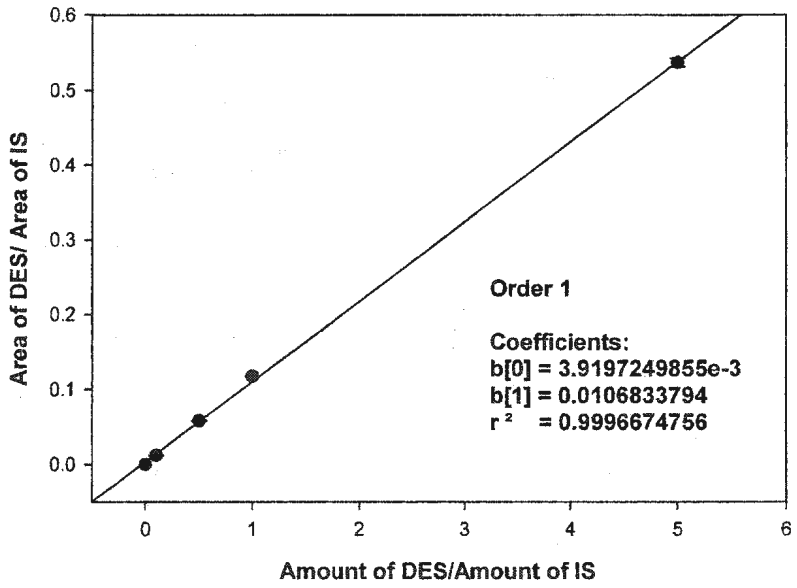
300
320
340
360
380


COPROSTANOL
AVG
355
370
313
327
341
378
386
392
397
300
310
320
330
340
350
360
370
380
390
400

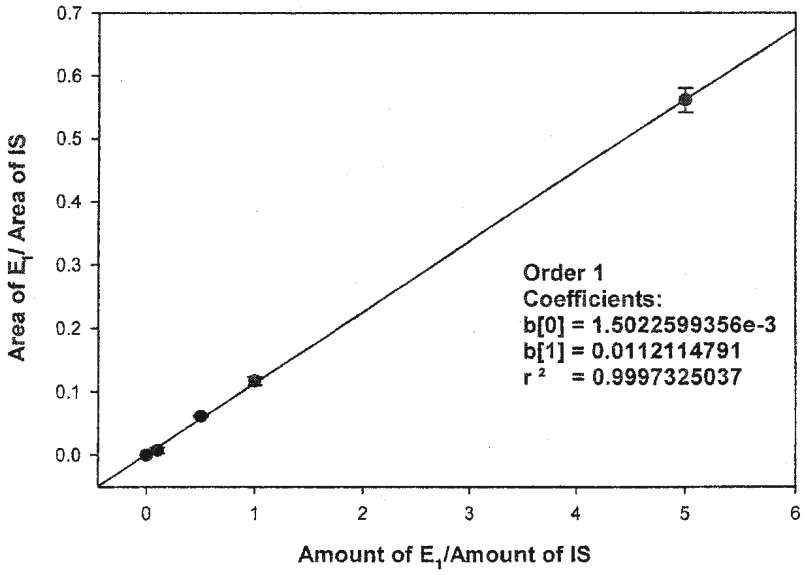
APPENDIX B

Calibration curve for some of the target analytes studied in this work. In each case, 10 ng of E_1-methyl ether is used as internal standard.

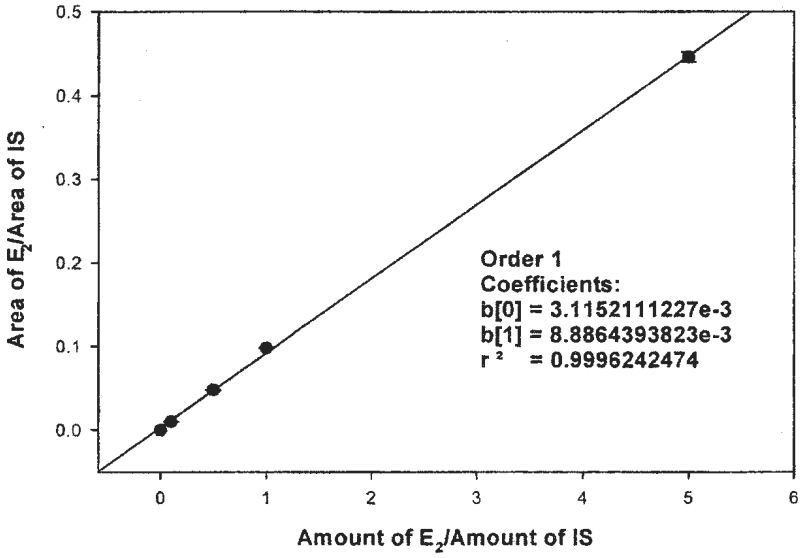
Calibration curve for DES



Calibration curve for E_1



Calibration curve for E_2



Calibration curve for EE_2