Impact of Endocrine Disrupting Chemicals (EDCs) on Estrone and Estradiol Content in Fish

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Summary: The objective of this paper is to investigate the exogenous chemicals that interfere with natural hormonal functions and considered endocrine disrupting chemicals (EDCs). Estrone (E1) and Estradiol (17β-estradiol or E2) are natural female hormones and the most potent of all xenoestrogens. The occurrence of estrone (E1) and 17*β*-estradiol (E2), in the tissues of different edible fish and shellfish, collected along the Virginia Coast was analyzed by solid-phase extraction and analysis by gas chromatography, mass spectrometry (GC–MS). Collective (E1 and E2) mean value was determined in all samples. E1 in male and female fishes were ranged as <LOD-3.04ng g^{-1} and 1.00-3.04ng g^{-1}, respectively. Similarly E2 determined in male and female fishes was ranged as <LOD -5.02ng g^{-1} and <LOD-2.30ng g^{-1}, respectively. However, in shellfish E1 and E2 varied as <LOD- 4.00ng/g and <LOD -5.024 ng/g, respectively. E1 and E2 reaches aquatic systems mainly through sewage and animal waste disposal. E1 and E2 concentrations in fish samples varied between <LOD and 5.02 ng g^{-1} (wet weight) . Highest E1 concentrations was determined in Stripped Bass (F) that is 3.37ng/g and highest E2 concentrations was determined in spotted Seatrout (male) fish the E2 concentrations in all the fish tested was 2.8–3.7 ng g^{-1}.

Introduction

A number of chemicals found in the environment have been shown to mimic or antagonize the actions of steroid hormones, an issue often described as "endocrine disruption/ modulation". There is very little evidence, however, to support the hypothesis that exposure to Endocrine-Disrupting Chemicals (EDC) is a global environmental health problem. However, feminization and any resulting effects on fish populations is not concern. Another potential effect might be immune system changes and perhaps greater susceptibility to infections and other diseases. It was fish kills and skin lesions on a number of fish species in the south branch of the Potomac River [1]. It was more than two decades ago when sexual disruption was first reported in male *Rutilus rutihs* (roach) a fish that was harvested from the Lea river, in southeast England [2]. This unusual finding initiated concern about Vitellogenenin (VTG) is normally synthesized by the liver in female oviparous (egg-laying) vertebrates and presence of VTG in male fish indicates that the fish have been exposed to Endocrine Disrupting Compounds. (EDC_S) or the pollution of disrupting chemicals [3]. The U.K. and European epidemiological is data sets have demonstrated that the occurrence of feminized fish associated with effluent discharges and that the incidence and severity is positively correlated with the proportion of treated sewage effluent in receiving waters. Based on an evaluation of existing information, the environmental agencies of England and Wales have concluded that the weight of evidence for endocrine disruption in fish is sufficient to develop a risk management strategy for estrogenically active effluents that are discharged to the marine environment [3].

Since the early 1980s, there has been growing concern about the ability of substances discharged into the environment to disrupt the normal endocrine (hormonal) function of aquatic life. This concern and subsequent investigations have been prompted by field observations of changes in the reproductive physiology and health of wildlife species from several taxonomic groups in many countries [3, 4].

Global concern about EDCs has been

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increasing due to sexual disruption in fish which is related to vitellogenesis (Fig. 1). EDCs bring about hormone imbalance in natural systems [4]. In recent years there has been considerable concern over the ability of substances discharged into the environment to disrupt the normal endocrine function of wildlife [5]. In particular, the apparent widespread feminization of male fish in rivers has received significant attention from regulators in the United Kingdom, the United States, Europe and Japan.

