Chapter 5

Injection Port Derivatization for GC/MS–MS: Analysis of Hormones in Water

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1 INTRODUCTION

Hormones have been a growing concern among the types of emerging contaminants since the late 1990s when it was first reported that fish were experiencing feminization from exposure to several hormones, including: 17-β-estradiol and 17-α-ethinylestradiol [1]. During the last decade, endocrine disruption in fish has been observed, including both wild- and caged fish [1–4]. Wastewater input has been implicated in these cases, and as a result, there has been a concerted effort to track down the compounds that cause feminization in fish, as well as other types of endocrine disruption. In particular, feminine hormones, such as 17-β-estradiol and 17-α-ethinylestradiol [1–4], have been implicated as prime targets for endocrine disruption. In

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one study, 17-α-ethinylestradiol (a human birth control pill) was added to a lake in Canada, and endocrine disruption and collapse of the fish population were observed at exposure concentrations of 5 ng/L [5].

Furthermore, the hormone 17-β-estradiol is produced and excreted by humans each day at levels of 2–100 μg per person [6]. Pregnant women may excrete much more than this, up to 30 mg/day [7]. Thus, between natural excretion and oral contraceptive use of 17-α-ethinylestradiol, wastewater receives large inputs of possible endocrine active substances. Agricultural wastes are another source of endocrine active substances. This includes feedlots for cattle, sheep, hogs, and other livestock. In these cases, both the addition of growth hormones and natural excretion of hormones, such as 17-β-estradiol, are involved [8]. Thus, the analysis of hormones in wastewater effluents, surface water that is impacted by wastewater, and groundwater that may receive these compounds is of widespread importance. Other compounds that are not hormones, but that may have the potential to behave as endocrine disruptors because of their chemical structure, include compounds such as nonylphenol and bisphenol A [9]. Thus, methods that address these compounds are environmentally important and significant.

Many methods have appeared over the past decade that address these compounds in water samples. These methods have been reviewed by several researchers [10,11]. They include extraction steps by solid-phase extraction or liquid/liquid extraction followed by mass spectrometry. There are basically two approaches for mass spectrometry, either gas chromatography/mass spectrometry or liquid chromatography/mass spectrometry. There are positive aspects for each approach. Gas chromatography/mass spectrometry offers the power of excellent separation because of the long columns of fused silica that have literally hundreds of thousands of theoretical plates, which allow excellent separation of hormones from their isomers or interfering substances. Nowadays, with the increase in the sensitivity of instrumentation and the use of mass spectrometry/mass spectrometry with multiple reaction monitoring (MRM), the instruments are both sensitive and reliable. The two major drawbacks with gas chromatography/mass spectrometry, however, are that the solvents used for GC/MS are not compatible with the most popular extraction method, which is solid-phase extraction or SPE. Second, the hormones themselves are not volatile in the GC/MS instrument. In past times, liquid–liquid extraction (LLE) was used, which is compatible with GC/MS. However, LLE is an environmental health hazard that requires large volumes of toxic solvents such as methylene chloride, which is also toxic to our atmosphere. Thus, SPE has nearly replaced LLE as the method of choice for sample preparation for hormone analysis and endocrine-disrupting substances in general.

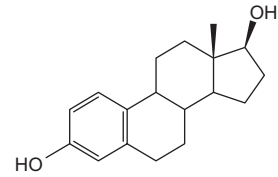
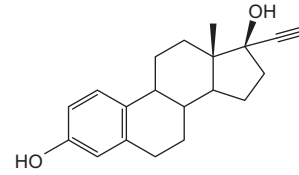
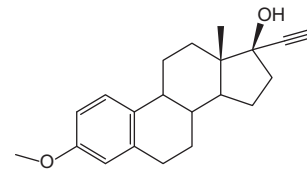
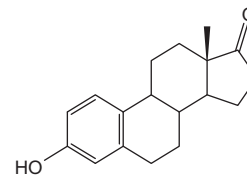
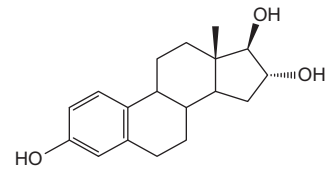
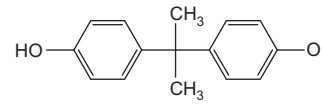
Because SPE directly allows the water sample to be stripped of hormones onto the solid support, it is a popular technique. Typically, 1–5 g of SPE material is used to remove hormones from a 200- to 1000-mL water sample.

Different types of SPE support have been used, the most popular being either C-18 or the hydrophobic lipophilic polymer, Oasis-HLB. Both are effective at the enrichment of the hormones from water and wastewater. The hormones are then removed from the sorbent by elution with a polar solvent, such as methanol or acetonitrile. These two solvents are not readily compatible with GC/MS analysis but are easily analyzed by liquid chromatography/mass spectrometry (LC/MS). Thus, the emergence of LC/MS has occurred with electrospray ionization to analyze hormones in water samples. Electrospray is a technique invented in the 1980s [12] to analyze proteins and works by the addition of either a proton to the molecule or the removal of a proton from the molecule, working on either weak acids or bases. Hormones, because of their phenolic hydroxyl, work in negative ion. In cases where there is no phenolic group, positive ion electrospray is used. Because of the structure of the hormones, the sensitivity of ionization is low, making it difficult to reach the low concentrations of endocrine disruption that have been observed in wild fish, that is, levels of 5–10 ng/L. Thus, at the moment, there is no simple method for hormone analysis that has all the attributes of an ideal method.

Given the fact that SPE is now the preferred choice for sample preparation, there is the problem of how to use this method if one chooses the option then of using GC/MS or GC/MS–MS for hormone analysis. How does one overcome the shortcoming of the volatilization of the hormones? This problem is exacerbated by the fact that although there are derivatization reagents that are effective for hormones, there is the issue of poor derivatization caused by the matrix, in this case, the wastewater organic compounds, and the salts and water associated with the isolation of the hormones themselves. Analysts have tackled this problem with the use of deuterated internal standards of each of the compounds that they are analyzing. This is an effective method, typically, but does increase the cost and labor of analysis. It would be quite useful if that were not necessary, as a typical deuterated standard costs 10 times more than the nonlabeled standard.

The common derivatization reagents that are used for the hormones are silylating reagents, such as BSTFA (*N*,*O*-bis(trimethylsilyl)trifluoroacetamide) or *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA). These reagents silylate the hydroxyl groups of the hormones. Table 1 shows five commonly analyzed hormones and one endocrine disruptor in wastewater samples. They include: 17-β-estradiol, 17-α-ethinylestradiol, mestranol, estriol, estrone, and bisphenol A. Compounds such as testosterone and progesterone do not contain hydroxyl groups and do not need derivatization and will chromatograph on the GC/MS directly. The silylation reaction is shown in Figure 1. Here, the silylating reagent adds 72 mass units to the molecular mass of the hormone. This increase in mass is then used to target the precursor ion for GC/MS–MS analysis, or if one is doing GC/MS only, to find the molecular ion of the hormone. These silylation compounds are easily

TABLE 1 List of Hormones and Bisphenol A Used in this Study of On-Column Derivatization

Compound Name	Elemental Composition	Molecular Exact Mass (M)	Chemical Structure
17-β-Estradiol	$C_{18}H_{24}O_2$	272.1776	
17-α-Ethinylestradiol	$C_{20}H_{24}O_2$	296.1776	
Mestranol	$C_{21}H_{26}O_2$	310.1933	
Estrone	$C_{18}H_{22}O_2$	270.1620	
Estriol	$C_{18}H_{24}O_3$	288.1725	
Bisphenol A	$C_{15}H_{16}O_2$	228.1150	

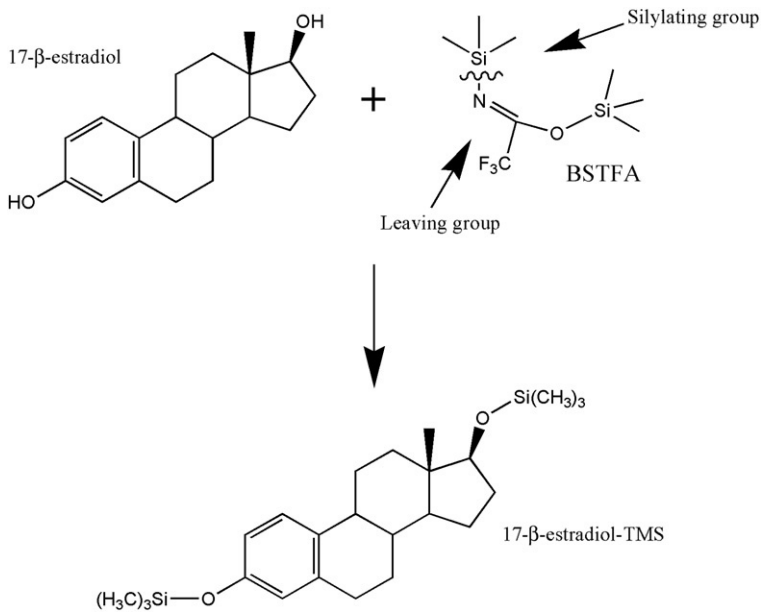


FIGURE 1 Derivatization of 17-β-estradiol with BSTFA, mechanism of action.

degraded by water or salt that may be present in the extracts. Because the natural organic matter is present in the extracts due to co-isolation by SPE, they will be present during the derivatization process.

