Application Brief 160

Measurement of Environmental Estrogens in Water Using a Gradient HPLC-EC-UV Method

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Key Words
Hormones, Estrogen, Endocrine function, Xenoestrogens, HPLC-EC-UV

Summary
This application describes a sensitive, selective and stable gradient-HPLC-EC-UV method capable of simultaneously measuring numerous endocrine disruptors in environmental waters.

Introduction
A hormone is a chemical substance that is secreted by an endocrine gland. It travels through the blood stream and can bind to specific receptor sites in various organs and tissues, sometimes far removed from its site of production. Hormones regulate a variety of processes such as metabolism, reproductive function, maintenance of blood pressure, glucose and ion levels, and other endocrine, muscle and nervous system functions.[1] The faithful transmission of the correct type and concentration of hormone(s) to the intended tissue or organ is vital to an organism's health.

For many years humans have been adding chemicals and pollutants to the environment. Some of these chemicals can alter endocrine function e.g., a number (called anti-estrogens) can mimic estrogen hormones by binding to the estrogen receptor and negating the normal effects of this hormone. Others can alter the synthesis, catabolism and action of natural hormones and their corresponding receptors. These substances are often termed environmental estrogens or "xenoestrogens" but are also sometimes referred to as endocrine disruptors or endocrine modulators. Many of these chemicals (which include pesticides, plasticizers, household products and detergents, pharmaceuticals and industrial chemicals) are now ubiquitous in nature and are in the air we breathe, the water we drink and the soil in which we grow crops. In addition, man is exposed to these chemicals through the food chain via bioaccumulation.

Examples of the effects of environmental estrogens on aquatic organisms include blocking the development of frog egg maturation;[2] decline in alligator populations[3] and male fish possessing male and female characteristics.[4] In humans, environmental estrogens have also been linked to declining male reproductive health,[5] and breast cancer;[6] and miscarriages[7] in women.

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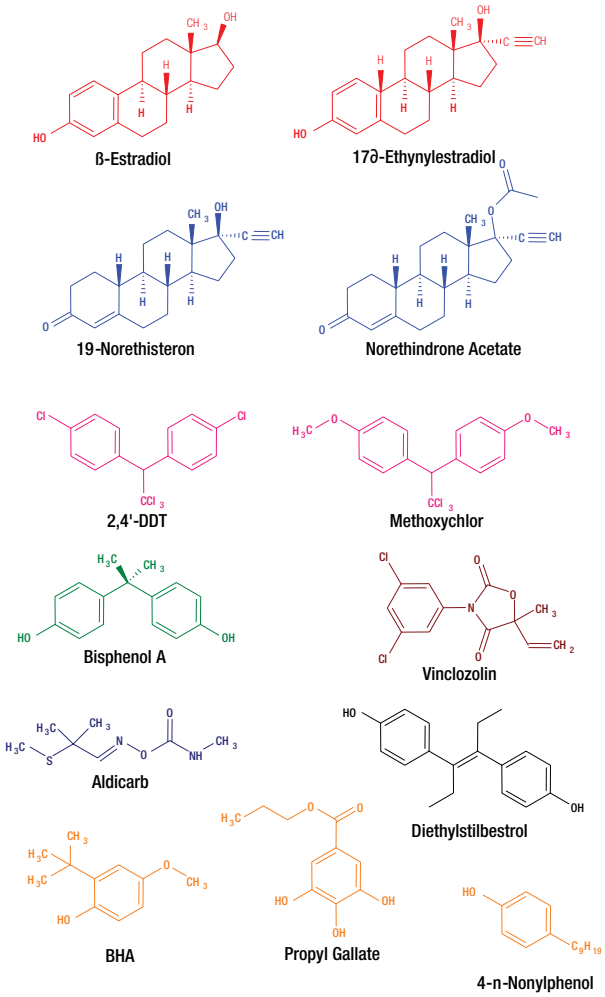

Figure 1. Structures of the environmental estrogens measured using this method.