Exposure to xenoestrogens is also associated with abnormal physiological changes and reproductive impairments in birds, fish, shellfish, turtles, gastropods, and mammals [6]. Feminization has been observed in the early life-stages of roach (*Rutilus rutilus*) exposed to estrogenic [7] effluents during periods of sexual differentiation. EDCs also created problem for human beings such as environmental estrogens have been suggested as the cause of increased incidence of male reproductive tract disorders and reduced sperm counts, and also for an increase in the frequency of female breast cancer [8]. Furthermore, intersex imposition (simultaneous presence of both testicular and ovarian characteristics), which is believed to be a consequence of exposure to estrogens, has been observed in the gonads of wild populations of roach [8]. Data suggest that low concentrations of estrogen can also induce oocytes in the testis of the Japanese medaka [9, 10]. E2 concentrations as low as 1–5 ng *l−1* have the ability to induce the production of a female-specific egg-yolk protein precursor VTG in male fish [11]. VTG was found absent in male fish under natural conditions [12]. E2 concentrations can vary due to factors such as dilution, sorption by sediments and organic matter, and photo-degradation [13]. E2 is one of the most potent estrogens; the relative potency of 17β-estradiol is 104 to 106 times that of six. E2 reaches aquatic environments mainly through sewage and animal waste disposal [14].

Impact of Endocrine Disrupting Chemicals

The objective of this study was to measure the concentration of estrone and estradiol in tissue samples of fish (8 edible fishes) and shellfish, including oyster (*Crassostrea ariakensis*) and white shrimp (*Litopenaeus vannamei*), that is not a native to the Chesapeake Bay. Their natural habitat extends from north America to south America in the warmer waters of the Pacific Ocean. Blue crab (*Callinectes sapidus*) and clams (mercenaries) are most common marine products from Virginia (Virginia Sea Food Board 2004).

Results and Discussion

Biometric data for the various fish analyzed was studied. The physiological impact described with the help of anatomical diagram (Fig.1) shows % recoveries obtained in the for each compound, which were 89.27% ± 2.6% and 91.5% ± 2.9% for estrone and 17β-Estradiol, respectively.

Mussel samples were found to contain high levels of cholesterol, whose retention time was close to that of estradiol-17β and strone Spectra of derivative estrone in full scan GC-MS and negative Cl GC-MS ion spectra of estrone derivative The QA-QC results were summarized (Table-1) for recovery, lower detection limit (LOD), and lower reportable limit (LOR) of the method. The quantification limits (by using internal standard) for detection were 1.02ng/g for E1 and 2.102ng/g for E2.

High concentrations of E1 and E2 E_1 & E_2 may have an impact on the determination method for these two compounds .

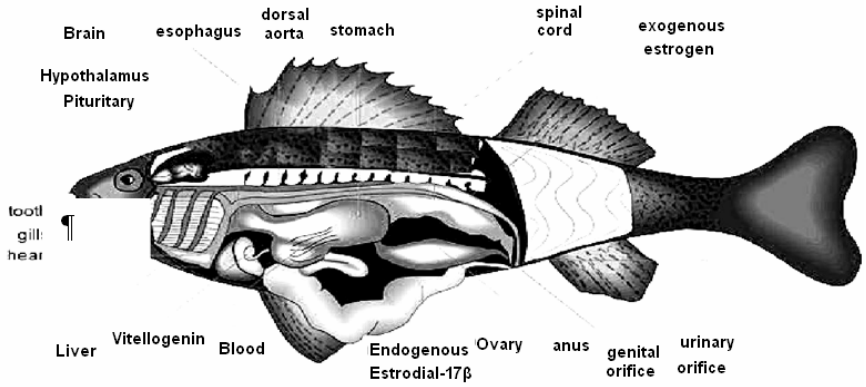

Fig. 1: A Diagram of fish showing route of exogenous estrone and estradiol.

Table-1: QA-QC study of selected steroids in fish and shellfish samples.