Typically, the derivatization process involves the extraction of the compound by SPE and elution by a solvent such as methanol and then the blowing to dryness of the solvent extract. The dried sample is then solubilized in a solvent such as pyridine and the derivatization reagent, let us say, BSTFA is added. The extracts are then heated to a temperature of 65°C for approximately 4 h. The extract is then transferred to a vial for analysis by GC/MS–MS or GC/MS. If vials are stored or left on the instrument, they have the tendency to degrade. The idea of the deuterated internal standard is to correct for this loss of analyte or, in some cases, for the poor derivatization that occurs in the first place.

For these reasons, analysts are always looking for better and easier ways to develop their methods. Also there are safety concerns for the analysts when they have to handle the derivatization reagents several times, as these compounds are toxic when on the skin or inhaled. This is where the idea of derivatization on the GC column comes into play. The idea of on-column derivatization is not a new idea. Recent papers in the 2000s [13–15] showed that on-column derivatization of pesticides was an effective technique for analyzing heat-labile compounds by GC/MS. Recently, papers have appeared that use on-column derivatization for a suite of organic compounds, including

organic acids, bases, and pesticides [13–15]. However, to date, hormones have not been included in this list of compounds.

This chapter describes the research that has been carried out to eliminate the tedious nature of the derivatization step so that the hormones may be analyzed by a robust method that is not analyst-dependent, that is, requiring special care during the derivatization step by the analyst so that the method has good recoveries. Once the reagents are added to the sample in the vial, the derivatization occurs in the injection port of the GC/MS. We have tested this method on a number of standards using GC/MS–MS analysis and compared our results with the standard method used by the U.S. Geological Survey for hormone analysis of water and wastewater samples to find good agreement. This chapter describes that work in detail.

2 EXPERIMENTAL METHODS AND SAMPLE PREPARATION

2.1 On-Column Derivatization Method for Hormones and Bisphenol A

2.1.1 Sample Preparation

Water samples were collected in baked glass containers as grab samples from Boulder Creek and the wastewater treatment plant in Boulder, Colorado. The samples were processed by solid-phase extraction using the Horizon automated solid-phase extraction disk system with C-18 disks [16]. This procedure allows for the total analysis of the water sample and is not plagued by problems with plugging of the solid-phase extraction cartridges. The Horizon disk system consists of an SPE-DEX 4790 automated extraction system with controller. The disks are Atlantic^{TM} C18 SPE disks 47 mm in diameter. The automated method is described in an Application Note of Horizon [16] that essentially consists of first wetting the disks and preparing them with a rinse of methanol followed by water. The disk is left wet in water so that there is good and even application of the water sample. A 1-L water sample can be applied to this disk with good recoveries, greater than 50–80%. This is an advantage of the disks over SPE cartridges in that the amount of sample applied can be 5–10 times larger, with fast processing times. The disks can process a 1-L sample with a 15-mL/min flow rate, or more, so that the sample is applied in approximately 1 h. The disks were eluted with 15 mL of methanol and evaporated to dryness in a Turbovap concentration workstation. The dry test tubes then received the derivatization solvents that consisted of the following.

2.1.2 Derivatization Reagents

The derivatization reagents BSTFA and trimethylchlorosilane (TMCS) were obtained from Sigma-Aldrich (St. Louis, MO). An aliquot of 500 μL of TMCS was added to 5 mL of BSTFA in order to have a 10% TMCS

derivatization reagent. In the same way, 2 mL of pyridine was mixed with 8 mL of BSTFA/TMCS to form a (BSTFA/TMCS)/pyridine (4:1 v/v) solvent mixture for addition to the dry extracts. This combination of BSTFA, TMCS, and pyridine was the mixture used not only for solubilizing the dry extract from solid-phase extraction but also for derivatizing in the GC/MS–MS instrument.

2.1.3 On-Column Derivatization

The (BSTFA/TMCS)/pyridine solvent was used for injection. Two hundred microliters of the reagent was used to solubilize the dry extracts after evaporation under nitrogen in 10-mL test tubes. The tubes were vortexed for 15 s, and the extracts were transferred to 2-mL vials for analysis by GC/MS–MS. The injection port of the GC/MS–MS system was set at 280 °C, and 1 microliter was injected on the column.

2.1.4 GC/MS–MS Instrumentation and Analysis

The identification of the hormones and bisphenol A was carried out on an Agilent 7890 gas chromatograph coupled to a triple quadrupole mass spectrometer, Agilent 7000 series (Agilent Technologies, Inc., Santa Clara, CA). The chromatographic separation was performed using an Agilent J&W HP-5 column (5% phenyl, 95% methylpolysiloxane), 30 m × 0.25 mm i.d., fused-silica capillary column. The carrier gas was helium at a constant flow rate of 1.2 mL/min held by electronic pressure control. Injector temperature was 280 °C, and a splitless injection mode was used. The oven temperature was programmed for 100 °C (held for 1 min) to 240 °C at 40/min and held for 1 min, then to 300 °C at 10/min and held for 4 min. The MS operating conditions were the following: positive electron ionization mode (EI+) using automatic gain control with an electron energy of −70 eV. The ion source temperature was 300 °C. Gain voltage was set to 30 V. A dwell time of 50 ms was used for each MRM transition. One microliter of the extracts was injected on the system. Mass Hunter software was used for instrument control and data analysis. Details of MRM and analysis are given in Section 3 of this chapter.

2.2 Comparison Method: U.S. Geological Survey Method for Hormones

2.2.1 Sample Preparation

Stream water samples were analyzed for hormones at the U.S. Geological Survey National Water Quality Laboratory in Denver, Colorado [17]. Filtered water samples were fortified with deuterated analogs of 13 analytes as isotope dilution standards, and the samples were poured into stainless steel extraction tubes equipped with a multigrade GFF over a 47-mm C-18 solid-phase

extraction (SupelcoENVI) disk [17]. The sample was passed through the GFF/C18 disk under pressure, as needed. Following compound isolation, the GFF/C18-disk was rinsed with 25% methanol in reagent water and dried with nitrogen, and the compounds were eluted with methanol. The methanol eluent was evaporated to dryness and reconstituted in a mixture of 5% methanol in dichloromethane (DCM/MeOH). The extract was passed through a 1-g Florisil SPE column and eluted with the DCM/MeOH solution. The eluent was reduced in volume and transferred to a 5-mL reaction vial, then evaporated to dryness.

2.2.2 Derivatization and Analysis

Ketone and alcohol groups on the analytes were derivatized to trimethylsilyl or trimethylenol ether analogs to make them stable for analysis by gas chromatography. Derivatization was accomplished by adding 200 μL of MSTFA activated with 2(trimethylsilyl)ethanethiol and ammonium iodide (NH_4I), then heating the MSFTA solution to 65 °C for 1 h. The analytes were separated by gas chromatography and quantified by tandem quadrupole mass spectrometry using an isotope dilution procedure. This procedure allowed for quantitation of 17 hormones and their deuterated analogues [17].

3 RESULTS AND DISCUSSION

3.1 Derivatization by BSTFA/TMCS/Pyridine

Six analytes were chosen for this method, which included the five hormones l7-α-ethinylestradiol, 17-β-estradiol, estriol, estrone, and mestranol and bisphenol A. Table 1 shows the structure, formula, and molecular mass of the investigated compounds. The five hormones are similar in structure with the four-membered ring system of the android hormones. Because of their structure, they contain between one to three hydroxyl groups, are not volatile in the gas chromatograph, and require derivatization. The reagent, BSTFA (Figure 1), was used as the reagent for this process. This reagent is highly reactive toward either amine or hydroxyl groups and reacts to form the derivative. Figure 1 shows the reaction for the natural human hormone, 17-β-estradiol. Both the hydroxyl groups are derivatized, which increases the volatility of the 17-β-estradiol so that it chromatographs easily for analysis by GC/MS–MS.