Although there is much interest in the effects of environmental estrogens on endocrine function, the study and the detection of these chemicals is a fairly new science. Due to the many different types and concentrations of chemicals that are suspected of being environmental estrogens, it has been difficult to develop a global methodology that can simultaneously measure a number of these chemicals in different matrices. This application note describes a gradient HPLC-EC-UV method capable of separating and detecting the estrogen metabolite estradiol and several classes of environmental estrogens. These include insecticides – 2,4'-DDT, aldicarb, and methoxychlor; the fungicide – vinclozolin; the plasticizer – bisphenol A; the synthetic estrogen – diethylstilbestrol; the oral contraceptive – norethindrone; synthetic antioxidants – propyl gallate and BHA; and the surfactant – 4-n-nonylphenol. Also illustrated is the potential use of this method for the measurement of these compounds in water samples.

Materials and Methods

The analytical system consisted of two pumps, a refrigerated autosampler, a thermostatic chamber, and an 8-channel Thermo Scientific™ Dionex™ CoulArray™ Coulometric Array Detector situated before a single wavelength UV detector. The UV detector extends the range of compounds measured by the CoulArray Coulometric Array Detector and supplies additional qualitative information.

Conditions

LC	
Column:	C18, 3.0 µm, 3.0 × 150 mm
Mobile Phase A:	50 mM Lithium acetate pH 4.8, acetonitrile (95:5 v/v)
Mobile Phase B:	50 mM Lithium acetate pH 4.8, acetonitrile, methanol (20:40:40 v/v/v).
Gradient Conditions:	Isocratic 50% B from 0–5 min. Linear increase of phase B from 50-100% from 5–22 min. Isocratic 100% B from 22–35 min. 50% B at 35 min. Isocratic 50% from 35–40 min.
Flow Rate:	0.6 mL/min.
Temperature:	40 °C
Injection Volume:	40 µL
Detector	
Electrochemical Detector:	Model 5600A, CoulArray
Applied Potentials:	200 to 900 mV in 100 mV increments (vs. Pd)
Detector Wavelength:	240 nm

Standard Preparation

Sample preparation (presented in this Application Note) initially used C18 SPE cartridges (200 mg, 3 ml) (Diazem, Midland, MI). The SPE cartridge was conditioned using one column volume (CV) acetonitrile, then one CV 50% methanol, one CV 100% methanol and finally, two CVs water (pH <3.0 using phosphoric acid). A 20 to 200 mL volume of sample was then applied to the conditioned SPE cartridge. The SPE cartridge was washed with one CV acidified water (as above). The sample was eluted with two 500 µL aliquots of acetonitrile:methanol (1:1 v/v). The eluent was then evaporated to dryness and then reconstituted ethanol (125 µL) and then water (125 µL).

A second approach, showing much greater promise, is currently being evaluated using a layered C2/C18 (EC) SPE cartridge (500 mg, 3 mL) (International Sorbent Technology, Mid Glamorgan, UK). The SPE cartridge was conditioned using two CV of methanol (MeOH) followed by two CV of acidified water (pH less than 2.0 with hydrochloric acid). The pH of the water samples was adjusted to less than 2.0 by the addition of HCl (approximately 0.5 mL/100 mL). In addition, 0.5 mL of MeOH per 100 mL of water was added in order to keep the SPE cartridge phase solvated. A 100 mL volume of acidified water was applied to the conditioned SPE cartridge. The SPE cartridge was then washed with 2 CV of water and allowed to dry under vacuum for 5 min. The sample was then eluted with two 500 µL aliquots of acetonitrile (ACN): MeOH (1:1 v/v). The eluent was evaporated to dryness and reconstituted in 125 µL of ethanol. A 125 µL aliquot of water was then added to the ethanol, the solution and 40 µL injected onto the HPLC.

Results and Discussion

Most compounds were well resolved in less than 27 min (Figure 2). The limit of detection for the assay ranged from 5–100 pg on column for the compounds detected by the EC-array and 200–4000 pg on column for those detected by UV (0.05 AUFS) (Table 1). The electrochemical array was 10-100 times more sensitive than UV, and had a linear response range of four orders of magnitude.