Sample (n=4)	Added amount (µg)	Sample N=4	Added amount (µg)
Fish	0.5	89.24±2.6	91.5±2.9
Shellfish	0.5	84.3±1.5	92.6±1.2
Blank	ND	95.2±2.3	94.3±1.6

ND= Not detected

Substantial data sets from laboratory studies have clearly confirmed that exposure to steroids at environmentally relevant concentrations can induce the effects observed in wild fish. Effective concentrations for vitellogenin induction have been reported as low as 5 ng/L for stradiol [15] and 3.2 ng/L for estrone [16]. Low levels of natural and synthetic estrogens have been detected in soil, water, and sewage treatments for many years and their ability to interfere with reproductive functions of living organisms has become a growing concern. Exposure to natural estrogens such as 17ß-estradiol and estrone in soil and water can cause sterility in fish, gastropods, and birds [17]. The intersex condition has been noted in various fish species following exposures to concentrations as low as 10 ng/L each for estradiol and estrone [18]. Mean estrone E1 and 17ß-estradiol E2 concentration in the fish samples ranged from 0-4.0 and 0-5.0 ng/g, respectively; these concentrations were not significantly different from each other as observed previously [19]. Similarly average estrone E1 and E2 concentration in the shellfish samples was ranged <LOD -4.00 ng/g and <LOD-0-5.02 ng/g (w.w) respectively. During the present study, estrone and estradiol-17β also analyzed in a mixture form . A GC chromatogram and mass spectra for a fish sample with estradiol is given in Fig 2. Similar concentrations of steroid estrogens upstream of Sewage Treatment Plants (STPs) have been reported [20] spawning fish can also contribute steroid hormones to surface waters [21].

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The amount of E2 was reported as15 ng g^{-1} in dried Canada geese waste [22]. E1 concentrations (4.00 ng g^{-1}) in croaker and crab and E2 concentrations (5.00 ng g^{-1}) flounder, flounder eggs and clam (Table-2, 3) were highest for the James River, which is considered as being not very polluted based on nutrients; however, metals, micro-organisms and similar pollutant concentrations have been reported (Fig. 3, 4). Although E1 mass spectra were found different from those of estradiol-17β (Fig. 5).

The sex ratio was female of biased at the effluent site in both the fall of 2003 and the spring of 2004 [23] the frequency of males at the effluent site (17–21%) was half that of the upstream site (36–46%). Intersex comprised 18–22% of the population at the effluent site. Intersex fish were not found at the upstream site. Chemical analyses determined that the WWTP effluent contained a complex mixture of endocrine of active chemicals, including 17β-estradiol (E_2) 17α-ethynylestradiol, alkylphenols, and bisphenol resulting in an estimated total estrogen equivalence of up to 31 ng/L of E_2 [24] These results indicate that the reproductive potential of native fishes may be compromised in wastewater of dominated streams [25].