Silylation, which is one of the most widely used forms of derivatization, works on active or exchangable hydrogens, such as those in either amines, hydroxyls, and phenolic hydroxyls. Thus, they are useful for the hormones since they contain both hydroxyl and phenolic hydroxyl groups. Silyl derivatives replace the active hydrogen with a trimethylsilyl group called TMS. Figure 1 shows an example for 17-β-estradiol, where both of the hydroxyl groups are replaced by a TMS group. The mechanism is thought to be a nucelophilic attack of the hydroxyl group upon the BSTFA molecule with the

leaving group shown in Figure 1, which is the larger half of the molecule with the bond breaking between the silicon and nitrogen atoms.

The typical procedure for derivatization involves adding an excess of BSTFA along with a catalyst, which is typically TMCS, between 1% and 10%. Ten percent was used in this work to help speed up the derivatization of hindered groups, such as the hydroxyl group next to the ethine group of 17-α-ethinylestradiol. Pyridine was also added to further help with the solubilization and catalysis of the reaction. The mixture used was 10% TMCS and 20% pyridine, as described in Section 2 of this chapter. The derivatization reagent does create a background peak in the chromatogram when the mixture is analyzed by GC/MS–MS. Figure 2 shows the full scan chromatogram for the derivatization of 17-β-estradiol. Notice that the first peak in the chromatogram is at 9.5 min, which has the mass spectrum also shown in Figure 2. This peak is most likely a phthalic acid contaminant based on the mass spectrum shown in Figure 2 with two major ions at *m/z* 149 and 167. This is not a surprising result since plastic pipette tips were used for transferring reagents during derivatization processes. Because of the reproducibility of this peak, it could be used as an internal reference for chromatography. This peak appeared in all of the derivatization reactions of each hormone standard. However, because it was not monitored in the MRM transitions of hormones, it is not seen in any of the MRM chromatograms. BSTFA and its by-products, including TMCS and pyridine, are volatile and appear early in the chromatogram, typically before the mass spectrometer is turned on and do not show up in the chromatograms, which makes this reagent a popular one for derivatization reactions. The hydrofluoric acid by-product of the BSTFA reaction is also not a problem and keeps the detector of the mass spectrometer from fouling.

The ease of derivatization of active hydrogen atoms follows the following order: alcohol > phenol > carboxylic acid > amine > amide. Steric hindrance also plays a role with alcohols in the following order: primary > secondary > tertiary; and for amines, the order is as follows: primary > secondary. As mentioned earlier, the use of TMCS can help with hindered sites. This will be addressed again as the various hormones are examined. BSTFA is a flammable and water-sensitive liquid. If properly stored, this reagent is stable indefinitely and can therefore be used quite easily over and over for on-column derivatization making the analyst work quite effortless.

The most likely problem with the BSTFA reagent is water that can decompose the reagent. However, when it is stored in a tight vial in the refrigerator with pyridine present the derivatization reagent does have a long half life. Typically, it is important to analyze a test compound with each day's analysis to test for repeatability. The addition of TMCS, which is a relatively weak silyl donor, to BSTFA will enhance the donor strength of the stronger donor, BSTFA, making the reaction go more quickly and efficiently for derivatization. The by-product of TMCS is hydrochloric acid. Thus, it is important to use a glass liner in the inlet of the GC/MS rather than metal. It is also

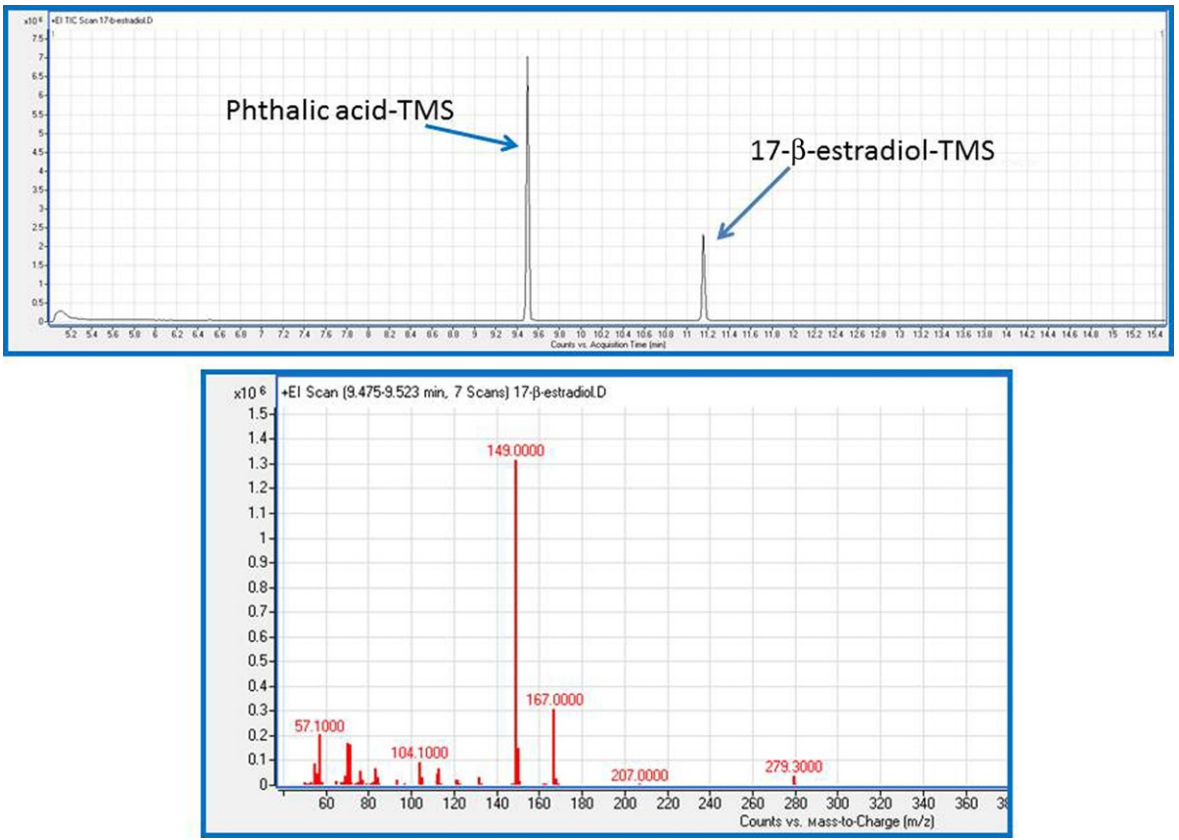


FIGURE 2 Chromatogram and mass spectrum of BSTFA phthalic acid eluting before 17-β-estradiol.

important to replace liners more often when doing on-column derivatization, as well as to use a relatively hydrophobic column such as the 5% phenylsilicone columns recommended in Section 2 of this chapter.

3.2 Derivatized Hormone Mass Spectra

The five hormones shown in Table 1 have different reactivities toward derivatization by BSTFA, which will be shown individually in the following sections. To summarize, several of the hormones derivatize completely with all hydroxyl groups being derivatized by the TMS group (trimethylsilane). This includes 17-β-estradiol and estrone. Several of the hormones have one peak that consists of a single TMS group because of the steric hinderance mentioned earlier, and a second smaller peak when both hydroxyl groups are derivatized. This includes 17-α-ethinylestradiol and mestranol. Estriol contains two major derivatization products, one with two TMS groups and one with three TMS groups. The fact that there is variable reaction suggests the importance of using a labeled standard for each of the hormones that undergo partial derivatization. The five hormones will be discussed in separate sections, beginning with the most effective derivatizations and progressing toward those that have multiple products. However, it is possible to easily derivatize and analyze the suite of hormones, as shown in Figure 3, with the extracted ion chromatogram for the set of five hormones, with their major precursor ion.

3.2.1 17-β-Estradiol

Figure 4 shows the mass spectrum for 17-β-estradiol. The major ion in the chromatogram is the *m*/*z* 386. This ion is the result of a loss of 30 mass units from the 17-β-estradiol with two TMS moieties, as shown in Figure 4.

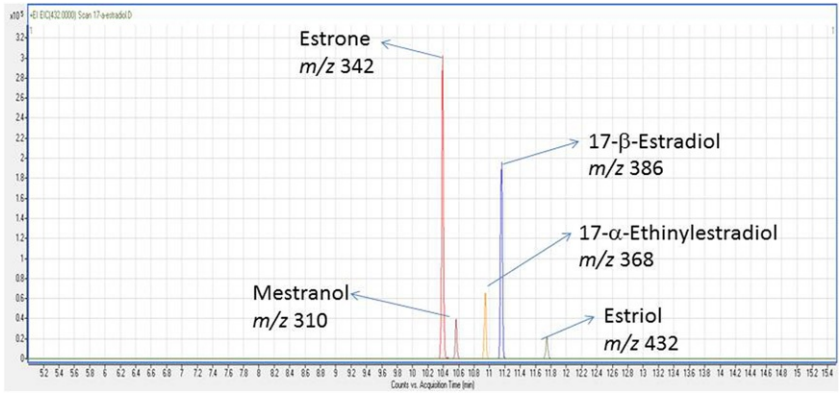


FIGURE 3 Extracted ion chromatogram of a hormone mixture with the major precursor ions shown.