The method was linear (in the range tested) with correlation coefficients >0.9985 for all compounds (40 µL repeats over an 8 h period; 100 ng/mL for EC active compounds and 1 µg/mL for UV active ones) (Table 1). The retention time variation for the method was 0.29% RSD. Peak height variation was <5.5% RSD (except vinclozolin; 9.2%) while peak area variation was <13.1% RSD (except vinclozolin; 15.6%).

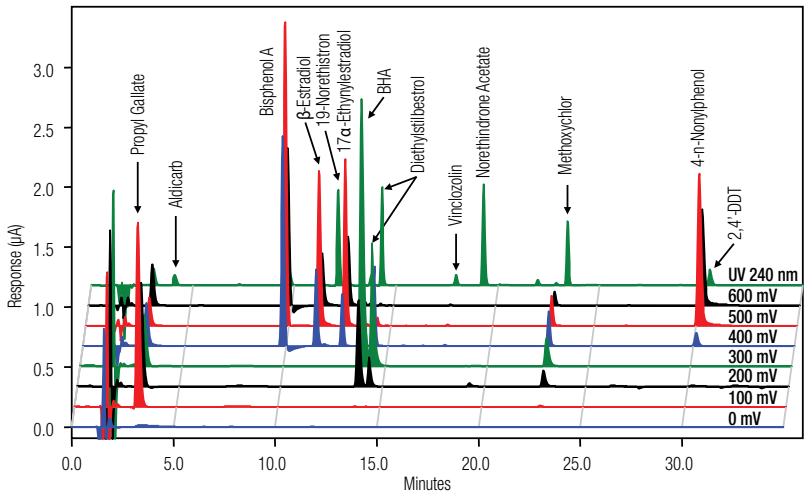

Figure 2. Chromatogram of a standard containing 100 ng/mL of EC-active compounds and 1000 ng/mL of UV active compounds.

Table 1. The limits of detection and linearities for the environmental estrogens measured using this method.

Analyte	Detection Source	Limits of Detection (pg)	Response Linearity (r^2)
Propyl Gallate	EC	100	0.9999
Aldicarb	UV	2,000	0.9998
Bisphenol A	EC	5	0.9985
β-Estradiol	EC	20	0.9986
19-Norethisteron	UV	200	0.9988
17α-Ethynylestradiol	EC	20	0.9991
BHA	EC	40	0.9999
Diethylstillbestrol	EC	40	0.9997
Vincolzolin	UV	4,000	0.9992
Norethindrone Acetate	UV	400	0.9999
Methoxychlor	UV	400	0.9999
4-n-Nonylphenol	EC	40	0.9995
2,4'-DDT	UV	2,000	0.9998

The SPE (Diazem) extraction method gave poor and variable results. Tap water spiked with standards (100 ng/L or 100 parts per trillion) showed extraction efficiencies ranging from 0 (aldicarb) to 50% (propyl gallate). A typical extracted spiked water sample is presented in Figure 3.

The IST SPE cartridge used here contains a C2 phase layered on top of a C18 phase. The short-chain C2 phase retains lipophilic compounds while the C18 phase retains the more polar compounds (that pass through the C2 packing). During the elution process, the compounds retained on the C2 are solvated and pass through the C18. Layered SPE columns allow for the retention and elution of analytes with greatly varying degrees of lipophilicity. The IST SPE cartridge under current investigation is showing much greater promise with typical extraction efficiencies of greater than 75%.