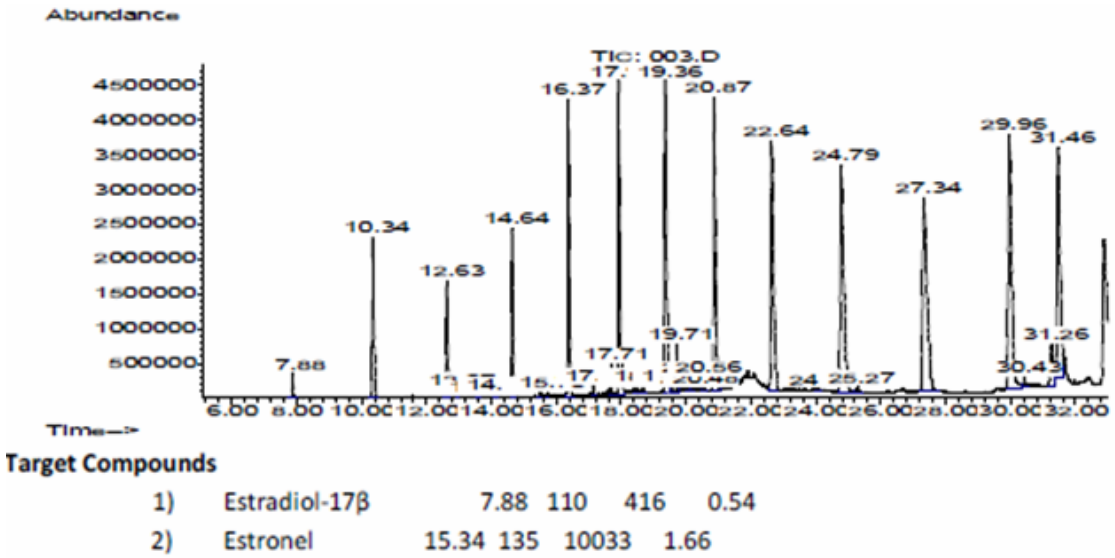


Fig. 2: Typical total ion chromatogram of oyster sample spiked with internal standard.

Table-2: Rusults for steroir compounds determined in edible fishes from Virginia Coast ng/g (wet weight).

S. No	Name of Samples	Sex	Estrone	Estradiol-17β
1.	Rock *(Momorone sexalities)*	M	1.00±1.02	2.30±1.023
2.	Stripped Bass(*Centropristis striate*)	F	3.37±2.03	<LOD
3.	Stripped Bass(*Centropristis striate*)	M	1.012±1.03	1.30±1.04
4.	Summer Flouder(*Paralichthys detatus*)	M	3.00±1.02	<LOD
5.	Blue fish(*Pomatomus saltarix*)	F	<LOD	1.630±1.05
6.	Blue fish(*Pomatomus saltarix*)	M	2.86±0.36	3.02±1.89
7.	Crocker (*Micropogon undulatus*)	M	1.23±1.00	3.00±1.04
8.	Crocker (*Micropogon undulatus*)	F	<LOD	<LOD
9.	Spotted SeaTrout (*Cynoscion nebulosus*)	M	3.04±0.23	5.20±2.56
10.	Spotted SeaTrout(*Cynoscion nebulosus*)	F	1.20±1.02	2.03±1.023
11.	White Perch (*Morone americana*)	M	1.03±3.21	4.25±1.24
12.	White Perch (*Morone americana*)	F	3.04±0.23	2.30±1.023
13.	Tilapia	ND	<LOD	<LOD

Table-3: Results for Concentration (w.w ng/g) of Polichlorinated Biphenyl(PCBs) determined in Fish from Virginia Coast.

S. No	Samples	Estrone	Estradiol-17β
1.	Oyster(*C.ariakensis*) (15)	2.056±1.23	2.0500±0.23
2.	Clam (*Callinectes sapidus*) (12)	<LOD	5.02±6.35
3.	Crab(*Litopenaeus vannameri*) (12)	4.00±1.96	<LOD
4.	White Shrimp (*Litopenous vannamei*)(30)	2.001±4.36	<LOD
5.	Eggs od flounder (04 pairs)	1.02±3.45	5.024±1.89

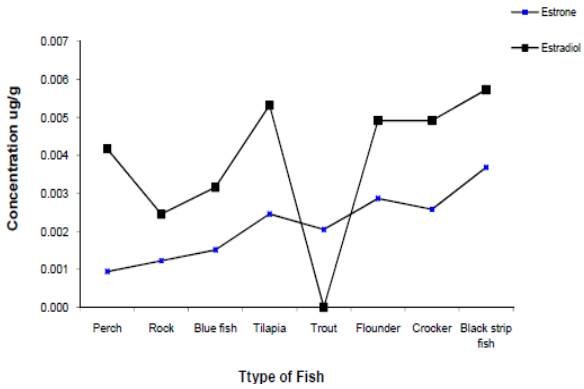


Fig. 3: Occurrence of estrone and estradiol in fish sample s from Virginia Coast.