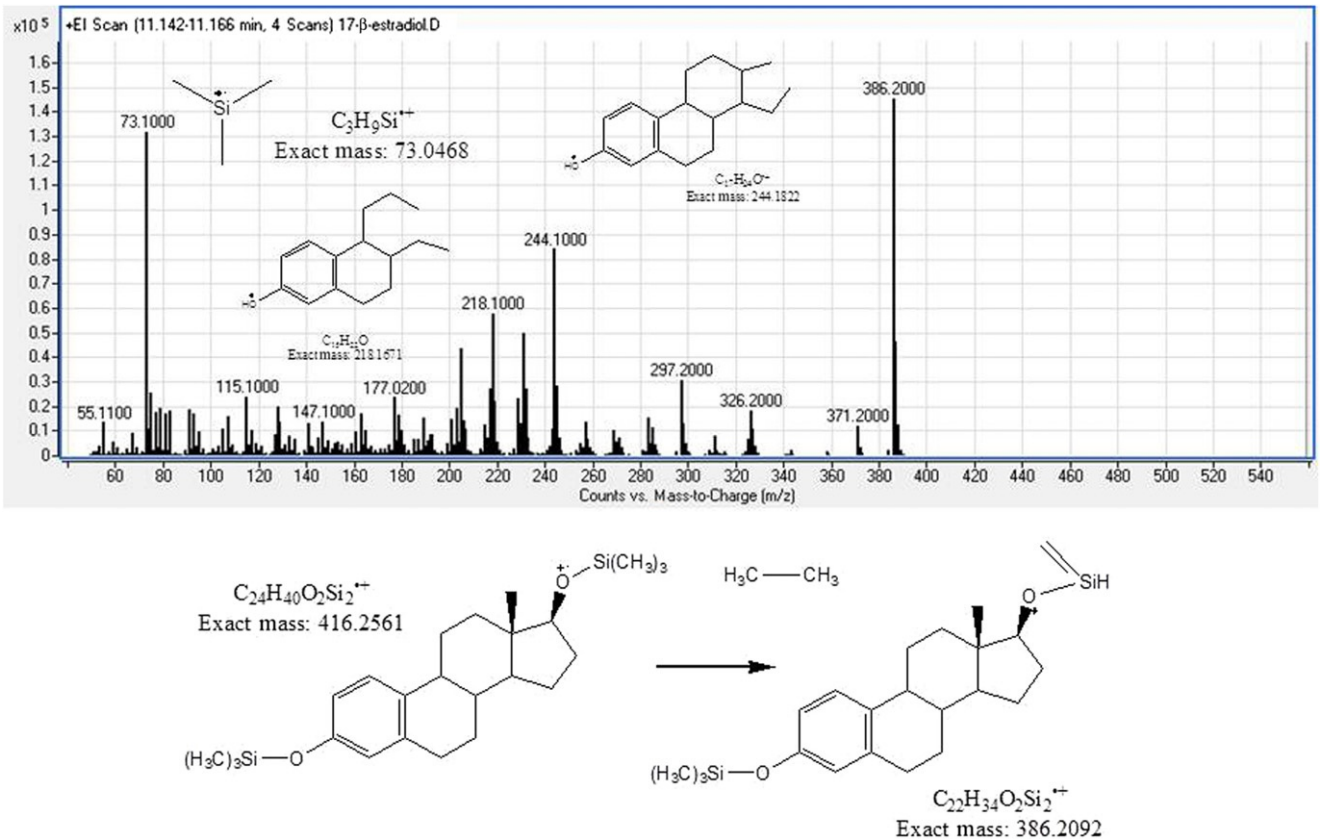


FIGURE 4 The mass spectrum of 17-β-estradiol as a TMS derivative.

Basically, there is a loss of two methyl groups from the *m/z* 416 ion to give rise to a stable and strong ion at *m/z* 386. The *m/z* 386 ion is then chosen as the precursor ion for the MRM transitions. The product ions include: the *m/z* 244 and 218 ions, which are structural to the hormone. It is never wise to choose the *m/z* 73 ion since it is the TMS derivatization reagent, which could originate from any compound that contains an active hydrogen atom. The *m/z* 73 ion is common and occurs in all of the derivatized hormones, often as a major component of the mass spectrum. Putative structures are shown for the major fragment ions at *m/z* 244 and 218. These two transitions from *m*/z 386 to 244 and from *m*/z 386 to 218 are the two transitions chosen for the analysis of 17-β-estradiol in MRM mode for GC/MS–MS analysis and give rise to a robust method for the 17-β-estradiol.

3.2.2 Estrone

Figure 5 shows the mass spectrum for estrone. The major ion in the chromatogram is the *m/z* 342. This ion is the result of a single TMS derivative to the estrone structure. Notice that the carbonyl group is not derivatized by the BSTFA, which is an expected result. The *m/z* 342 is chosen then as the precursor ion for estrone for the MRM transitions. The product ions include: *m/z* 257 and 218 ions, which again are structural to the hormone. Putative structures are shown for the two major fragment ions at *m/z* 257 and 218. The *m/z* 218 again occurs in the estrone structure because of its similarity to 17-β-estradiol. In fact, it differs only by the conversion of the hydroxyl group of the 17-β-estradiol to a ketone. These two transitions from *m/z* 342 to 257 and from *m/z* 342 to 218 are the two transitions chosen for the analysis of estrone in MRM mode for GC/MS–MS analysis and give rise to a robust method for estrone.

3.2.3 17-Alpha-Ethinylestradiol

Figure 6 shows the total ion chromatogram and mass spectrum for the major chromatographic peak at 10.9 min for 17-alpha-ethinylestradiol. This compound shows a different derivatization pathway than the first two hormones, 17-β-estradiol and estrone, which had only one chromatographic peak each. The 17-α-ethinylestradiol shows two chromatographic peaks because it has one for a single TMS group and a smaller peak at a longer retention time when there are two TMS groups. The derivatization favors the single TMS derivatization of the phenolic hydroxyl group. The alcoholic hydroxyl group is highly hindered by the ethyne group attached to the same carbon as the hydroxyl (Table 1). The ethyne group blocks the BSTFA from reacting with the alcoholic hydroxyl, and the product is the smaller chromatographic peak at 11.3 min with its highest mass being the *m/z* 425 ion, which is consistent with two TMS groups (Figure 7).

