WATER EXTRACTION TECHNIQUE FOR DETECTION OF ESTROGENIC ACTIVITY USING THE LUMI-CELL® ER BIOASSAY

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Introduction

For several years there has been an increasing concern over the strong association between the exposure and bioaccumulation of endocrine disruptor chemicals (EDCs) and their adverse effects on human and wild life populations[1,2]. These EDCs are largely synthetic organic compounds (synthetic hormones, phytoestrogens, pesticides, surfactants, ect.), which are introduced into the environment through human consumption of pharmaceuticals, personal care products, and other environmental contaminants. Compounds from these products have become ubiquitous with aquatic environments and wastewater effluents, as well as in surface and drinking water[3-5]. To this end the U.S. Congress passed legislation (Food Quality Act of 1996 and Safe Water Reauthorization Act Amendments of 1996), which mandated the EPA to investigate the exposure to environmental EDCs[16-18]. Some examples of the effects of EDCs are: decreased reproductive success and feminization of males in several wildlife species; increased hypospadias along with reductions in sperm counts in men; increase in the incidence of human breast and prostate cancers; and endometriosis[6-8]. In light of these detrimental effects from EDC exposure through water, there is an obvious need to screen wastewater and other water systems for EDCs.

Most techniques described to extract EDCs from water involve a solid phase step and extraction with several solvents, such as Ethyl Acetate, Acetic Acid, and Methanol[3-5,9-14]. We wanted to look at the possibility of extracting EDCs without a solid phase, which meant the use of solvents that were not ubiquitous with water, allowing for phase separation. To this end both Ethyl Acetate (EA) and Methyl tert-Butyl Ether (MTBE) were used in a water shake study to extract water samples spiked with 17β-estradiol.

Methods and Materials

Chemicals: All reagents used were 99% or greater in purity. The 17β-estradiol (E2) was purchased from Sigma Aldrich. The MTBE was purchased from J.T. Baker. The Methanol was purchased from Burdick & Jackson. The EA was purchased from Fisher.

Sample Preparation and Analysis Procedure: Durham, NC municipal tap water was used throughout the study. Five ml of tap water was spiked at 1 μg/ml E2. The spiked water or an un-spiked water sample (5 ml each) were then mixed with 5 ml Ethyl Acetate, Methyl tert-Butyl Ether or a 50:50 mixture of the two solvents and shaken for 5 min. The solvent extraction was repeated 2 additional times. Three solvents Methanol, EA and MTBE were also spiked at 1 μg/ml E2 to determine recoveries in the water extractions. A range finding assay was preformed on each extract using six dilutions (10, 2, 1, 0.5, 0.2 and 0.1 μg/L).

LUMI-CELL® ER Bioassay. The BG1Luc4E2 cell line was constructed as previously described by Rogers and Denison (2000). Briefly, BG1 cells were stably transfected with an estrogen-responsive luciferase reporter gene plasmid (pGudLuc7ere) and selected for G418 resistance[8]. For analysis, the BG1Luc4E2 cell clone resulting from this selection was grown in RPMI 1640 medium. The cells were transferred into flasks containing phenol red-free DMEM media supplemented with charcoal dextran stripped FBS and incubated for four days before harvesting for BG1Luc4E$_2$ bioassay plating. The cells were then plated in white clear-bottomed Corning 96 well microplates and incubated at 37°C for 24-48 hours prior to dosing. The media solution in each well was removed and two hundred microliters of phenol red-free DMEM supplemented with charcoal dextran stripped

FBS containing the indicated concentration of the desired chemical to be tested was added to each well. The plate was then incubated for 20 hours 37^{o}C before analysis of luciferase activity.

Measurement of Luciferease Activity. After lysing the cells (Promega lysis buffer), the luciferase activity was measured in a Berthold Orion Microplate Luminometer, with automatic injection of 50 micro liters of luciferase enzyme reagent (Promega) into each well. The relative light units (RLUs) measured were compared to that induced by the E2 standard after subtraction of the background activity.