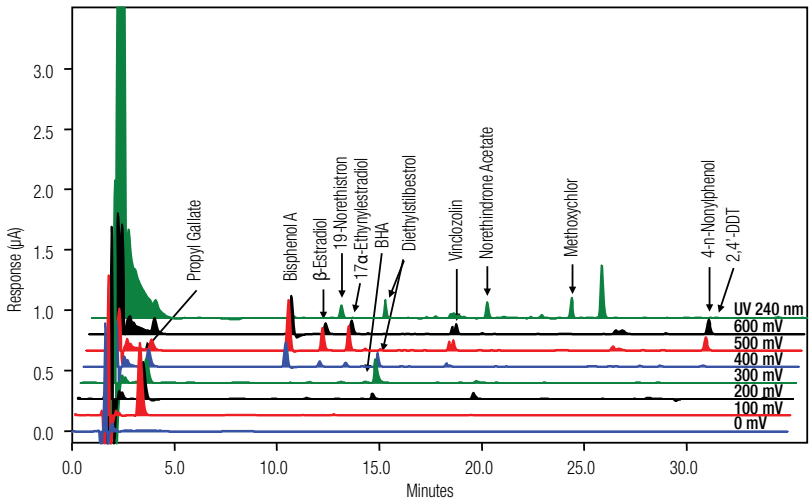

Figure 3. Chromatogram of spiked (100 ng/L) extracted tap water.

Conclusion

Described here is a sensitive, selective and stable gradient-HPLC method capable of measuring numerous endocrine disruptors simultaneously. Electrochemically active analytes are resolved both chromatographically and voltammetrically for added confidence in analyte identification. The range of analytes is extended to non-electrochemically active compounds by the use of UV detection. The routine use of this method to measure contaminated water samples is dependent on improving sample extraction. This is currently being addressed by using a novel C2 / C18 extraction cartridge.

Ordering Information

Description	Part Number
HPG-3400RS Biocompatible Binary Rapid Separation Pump with two solvent selector valves	5040.0046
WPS-3000TBRS Biocompatible Rapid Separation Thermostatted Autosampler	5841.0020
CoulArray Thermal Organizer	70-4340T
5600A CoulArray 8-Ch Detetector Inst 120 Vac	70-4324
Accessory Kit, CoulArray Detector to Thermo Scientific™ Dionex™ UltiMate™ 3000 System	70-9191

References

1 Crisp, T. M., Clegg, E. D., Cooper, R. L, Wood, W. P., Anderson, D. G., Baetcke, K. P., Hoffman, J. L., Morrow, M. S., Rodier, D. J., Schaeffer, J. E., Touart, L. W., Zeeman M. G., and Patel, Y. M. (1998). Environmental endocrine disruption: an effects assessment and analysis. Environ. Health Perspect., **106**, 11–56.

2 Pickford, D. B., and Morris, I. D. (1999). Effects of endocrine-disrupting contaminants on amphibian oogenesis: methoxychlor inhibits progesterone-induced maturation of *Xenopus laevis* oocytes *in vitro*. Environ. Health Perspect. **107**, 285–292.

3 Crane, D. A. Guillette Jr. L. J., Pickford, D. B., Percival H. F., and Woodward, A. R. (1998). Sex-steroid and thyroid hormone concentrations in juvenile alligators (*Alligator mississippiensis*) from contaminated and reference lakes in Florida, USA. Environ. Toxicol. Chem., **17**, 446–452.

4 Jobling, J. S., and Sumpter, J. S. (1993). Detergent components in sewage effluent are weakly oestrogenic to fish: an *in vitro* study using rainbow trout (*Oncorynchus mykiss*) hepatocytes. Aquatic Toxicol., **27**, 361–372.

5 Toppari, J., Larsen, J.C., Christiansen, P., Giwercman, A., Grandjean, P., Guillette, L.J. Jr., Jegou, B., Jensen, T. K., Jouannet, P., Keiding, N., Leffers, H., McLachlan ,J. A., Meyer, O., Muller, J., Rajpert-De Meyts, E., Scheike, T., Sharpe, R., Sumpter, J., and Skakkebaek, N E. (1996). Male reproductive health and environmental xenoestrogens. Environ. Health Perspect., **104**, 741–803.

6 Davis, D., Bradlow, H., Wolff, M., Woodruff, T., Hoel, D., Anton-Culver, H. (1993). Medical hypothesis: xenoestrogens as preventable causes of breast cancer. Environ. Health Perspect., **101**, 372–377.

7 Nollar, K. L., O'Brien, P. C., Colton, T., Kaufman R., and Melton, L. J. (1990). Medical and surgical diseases associated with *in utero* exposure to diethylstilbestrol. In: *Clinical Practice of Gynecology*. Elsevier, New York. Pp. 1-7.

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