APPLICATION NOTE

Preservation and Quantification of Hormones in Surface Water

Keywords: Androgens, Automation, Degradation, Endocrine Disruptors, Estrogens, GX-274 ASPEC™, Hormones, LC-MS/MS, Preservation, Progestogens, Runoff, Sample Preparation, Solid Phase Extraction, SPE, Surface Water, Water

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Introduction

The contamination of aquatic environments with hormones can disrupt the reproductive and developmental function of aquatic organisms such as fish (Jensen, K.M. et al., 2006; Orlando, E.F. et al., 2004). One source of these hormones is the natural and synthetic hormones that are present in manure from large-scale livestock operations. Approximately 130 billion pounds of manure are produced annually in the United States (USEPA, 2000). The hormones that are present in manure and in crop fields that have been amended with manure can be readily transported to surface and shallow ground water with events such as rain or snowmelt. There is a great deal of interest in studying the distribution and fate of these hormones in surface water.

Measuring the concentrations of natural and synthetic hormones in water can be challenging. Hormone degradation may occur during the storage of surface water prior to analysis in the laboratory (Baronti, C. et al., 2000; Vanderford, B.J. et al., 2006). This can result in the underestimation of hormone concentrations. Thus, a preservation protocol that inhibits the degradation of a large number of hormones and hormone metabolites during sample collection and storage is a necessary prerequisite for the accurate estimation of hormone occurrence in surface and ground water (Havens, S.M.et al., 2010).

This application note describes the use of different preservatives (sodium azide, hydrochloric acid and sulfuric acid) to inhibit the degradation of hormones in samples of surface water runoff from cattle manure-amended fields during storage at 4°C. The hormones and hormone metabolites were extracted from surface water using the automated Gilson GX-271 ASPEC™ System (Figure 1). Details of this procedure are described below. Hormone and hormone metabolite levels were determined using HPLC tandem mass spectrometry (LC-MS/MS).

Figure 1. Gilson GX-271 ASPEC™ System with 406 Single Syringe Pump (Part no. 2614007).

Experimental Conditions

Materials

The large suite of hormones chosen for the study included natural and synthetic estrogens, androgens and progestogens that have previously been detected or could be present in surface water (Table 1).

Table 1. Analytes Tested

Analyte	Chemical Abstracts (CAS) Number	Isotope Analog
17ß-Estradiol	50-28-2	17ß-Estradiol-d5
Estrone	53-16-7	17ß-Estradiol-d5
Estriol	50-27-1	Estriol-d3
α-Zearalenol	36455-72-8	α-Zearalenol-d4
Zearalenone	17924-92-4	α-Zearalenol-d4
Zearalanone	5975-78-0	α-Zearalenol-d4
Androsterone	53-41-8	Testosterone-d5
5α-Androstan-17ß-ol-3-one	521-18-6	Testosterone-d5
5α-Androstane-3, 17-dione	846-46-8	Testosterone-d5
4-Androstene-3, 17-dione	63-05-8	Testosterone-d5
1-Dehydrotestosterone	846-48-0	Testosterone-d5
17ß-Nortestosterone	434-22-0	Testosterone-d5
Testosterone	58-22-0	Testosterone-d5
17ß-Trenbolone	10161-33-8	17ß-Trenbolone-d3
Progesterone	57-83-0	Progesterone-d9
17,20-Dihydroxyprogesterone	1662-06-2	Progesterone-d9
Melengestrol	5633-18-1	Melengestrol-d3
Melengestrol acetate	2919-66-6	Melengestrol acetate-d3

All the analytical standards were of high purity (> 98%) and were obtained from Sigma-Aldrich (USA) with the exception of 17α-trenbolone, which was purchased from Hayashi Pure Chemical Inc. (Japan). Isotopically labeled standards were obtained from C/D/N Isotopes (Canada) or the European Union Reference Laboratory at the National Institute for Public Health and the Environments (RIVM, Bilthoven, The Netherlands). All solvents used were HPLC grade. All the runoff and ultra-pure water (control) samples were stored in 60 mL amber glass vials (I-CHEM, USA) that received a silanization treatment to deactivate the glass surface to prevent hormone adsorption to the vial wall. The silanization treatment included: one rinse with 5% dimethyldichlorosilane (in toluene; Supelco, USA), two rinses with toluene and three rinses with methanol.