Results and Discussion

Current wastewater treatment techniques are at least partially inefficient in removal of all EDCs[3-5,9-14]. Therefore there is a need to test water for potential EDCs. To this end we have developed a new simple non-solid phase extraction technique for testing estrogenic EDCs in conjunction with the LUMI-CELL® ER bioassay.

Several solid phase extraction techniques such as liquid chromatography (LC), gas chromatography (GC), mass spectrometry (MS) and molecular imprinting have been described for extraction of EDCs from water. The recoveries for these techniques usually range from 49% to 99% of spiked EDCs[3-5,9-14]. In this study, water samples were spiked with 1 μg/ml E2 and were extracted with EA, MTBE or a 50:50 mixture of the two solvents.

Ethyl Acetate

Figure 1 demonstrates the water extracted with EA. Samples of the EA and Methanol were also spiked at 1 μg/ml E2 for recovery determinations. The non-spiked water sample demonstrated no estrogenic activity when extracted with EA. Estrogenic activity in the spiked water sample was detected with a recovery of 85% when compared to the spiked EA and 86% when compared to the spiked Methanol (Table 1).

Methyl tert-Butyl Ether

Figure 2 depicts the water sample extracted with MTBE. Samples of the MTBE and Methanol were also spiked at 1 μg/ml E2 for recovery determinations. The non-spiked water sample demonstrated no estrogenic activity when extracted with MTBE. Estrogenic activity in the spiked water sample was detected with a recovery of 95% when compared to the spiked MTBE and 90% when compared to the spiked Methanol (Table 1).

50:50 mixture of Ethyl Acetate and Methyl tert-Butyl Ether

Figure 3 demonstrates the water extracted with a 50:50 mixture of EA and MTBE. Samples of the 50:50 mixture of EA and MTBE and Methanol were also spiked at 1 μg/ml E2 for recovery determinations. The non-spiked water sample demonstrated no estrogenic activity when extracted with the 50:50 mixture of EA and MTBE. Estrogenic activity in the spiked water sample was detected with a recovery of 105% when compared to the spiked 50:50 mixture of EA and MTBE and 97% when compared to the spiked Methanol (Table 1).

Previous studies using analytical and chemical methods have shown the need for a solid phase in the extraction of ECDs from water[3-5,9-14]. The recoveries seen using this non-solid phase extraction were 85% or greater in all cases and were in the upper range for recoveries determined using LC/MS and GC/MS and molecular imprinting (between 49% and 99%[3-5,9-14]). These results demonstrate the effectiveness of the extraction of E2 from water using a non-solid phase extraction technique and the effectiveness of the LUMI-CELL® ER bioassay to detect activity in these extracts. The use of a non-solid phase extraction technique, coupled with a bioassay will make a quick inexpensive method for municipalities to test wastewater and drinking water sources and address a growing public health concern.