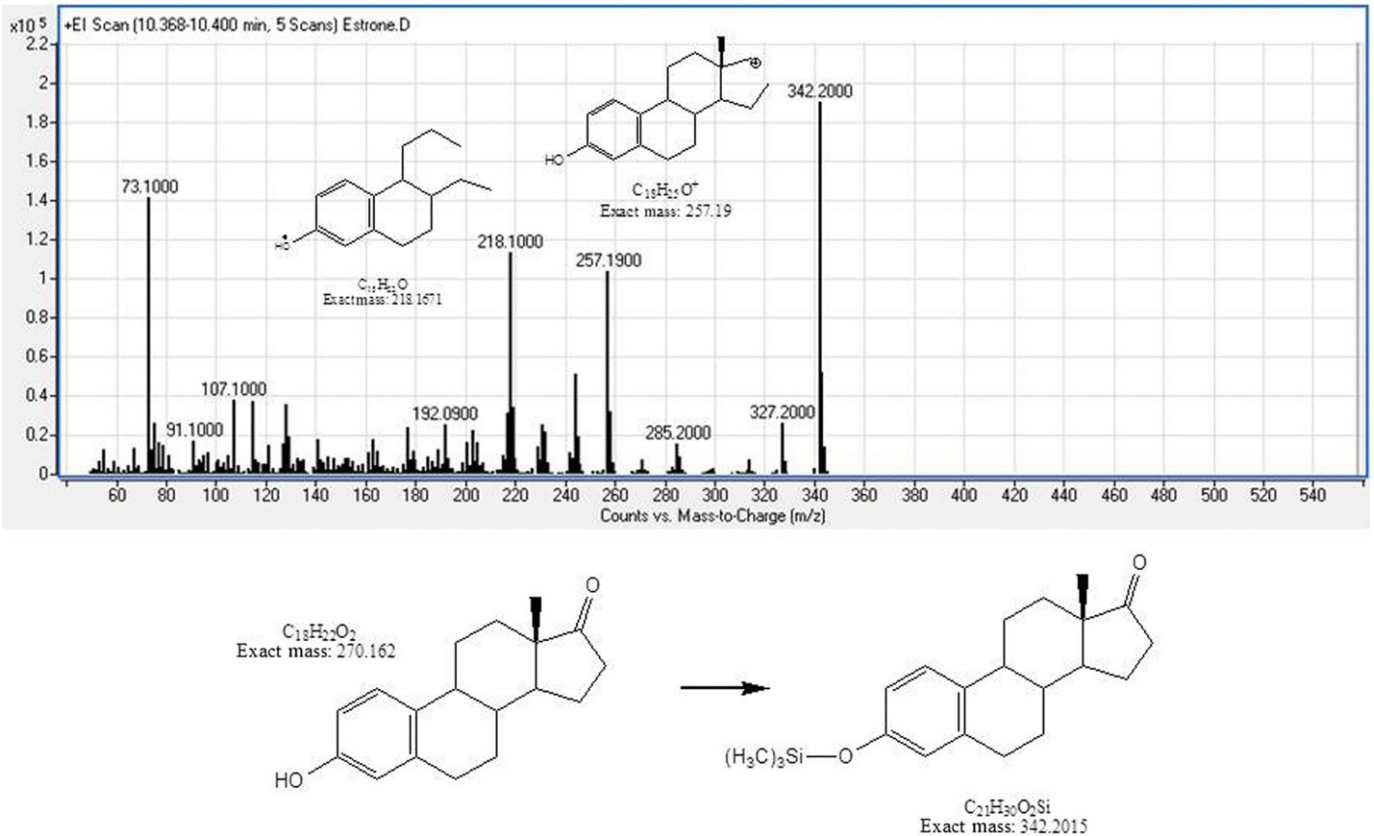


FIGURE 5 The mass spectrum of estrone as a TMS derivative.

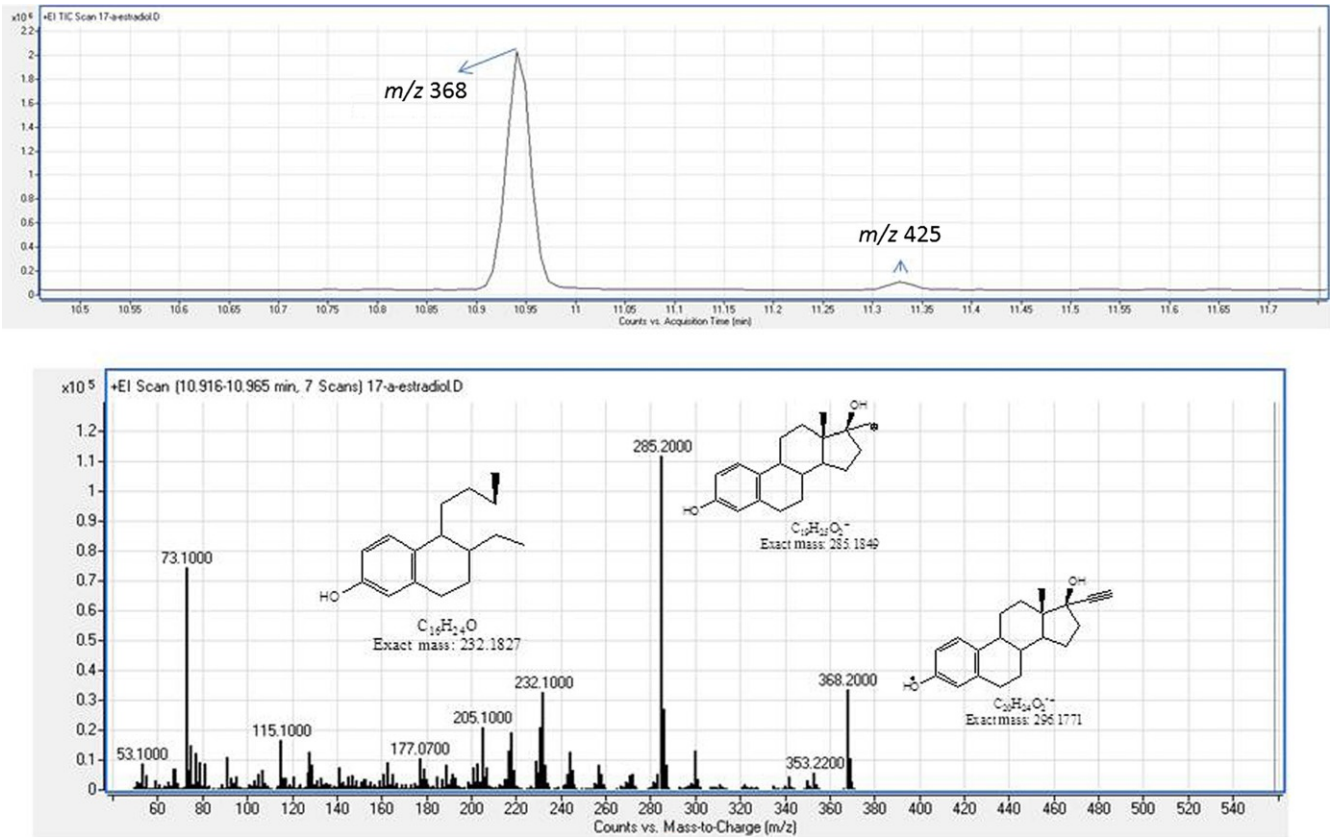


FIGURE 6 The mass spectrum of 17-α-ethinylestradiol as a TMS derivative.

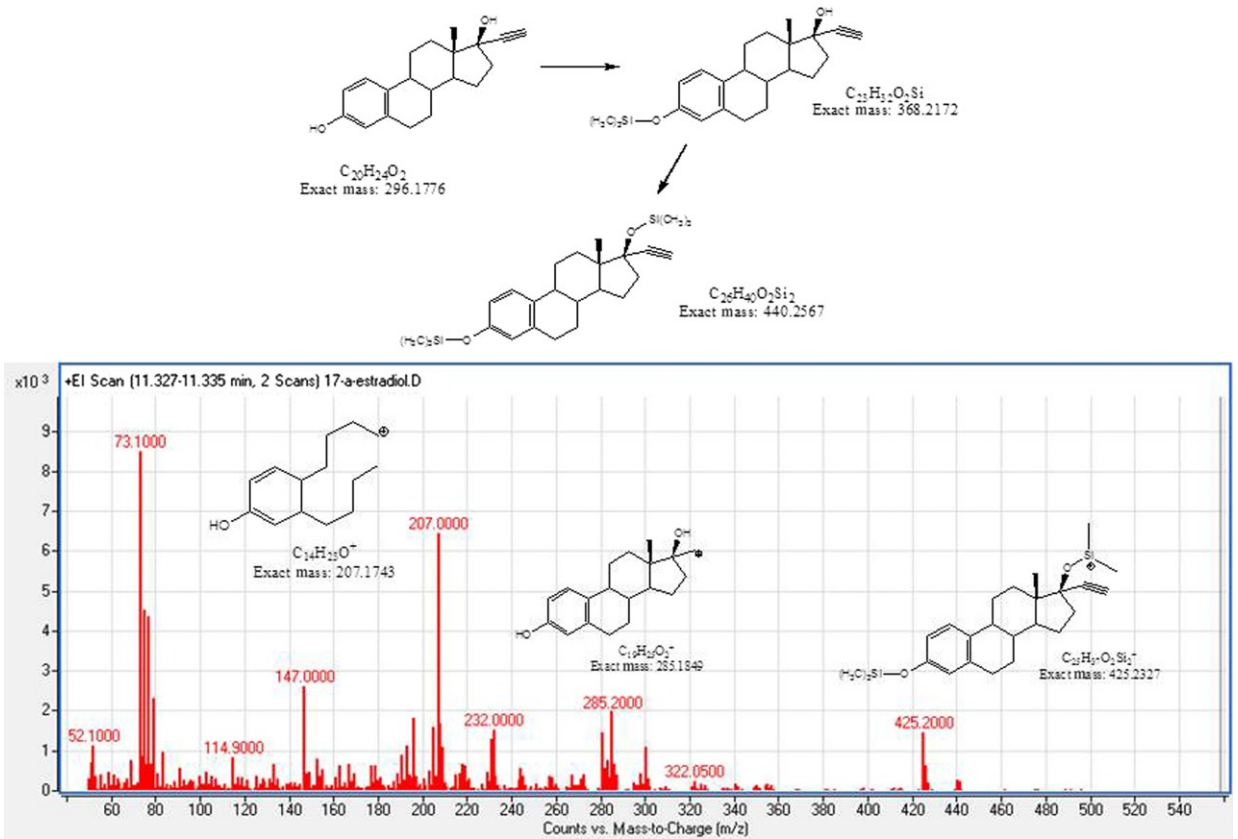


FIGURE 7 The mass spectrum of the two TMS deivatives of 17-α-ethinylestradiol with two TMS groups.