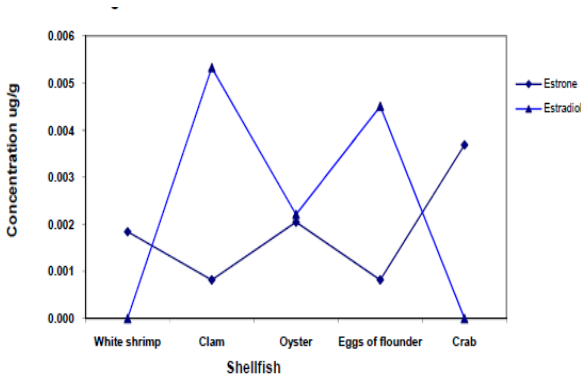


Fig. 4: Occurrence of estrone and estradiol in Shellfish.

Amount of estrone and estradiol was compared between fish and shellfish and concentration was significant during GC-MS (Fig. 5). These results show that E1 and E2 concentrations in the various fish samples from the Chesapeake Bay watershed on the Eastern Shore of MD found to be more than 1 ng g^{-1}. This small concentration, however, appears in sufficient to induce estrogenic effects in aquatic organisms [26].

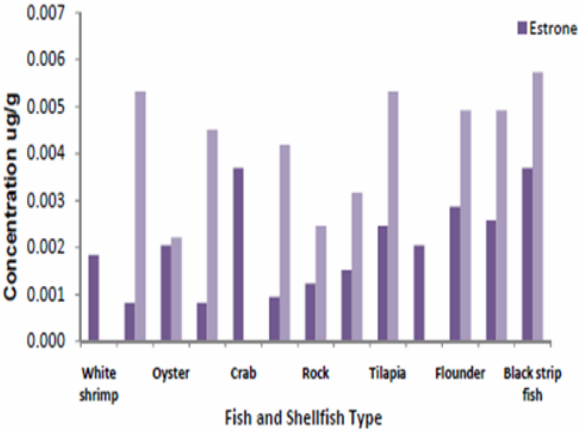
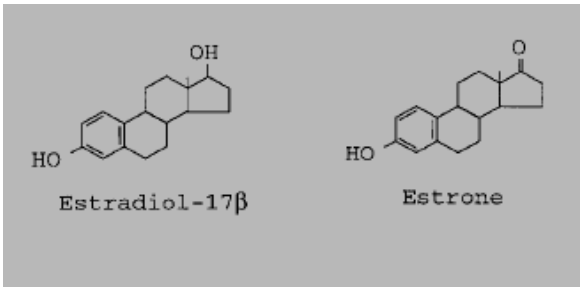


Fig. 5: Comparison of estrone and estradiol in fish and Shellfish.

Experimental

Chemicals, Reagents and Materials

Standards of estrone and 17β-estradiol were purchased from Chem Services.



The internal standard (Dodecylbenzene) was purchased from Fisher Scientific at 99% purity. For derivatization. Trimethylchlorosilane (TMCS) or N-methyl-N-trimethylsilyltri-fluoroacetamide +1%

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Trimethylchlorosilane and N, O-Bis (trimethylsilyl) trifluoroacetamide (BSTFA) PI-

48915, Pierce Chemical Company, No.:48915, all purchased form Pierce.

Study Area and Sampling Sites

The Department of Civil and Environmental Engineering of Virginia Tech; has undertaken a study (2004-2005) to measure the ecotoxicological effect of estrone and 17β-estradiol as endocrine disrupters in commonly edible fish and shellfish . A number of fish and shell fish were procured along Virginia Beach, the third largest seafood producer on America's Atlantic Ocean coast. The Chesapeake Bay is the largest and biologically most diverse estuary of America, yielding more fish than the other 840 estuaries in America. The fish and shellfish includes, Crab (Callinectes sapidus), Clam Merccnaria), Oyster (C. ariakensis), White Shrimp (Litopenaeus vannamei) and eggs of flounder Plus eight fish species, striped bass (Morone saxatilis), Atlantic mackeral (Centropristis striate), Summer Flounder, (Paralichthys dentatus), bluefish (Pomatomus saltatrix), croaker (Micropogon undulatus), spotted sea trout (Cynoscion nebulosus), White perch (Morone americana), as well as Tilapia sp. and white shrimp from aquaculture center of Virginia Tech.