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References

1. Jarry H,, Christoffel J., Rimoldi G., Koch L., Wuttke W. (2004). Toxicology. 205(1-2):87-93.
2. Jefferson W.N., Padilla-Banks E., Clark G., Newbold R.R. (2002). J Chromatogr B Analyt Technol Biomed Life Sci. 777:179-189.
3. Le Noir, M., Lepeuple, A.S., Guieysse, B., and Mattaisson, B. (2007). Water Research 41(12):2825-2831.
4. Jones, O.A., Lester, N.J., and Voulvoulis, N. (2005). Trends Biotechnol. 23:163.
5. Ternes, T.A., et. al. (2002). Environ. Sci. Technol. 36:3855.
6. Markey C.M., Coombs M.A., Sonnenschein C., Soto A.M. (2003). Evol Dev. 5:67-75.
7. Safe S.H. (2002). Health Perspect. 110: 925-929.
8. Rogers J.M, and Denison M.S. (2000). In Vitro Mol Toxicol. 13:67-82.
9. Gomez, M.J., et. al. (2006). Intern. J. Environ. Anal. Chem. 86:3-13.
10. Ballesteros, O., Zafra, A., Navalon, A., Vilchez, J.L. (2006). J. Chromatrography A. 1121:154-162.
11. Salste, L., Leskinen, P., Virta, M., and Kronberg, L. (2007). Science of The Total Environment. 378:343-351.
12. Le Noir, M., Plieva, F., Hey, T., Guieysse, B., and Mattiasson, B. (2007). J. Chromatrography A. 1154:158-164.
13. Gibson, R., Becerril-Bravo, E., Silva-Castro, V., and Jimenez, B. (2007). J. Chromatrography A. 1169:31-39.
14. Sonneveld E., Riteco J., Jansen H., Pieterse B., Brouwer A., Schoonen W., Burg B. (2005). Toxicol Sci. 89:173-187.
15. de Johgh, G. (1999). http://www.bio.net/bionet/mm/methods/1999-May/075278.html
16. Nomination of the LUMI-CELL® ER bioassay High Throughput System for Screening Estrogen-Like Chemicals for Validation Studies. http://iccvam.niehs.nih.gov/methods/endocrine/endodocs/XDSeval2.pdf
17. Current Evaluation of In Vitro Test Methods for Detecting Potential Endocrine Disruptors (May 2003). ICCVAM, NTP, NICEATM, NIEHS. NIH Publication No. 03-4503.
18. Current Status of Test Methods for Detecting Endocrine Disruptors: In Vitro Estrogen Receptor Transcriptional Activation. http://iccvam.niehs.nih.gov/docs/docs.htm#endocrine and http://iccvam.niehs.nih.gov/methods/endodocs/final/erta_brd/erta_all.pdf.

Table 1. Recovery determinations of the extraction procedures.

	EA Extracton	MTBE Extraction	50EA:50MTBE Extraction
EA	85%	-	-
MTBE	-	95%	-
50EA:50MTBE	-	-	105%
Methanol	86%	90%	97%

Figure 1. EA extraction of water samples.

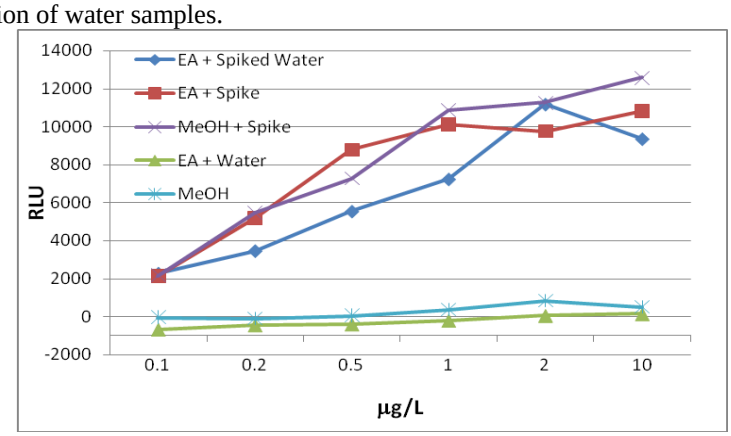

Figure 2. MTBE extraction of water samples.

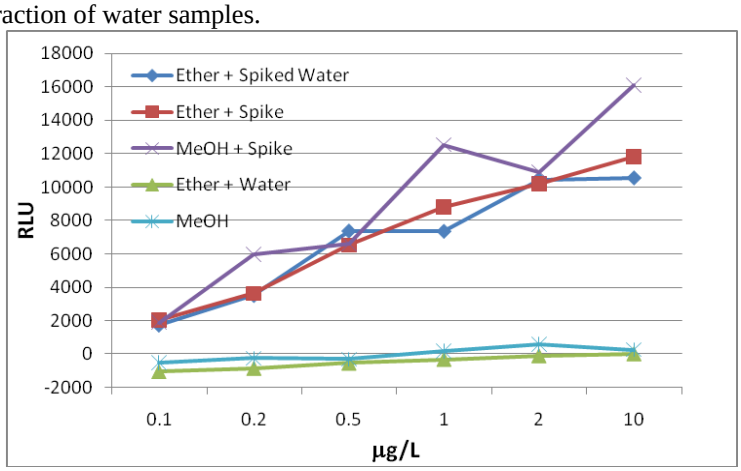

Figure 3. 50:50 mixture of EA and MTBE extraction of water samples.