The larger chromatographic peak has the highest mass of *m/z* 368, which is the correct mass for the addition of one TMS group (Figure 7 pathway at the top of the figure). The *m/z* 368 is not the base peak ion; rather, the *m/z* 285 ion is the major peak in the mass spectrum, with the putative structure shown in Figure 6. Again, it is consistent with the core part of the hormone and is a good product ion for the MRM transition, *m/z* 368–285. The second MRM comes from the transition of *m/z* 368–232, which is again a structural component of 17-α-ethinylestradiol.

Figure 7 shows the mass spectrum for the second smaller peak at 11.3 min with the highest mass of *m/z* 425. This mass is consistent with the addition of two TMS groups followed by the loss of 15 mass units, a $—CH_3$ group. In this case, the *m/z* 425 ion is not the base peak ion but the *m/z* 207 ion (the *m/z* 73 ion is not considered here because it is the silyl ion). The putative structure for the *m/z* 425 ion is shown in Figure 7. This ion looses both TMS groups to give rise to its two products ions, *m/z* 285 and 207. The *m/z* 285 ion was found also in the preceding example of the *m/z* 368 ion where there is one TMS group attached to the 17-α-ethinylestradiol. The putative structures for both of the product ions are shown in Figure 7 and consist of the basic core of the hormone structure.

3.2.4 Mestranol

Figure 8 shows the total ion chromatogram and mass spectrum for the major chromatographic peak at 10.5 min for mestranol. This compound also shows a different derivatization pathway, which is actually similar to 17-α-ethinylestradiol. Because the phenolic oxygen is not present, rather there is a methoxy group, the major chromatographic peak derivatized by the TMS corresponds to the mass at *m/z* 310. The ethyne group again blocks the derivatization of the alcoholic hydroxyl group. But not only does it block derivatization it also sterically hinders the hydroxyl group from reacting and sorbing to the inlet of the GC, which creates a large tailing chromatographic peak or complete retention in the inlet. Thus, the dual role of the ethyne group leads to an underivatized chromatographic peak for mestranol as the major component of the hormone.

The second chromatographic peak at 10.95 min is the case where there is one TMS group derivatized to the alcoholic hydroxyl group that is protected by the ethyne group. It is a small peak since it is poorly reactive, which is nearly identical to the result that was seen for 17-alpha-ethinylestradiol in Figure 6. The single TMS derivative of mestranol gives rise to a ion at *m/z* 367, which is consistent with the addition of one TMS group followed by the loss of a $—CH_3$ group (Figure 9). This is the same reaction that was seen in the previous hormone, 17-α-ethinylestradiol, for the *m/z* 425 ion. Apparently, the similarity in structure gives rise to similar pathways of fragmentation. The base peak in Figure 9 was the *m/z* 207 ion, again a similarity to

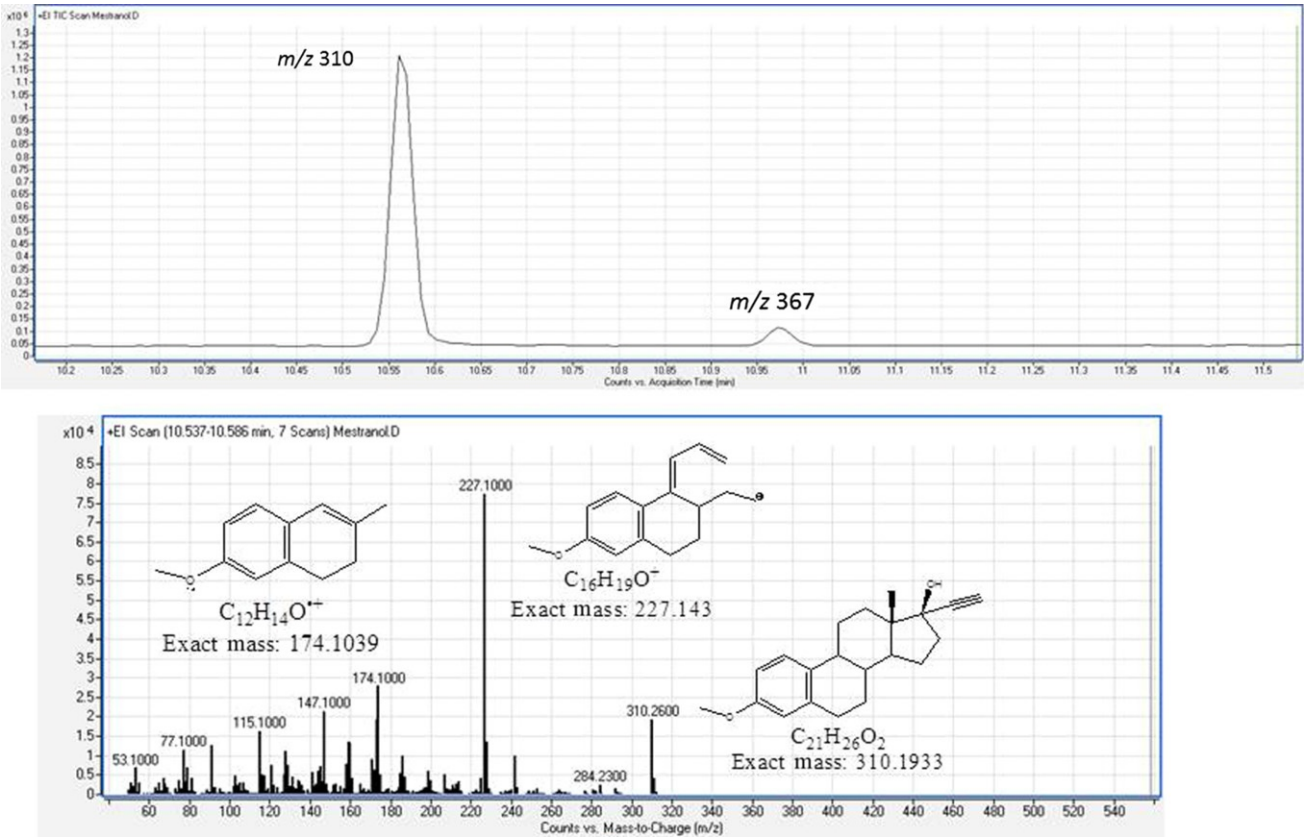


FIGURE 8 Total ion chromatogram for mestranol showing two chromatographic peaks at 10.55 and 10.97 min.

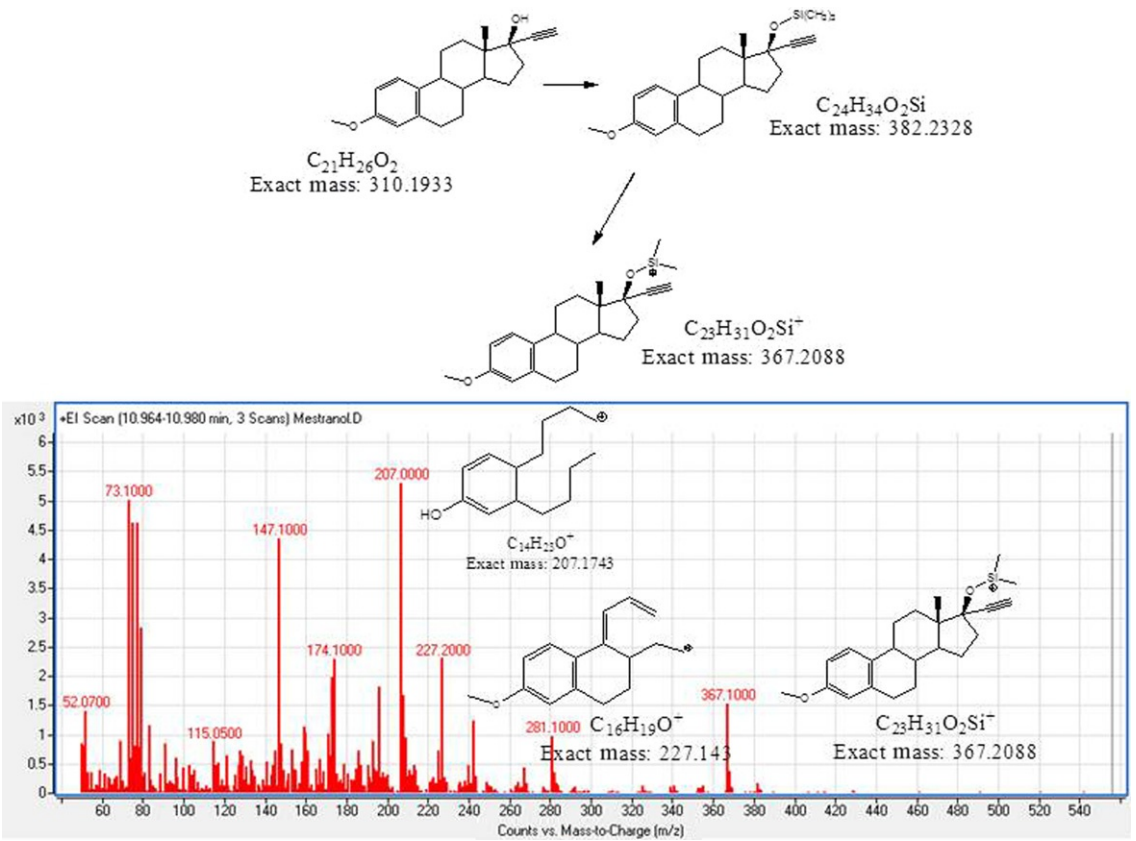


FIGURE 9 The mass spectrum and pathway of derivatization for a single TMS derivative of mestranol.