Sample Preparation

Sample storage and preparation was done in the laboratory of the Food Science and Technology (FST) and extraction, purification and identification were performed at the Pesticides Residues Laboratory and Civil and Environmental Engineering Departments. After dissection, samples of some tissues and organs were taken to Pesticides Residues Laboratory and Civil and Environmental Engineering Departments for analyses. The samples were wrapped in aluminum foil and stored in deep freezer (−20 °C) until analysis.

Fish samples were thawed and cut into small pieces with a stainless steel knife. Finely chopped fish tissue (0.5 g) was weighed in a small plastic weigh boat. The weighed tissue was transferred to a glass mortar and pestle along with 2g Bondesil® C-18 sorbent (Varian Inc. CA). Using the pestle, the tissue and C-18 sorbent were thoroughly ground in the mortar to a homogenous consistency with the appearance of small grains [27]. A 20 ml bond Elute® Florisil solid phase extraction cartridge (Varian Inc. CA) was affixed to a vacuum manifold (Supelco, PA) and the sample/C-18 mixture was transferred to the cartridge using a metal spatula. A polyethylene frit (Varian Inc. CA) was firmly tamped in to the place above the sample /C-18 mixture using a disposable syringe plunger. The sample was eluted from the cartridge under vacuum with successive washes (3x 5 ml) of dichloromethane.

The eluent was collected in a glass, 25ml test tube, evaporated to about 1 ml using a nitrogen evaporator [28], and quantitatively transferred to a centrifuge tube using about 10 ml hexane. The sample was evaporated a second time, diluted to thefinal volume, and transferred to an auto sampler vial for gas chromatographic analysis. The hexane extract was transferred to benzene and preserved for silylation [29].

Samples were spiked with 50 µL of the internal standard (0.5 pg of estradiol-17J3-d 2) and 100µL of the spiking solution that contained target compounds. Samples were then stirred for 5 min and set aside for 1 hour before extraction. The internal standard was used to determine the effectiveness of the extraction technique and chosen to quantify the class of steroids and sterol in fish samples.

Silylation

Silylation is a widely used technique; it involves the replacement of an acidic hydrogen with an alkylsilyl group, analysis by which, an active hydrogen is replaced by an alkylsilyl group such as Trimethylchlorosilane (TMCS) or n-methyl-n-trimethylsilyltri-fluoroacetamide+1% trimethylchlorosilane and N,O- Bis

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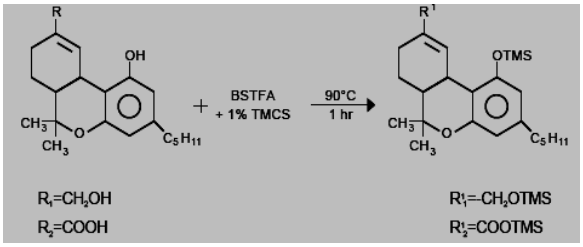
(trimethylsilyl) trifluoroacetamide (BSTFA) compared Compared to their parent compounds, silyl derivatives are more volatile, less polar, and more thermally stable. As a result, GC separation is improved and detection is enhanced. (then dodecylbenzene re-dissolved in methanol containing as an internal standard).

The benzene extract was added to 100 µL of trimethylchlorosilane (TMCS) /benzene(v:v) solution, and 50 µL Bis (trimethylsilyl) trifluoroacetamide (BSTFA). The mixture was heated at 65°C for 15 minutes, and then the reaction was quenched with 1 mL of 5% ammonia solution. After the emulsion was allowed to settle out, the benzene layer was collected, evaporated to dryness with nitrogen, and re-dissolved in 1 mL dichloromethane (7:3) benzene mixture for analysis. The mass spectra of estradiol-17 and estrone contained significant

peaks at m/z 181, which corresponded to TMCS-BSTFA as shown below.