Water Sample Collection, Preparation and Preservation

Three types of water were utilized for the study- ultra-pure water, a simulated fresh surface water runoff (a surrogate runoff), and an aged surface runoff sample collected from six edge-of field weirs at three anonymous cattle farms and stored for four months at 4°C. The simulated fresh water runoff sample was prepared by suspending freshly collected cattle manure and soil into groundwater collected from a tile drain on a dairy farm. This mixture was brought to a final volume of 5 Liters with dechlorinated tap water and then filtered through a 1µm glass fiber filter to remove suspended particles. 50 mL aliquots of each sample type were distributed into silanized 60 mL amber vials prior to SPE extraction. For more details on preparation of these samples, see Havens et al., 2010.

Aged runoff, filtered simulated fresh water runoff and ultra-pure water received either no preservative, sulfuric acid (H_2SO_4, 90 µl, pH = 2), HCl (200µl, pH = 2) or sodium azide (NaAz; 1 $g \cdot L^{-1}$). Isotopically labeled standards (ISTD) and target analytes (Target) were spiked at 50 µl of $1x10^3$ $ng \cdot ml^{-1}$ (in methanol). Spiked and ambient analytes were extracted (in triplicate) immediately (t = 0), after 24 hours (t=1 d) and after 14 days (t=14 d) of storage at 4°C. The samples were sequentially extracted on the Gilson GX-271 ASPEC solid phase extraction system. (Gilson Inc., USA). Simulated fresh water runoff samples were extracted first, followed by the aged surface runoff samples and then the ultra-pure water.

Automated Solid Phase Extraction

The Gilson GX-271 ASPEC System was configured as follows:

Description	Part numbers
GX-271 ASPEC w/ Single 406 Syringe Pump	2614007
10 mL Syringe	25025345
SPE Pressure Reg. Assembly and Plumbing package for gas + 10 mL Plumbing Package	25051376, 2644703, and 2644701
221 x 1.5 x 1.1 BV Tapered Probe and Guide Assembly for 1.5 mm Probes	27067374 and 26046228
Rinse Stations	26034551 and 26034555
Locator Tray for three 200-series Racks	26041035
Disposable sealing caps for 6 mL SPE cartridges, package of 1000	2954730
Rack Code 211 for 9 – 48 x 113mm (125 mL) bottles	2504611
Rack Code 222 for 27 30 x 115mm (50mL) conical bottom tubes	2504622
Rack Code 376 ASPEC 6 mL, 15 Position	260440046
Custom Adaptor – Mounting Code 3xx Rack to 200 – Series tray	Special 1284: Custom adapter and 210630CR, TLH Rack File for Special 1284
Solvent Reservoir Tray Insert for 700 mL bottles and pkg of four 700 mL solvent bottles	260440005 and 543701700
Viton tubing, .313 ID x .438 OD, 20 ft	4701438630
Safety Shield Assembly, GX27X	2604706
TRILUTION® LH Software Package	21063020, 210630R20 and ORACLE10GXE

The fractionation procedure used 200 mg/ 6mL ISOLUTE™ + polypropylene solid phase extraction cartridges (Biotage, USA). The cartridges were sealed using Gilson 6 mL Sealing Caps. Note: for Bioassay studies, glass cartridges should be substituted for polypropylene cartridges to eliminate effects of hormone mimicking plasticizers. In this case, Gilson Special 1778 for 6 mL glass cartridges can be substituted for the standard 6 mL cartridge SPE racks. Risers may be necessary when using this rack with the 200-series racks.

The solid phase extraction protocol is entirely automated using the Gilson GX-271 ASPEC system. The SPE steps are summarized with the general schematic provided in the GX-271 ASPEC control software, TRILUTION LH (Figure 3).