17-α-ethinylestradiol. The putative structures are shown for both the product ions for MRMs of mestranol with MRM transitions of *m/z* 367–207 and *m/z* 367–227.

3.2.5 Estriol

Figure 10 shows the total ion chromatogram and mass spectrum for two major chromatographic peaks at 11.75 and 11.85 min for estriol. This compound has three alcoholic hydroxyl groups, two of which are positioned on adjacent carbon atoms and apparently experience some steric hinderance. The first chromatographic peak has a mass of *m/z* 432, which is the addition of two TMS groups to the molecule, taking it from a mass of *m/z* 288 to 432 with the addition of two groups of 72 mass units each. The molecular ion at *m/z* 432 is not the base peak, rather the ion at *m/z* 345 and 129 are larger. The putative structures for each of these two product ions are shown on Figure 10. Thus, the two transitions chosen for MRM study are the *m/z* 432–345 and *m/z* 432–129. The *m/z* 345 ion is simply the basic structure of estriol and one TMS group that has subsequently lost a —CH_3 group. The *m/z* 129 ion is a highly fragmented component of the estriol structure for which a structure can be easily drawn, but without accurate mass data, it is not possible to be sure of its exact formula or structure.

The second chromatographic peak at 11.85 min is the larger of the two peaks and has a molecular ion at *m/z* 504, which is consistent with three TMS groups each derivatizing one of the three hydroxyl groups of estriol. This derivative has ions at *m/z* 345 and 129, which are the same ions that occur in the previous derivative containing two TMS groups.

3.2.6 Bisphenol A and d-16 Bisphenol A

Bisphenol A is an important endocrine-disrupting compound and is included in this chapter along with the hormones that have been implicated in endocrine disruption in fish. Along with the bisphenol A is an example of the use of a labeled standard, which is quite useful for quantitative analysis of derivatized hormones and endocrine-disrupting compounds. These two compounds are shown as examples of the approach to use for labeled standards by the method called isotope dilution.

Figure 11 shows the mass spectra for both the derivatized bisphenol A and its d-16 label. Note that all of the hydrogen atoms of the bisphenol A have been replaced by deuterium atoms. However, the mass increase is 14 mass units rather than 16 mass units because two of the deuterium atoms are active and replaced by TMS groups. Bisphenol A is unstable as a derivative and fragments by the loss of the CH_3 group in the center of the molecule. This is easily determined because of the loss of CD_3 on the labeled compound, where the mass of the molecular ion goes from *m/z* 386 to 368 with the loss of 18, while the nonlabeled compound goes from *m/z* 372 to 357, the loss

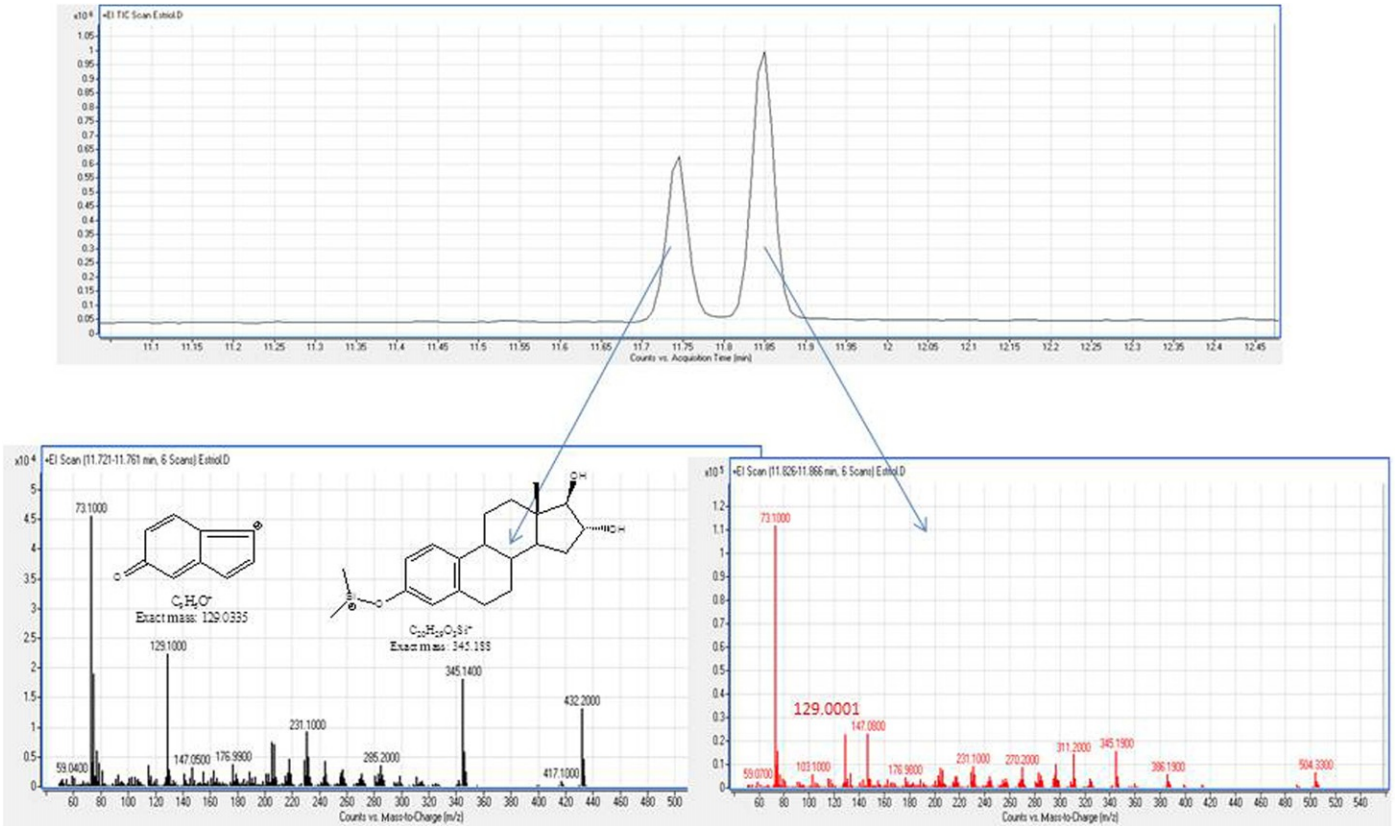


FIGURE 10 The mass spectrum and major ions for both the two and three TMS derivatives of estriol.