Finally, the extracts (2 ml) were analyzed by capillary gas chromatography-mass spectrometry (GC/MS) in scan mode. (Fig. 2 and 3)

Gas Chromatographic Separation

The instrumental conditions and specifications for present work is reported is Table-4. The instrument used was an Agilent 6890 series GC with an Agilent 5973 Mass Selective Detector (EI 70 eV) coupled with the (computer data system) Agilent Chemical Station. The GC column, a 30 m DB-5MS (0.25 mm i.d.; 5% biphenyl, 95% dimethylpoly-siloxane), was connected directly to the MS detector. The transfer line was kept at 280 °C. The sample (1 ul) was injected in a split less mode at 260 °C. The temperature program was: 70 °C (2 min), 10 °C per min up to 240 °C per 2 °C per min up to 260 °C than 20 °C per min up to 300 °C (2 min); make up gas hydrogen, was combined with nitrogen to serve as a carrier gas at a flow rate of 60 mL/min. Hewlett Packard Chemstation software (G 2070AA Rev.A.10.02) was used for data acquisition and analysis.

Extraction Recoveries and Reproducibility

Performance and Quality Control

Good Laboratory Practices (GLP) were applied throughout and procedural blanks were analyzed. All blanks had values below the detection limit of the method. Recovery was calculated. The quality of the analytical

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Measurements was investigated using QA-QC samples, i.e., method blanks and control samples. The certified reference material (CRM) was not included in the QA-QC samples because it could not be supplied by a recognized certifying agency. Properly washed and dried glassware was used, contact with any rubber or plastic items was avoided during the entire study, and periodically "Blanks" were analyzed to ascertain contamination free analysis. The blank, which consisted of reagents and internal standard without tissue, and the control sample, which was a blank sample spiked with model compounds and internal standard, were tested parallel to the samples. Internal spiking and reagent blanks were used to determine recovery values.

Table-4: Instrument Parameters.

GC operating condition (Agilent 6890)		
	Column	**DB-5MS,0.25mmx30m.(.25ui.d:5% biphenyl.95% dimethylpolysiloxane)**
	Oven	**70º(2mm)10Cper min up to 240 C2 per mm up to 260º C- 20ºC per min up to 300ºC (2min)Negative**
	GC-MS parameters (Agilent 5073) Mass Selective Detector EI 70eV	
1.	**Ionization mode**	**Chemical Ionozation**
2.	**Gas flow**	**1.1mL/min**
3.	**Split injection Injector**	**Temperature:255C**
4.	**Split flow**	**20mL/min**
5.	**Constant flow of 1.0 mL/min**	
6.	**Injection volume**	**2µL**

Calibration standard solutions were used to generate response factors in relation to the internal standard. The concentration of each steroid was calculated by comparing the ratio of integrated peak areas with that of the internal standard, and use of a relative response factor. Further dilutions were made using an analytical grade solvent for calibration. Identified compounds were quantified using the external standard technique.

Further dilutions were made using analytical grade solvent for calibration. 1.00µl of each standard solution (estrone and 17β-estradiol) was added to each of the 0.5g samples. The recoveries were calculated relative to the internal standard by comparing spiked amounts of the surrogate standards to values for samples minus blank values.

It was evaporated a second time, diluted to final volume, and transferred to an auto sampler vial for GC-MS analysis.

The method detection limit (MDL), which is dependent on sample size, final extract volume, instrument detection limit, and level of confidence, was established analyzing by three method controls. No significant amount of model compounds was detected in the blank samples of water and mussel within instrument detection limits.

Statistical Analysis

All experiments were replicated three times. Statistical analysis was done using Tukey's mean comparison test. All calculations were carried out by using Excel for Windows on the basis of linear equations.

Conclusion

Estrone and estradiol determined were not at a very high level in fish and shell fish.

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