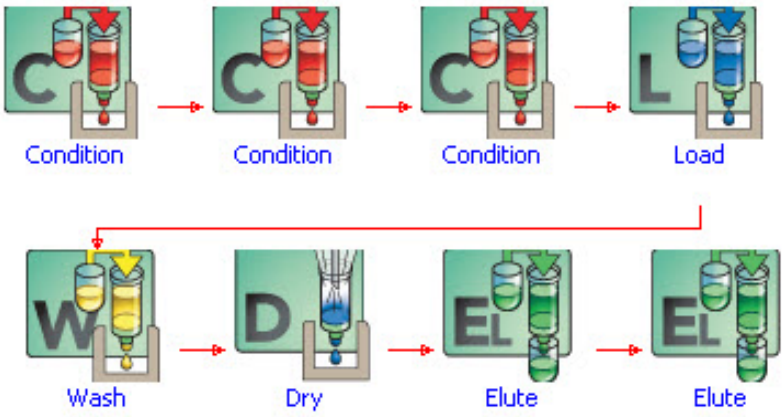

Figure 3. TRILUTION LH Basic SPE Tasks for Solid Phase Extraction of Hormones from Water

The summary of each step are as follows:

- Initialization Step: Gilson Mobile SPE Racks are moved above the waste rack (Figure 4)
- Condition the cartridge with 3 mL of methanol:ethyl acetate (1:1, v/v) at 3 mL/min
- Condition the cartridge with 3 mL of methanol at 3 mL/min
- Condition the cartridge with 3 mL of ultra-pure water at 3 mL/min
- Load 50 mL of sample onto the SPE cartridge at a flow rate of 3 mL/min
- Wash the cartridge with 10 mL of ultra-pure water at a flow rate of 3 mL/min
- Dry the cartridge with a stream of air for 5 minutes
- Move the Gilson Mobile SPE Rack over the collection tubes
- Elute with 4 mL of methanol at a flow rate of 0.5 mL/min
- Elute with 4 mL of methanol: ethyl acetate (1:1, v/v) at a flow rate of 0.5 mL/min
- Concentrate the fractions with a gentle stream of nitrogen gas to a volume of approximately 100 µL and reconstitute to a volume of 1.0 mL using methanol for HPLC-MS/MS analysis.

Figure 4. Gilson Mobile SPE Rack

LC-MS/MS Analysis

The hormone concentrations in the extracts were analyzed using high-performance liquid chromatography (Agilent Technologies 1100 HPLC, USA) with tandem mass spectrometric detection (Applied Biosystems/MDS SCIEX API 4000 USA; HPLC-MS/MS) operating in positive Atmospheric Pressure Chemical Ionization mode. A sample injection volume of 15 µL was applied to a 4 micron, 4.6 x 250 mm Synergi™ MAX-RP column (Phenomenex, USA) and separated with a reversed phase binary mobile phase gradient (Havens et al., 2010) at 0.8 mL/min. Relevant multiple reaction monitoring (MRM) mass spectrometer settings include TurboIonSpray™ voltage at 5500 Volts, collision gas at 6 arbitrary units, curtain gas at 25 psig, nebulization gas at 40 psig, drying gas at 15 psig, corona discharge current of 3 volts and source temperature at 450°C.

In order to determine the concentration of each target analyte, normalized to ISTDs, the instrument was calibrated by generating a curve based on the relative response ratios of peak areas between variable target analyte concentrations (1.0, 2.0, 5.0, 10, 25, 50, 100, 250 and 500 ng/mL) and ISTDs added to each calibration point at a concentration of 50 ng/mL. This corresponded to the ISTD concentration spiked into all of liquid chromatography samples. Linear or quadratic regression with $1/x^2$ weighting was used to generate calibration curves for all analytes. The calibration coefficients always exceeded 0.990. The target analyte concentrations in all the sample extracts (spiked and ambient) were calculated by normalizing the relative response ratio in the sample extract to those in the calibration curve. Detection limits for the extracted samples are in the 1.0 ng/L range, but this is dependent upon the amount of interference due to co-eluted matrix components present in the sample.