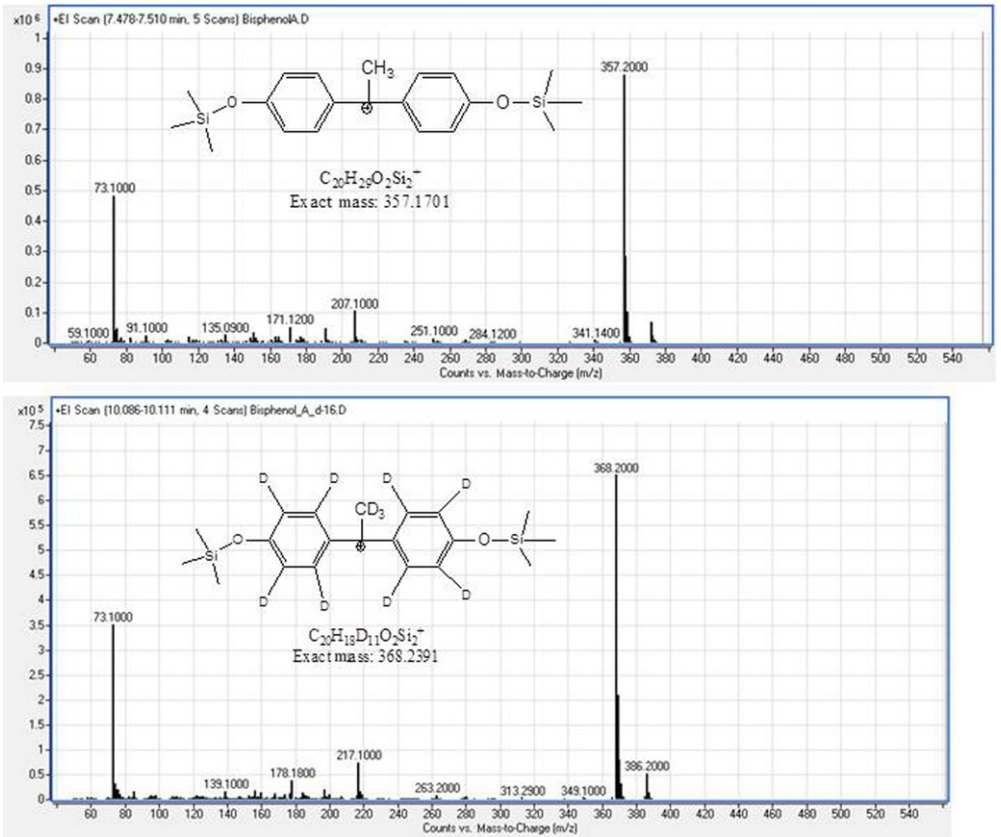


FIGURE 11 The mass spectra and major ions for both bisphenol A and its d-14 deuterium label as TMS derivatives.

of 15 mass units. Thus, we know that the methyl group lost must contain three deuterium atoms. The chromatography of the two compounds is different. Bisphenol A elutes at 7.5 min and the deuterium bisphenol A at 10.1 min. The addition of 14 deuteriums increases the boiling point of the compound considerably. The MRM transitions were from *m/z* 357 to 207 and 357 to 171 for bisphenol A and from *m/z* 368 to 217 and 368 to 178 for the d-14 bisphenol A.

3.3 Building the MRM Table for Hormones and Endocrine Disruptors

The MRM table for the five hormones and bisphenol A and its deuterated analogue is shown in Table 2. The larger intensity ion (in bold) is used for quantitation and the smaller intensity ion for confirmation. These MRM transitions give a robust method for the analysis of hormones and endocrine disruptors, such as bisphenol A. For a complete method, it is important to also obtain labeled standards for each of the hormones. This is our future work using the on-column derivatization technique, similar to what was done for bisphenol A. With the isotope dilution method, one has only to add a known amount

TABLE 2 MRM Transitions for Hormones and Bisphenol A

Compound Name	Precursor Ion	Product Ions	Retention Time (min)
17-β-Estradiol	**386**	**244** 218	11.1
17-α-Ethinylestradiol	**368**	**285** 232	10.9
Mestranol	**310**	**227** 174	10.5
Estrone	**342**	**257** 218	10.4
Estriol peak 1	**432**	**345** 129	11.7
Estriol peak 2	**504**	**345** 129	11.8
Bisphenol A	**357**	**207** 171	7.5
d-16 Bisphenol A	**368**	**217** 178	10.1

Quantitation ion is in bold and Confirmation Ions are in regular type.

of the labeled compound and use this to develop a standard curve for each of the hormones, although it is possible to identify hormones and to be semi-quantitative using the on-column method without labeled standards.

3.4 Analysis of Water Samples

3.4.1 Sample Collection

Surface water samples were taken from Boulder Creek (Boulder, CO) upstream and downstream of a WWTP. A U.S. Geological Survey spiking mixture was used for the SPE recovery experiment, which contained four-labeled hormones ($^{13}C_6$-estradiol, $^{13}C_6$-estrone, D_4-ethynylestradiol, and D_4-mestranol). No additives were added to the water samples, and no filtration of samples was needed. This part of the study involved the use of the U.S. Geological Survey Laboratory in Denver, Colorado, and the use of their hormone method described in Section 2 [17]. The purpose was to evaluate both the SPE isolation procedure and to compare the on-column derivatization for hormones with the U.S. Geological Survey published method.

3.4.2 Hormone Recovery by SPE

The SPE extracts were analyzed at the U.S. Geological Survey Laboratory by GC/MS–MS using a Quattro-micro-GC® instrument (Waters Corp., Milford, MA) with an Agilent 6890 gas chromatograph. Chromatography was on a 30 m × 0.25 mm internal diameter Rxi XLB gas chromatography column with a 0.25-μm film thickness (Restek Corp., Bellefonte, PA) and a helium flow rate of 1 mL/min with the injection port maintained at 275 °C. The gas chromatograph was programmed on a variable temperature gradient from 100 to 310 °C. For each target compound, the most abundant diagnostic ion in the full scan spectrum was selected as a precursor, and appropriate conditions were selected to maximize the signal for three precursor-product transitions. The recoveries for all five hormones were obtained by comparing the chromatographic areas to an external labeled standard. The other hormones were quantified relative to the isotopic dilution standards based on the absolute method recovery of the isotopic standards (i.e., U.S. Geological Survey method in Ref. [17]). The recoveries for the five hormones and bisphenol A are presented in Table 3. In general, acceptable recoveries of extraction were obtained for the compounds studied, approximately 47–80%. Also, the recoveries were very consistent in the three different water matrices studied, showing that the Horizon automated disk solid-phase extraction procedure is reliable, reproducible, and comparable to the U.S. Geological Survey method [17].

3.4.3 Hormone Analysis in Water Samples

Three surface water samples (downstream, upstream, and near a wastewater source) were analyzed with the on-column derivatization method described

TABLE 3 Recovery Results for Five Hormones Using the Horizon Automated Solid-Phase Extraction Disk with C-18 Disks

Recoveries for Hormones by SPE % Using Solid-Phase Extraction Disks			
Compound Name	*Surface Water Upstream*	*Surface Water Downstream*	*Canyon Water Site*
17-β-Estradiol	60	59	54
Estrone	72	80	63
17-α-Ethinylestradiol	64	70	57
Estriol	47	66	49
Mestranol	72	74	63

TABLE 4 Concentrations in Nanogram per Liter for Five Hormones Identified in Surface Water Samples By the On-Column Derivatization Method, Which Compared Well with the U.S. Geological Survey Method for the Five Hormones Below (Values Within ±25% Data not Shown)

Compound Name	Surface Water Upstream (ng/L)	Surface Water Downstream (ng/L)
17-α-Ethinylestradiol	–	0.2
17-β-Estradiol	–	0.5
Estriol	–	–
Estrone	0.6	9.5
Mestranol	–	–

in this work coupled to disk solid-phase extraction, and several hormones were successfully identified. The results are shown in Table 4. These results are preliminary at this time and show that the on-column derivatization method coupled to solid-phase disk extraction is a viable method for the five commonly studied hormones and bisphenol A. Future work will examine the five labeled hormones (deuterated analogues of the five compounds studied herein) to complete a robust method. Given the results at this time, this should be a straightforward method and allow complete quantitation from sample concentration to final analysis.

4 CONCLUSIONS

Hormones are an important class of emerging contaminants that are not easily analyzed by either LC/MS or GC/MS. They have been implicated in the feminization of fish, especially in water that receives wastewater downstream. They do not ionize easily by electrospray LC/MS and have a low sensitivity. With GC/MS, they are not volatile unless they are derivatized. Derivatization creates another step in analysis that is difficult and time-consuming, and often there are losses of analyte in the process. Thus, methods that make this task easier are important for the analysis of these compounds in water and wastewater. We have developed and tested an on-column derivatization method for five major hormones that is quite simple and takes the work out of derivatization for GC/MS analysis. The method consists of concentrating the hormones by solid-phase extraction from water followed by transferring the extract to a tube, drying the extract, and adding derivatization reagent to the tube. The reagent and sample are injected into the GC/MS injection port, where the derivatization occurs, and then the hormones are chromatographed. This on-column derivatization removes the tedious steps from off line and puts them online for higher recoveries. The hot temperature of the inlet, 280 °C, is sufficient for instantaneous derivatization of the hormones. The method will also work with phytoestrogens and bisphenol A, two other classes of compounds that fit the emerging contaminants list and show possible feminization potential. The combination of C-18 disk solid-phase extraction followed by on-column derivatization was successful for the analysis of five hormones, 17-α-ethinylestradiol, 17-β-estradiol, estrone, estriol, mestranol, and the endocrine disruptor, bisphenol A.

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