Results

Table 3. The recovery range of each analyte normalized to its corresponding isotopically labeled standard in simulated fresh water runoff (surrogate) with either no preservative, sulfuric acid or sodium azide after 14 days of storage at 4ºC.

Analyte	No Preservative	Sulfuric Acid	Sodium Azide
17ß-Estradiol	< 10%	80 - 120%	< 10%
Estrone	< 40%	40 - 59%	40 - 59%
Estriol	80 - 120%	80 - 120%	80 - 120%
α-Zearalenol	< 20%	60 - 79%	60 - 79%
Zearalenone	< 40%	60 - 79	40 - 59%
Zearalanone	40 - 59%	60 - 79%	60 - 79%
Androsterone	non detected	60 - 79%	< 40%
5α-Androstan-17ß-ol-3-one	< 10%	60 - 79%	< 10%
5α-Androstane-3, 17-dione	none detected	60 - 79%	< 10%
4-Androstene-3, 17-dione	none detected	60 - 79%	80 - 120%
1-Dehydrotestosterone	none detected	60 - 79%	< 10%
17ß-Nortestosterone	< 10%	60 - 79%	< 10%
Testosterone	none detected	60 - 79%	< 10%
17ß-Trenbolone	40 - 59%	80 - 120%	60 - 79%
Progesterone	none detected	80 - 120%	< 40%
17,20-Dihydroxyprogesterone	< 10%	60 - 79%	60 - 79%
Melengestrol	60 - 79%	60 - 79%	60 - 79%
Melengestrol acetate	40 - 59%	60 - 79%	40 - 59%

Table 4. The recovery range of each analyte normalized to its corresponding isotopically labeled standard in aged runoff preserved with either no preservative, sulfuric acid or hydrochloric acid after 14 days of storage at 4ºC.

Analyte	No Preservative	Sulfuric Acid Preserved	Hydrochloric Acid Preserved
17ß-Estradiol	< 40%	60 - 79%	80 - 120%
Estrone	> 160%	80 - 120%	80 - 120%
Estriol	< 40%	80 - 120%	80 - 120%
α-Zearalenol	< 40%	40 - 59%	60 - 79%
Zearalenone	40 - 59%	80 - 120%	80 - 120%
Zearalanone	> 160%	60 - 79%	60 - 79%
Androsterone	< 20%	80 - 120%	80 - 120%
5α-Androstan-17ß-ol-3-one	< 20%	80 - 120%	80 - 120%
5α-Androstane-3, 17-dione	none detected	60 - 79%	80 - 120%
4-Androstene-3, 17-dione	< 20%	60 - 79%	60 - 79%
1-Dehydrotestosterone	none detected	60 - 79%	60 - 79%
17ß-Nortestosterone	< 10%	80 - 120%	80 - 120%
Testosterone	< 20%	80 - 120%	80 - 120%
17ß-Trenbolone	40 - 59%	60 - 79%	60 - 79%
Progesterone	< 10%	60 - 79%	60 - 79%
17,20-Dihydroxyprogesterone	< 40%	80 - 120%	80 - 120%
Melengestrol	60 - 79%	60 - 79%	80 - 120%
Melengestrol acetate	60 - 79%	60 - 79%	60 - 79%

Conclusion

Significant degradation of estrogenic, androgenic and progestogenic hormones occurs in water samples within hours of sample collection. Much of this degradation is due to microbial activity (Havens et al., 2010). Adding sodium azide did not adequately inhibit androgen degradation at the concentration used. Acid preservation (HCl or H_2SO_4, pH = 2) stabilized the hormones in the water samples. Coupling acid preservation with the use of internal standards resulted in reliable and accurate recovery of a suite of androgens, estrogens and progestogens in surface water stored up to 14 days at 4ºC.

Using the Gilson GX-271 ASPEC for automation of the solid phase extraction (SPE) process increased sample throughput, reduced solvent usage and reduced the potential errors that may occur in during manual processing of samples. Automation also permitted scientists to spend more time planning scientific experiments and developing new methods for the analysis of compounds of interest to the laboratory.

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