DEVELOPMENT OF A MAGNETIC PARTICLE ENZYME IMMUNOASSAY AND ITS APPLICATION TO THE MEASUREMENT OF TRICLOSAN IN WATER AND WASTEWATER

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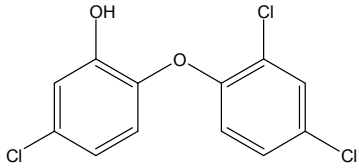
Introduction



Triclosan

Triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol) is a broad spectrum antibacterial that is incorporated into numerous home use products such as soap, deodorants, toothpastes, toys, bedding, socks, and trash bags to decrease bacterial contaminations. Structurally, triclosan can be related to environmental contaminants such as polybrominated biphenyl ethers or dioxins; therefore, numerous reports of triclosan in sewage and wastewater treatment effluents have raised concerns. Because of its high degree of lipophilicity and non volatility, triclosan has been demonstrated to bioaccumulate and was found at concentrations ranging from 750 to greater than 10,000 pg/g in fish plasma from the Detroit river[1]. Breakdown products produced in river water by the action of sunlight include dioxins[2]. Methyl triclosan, presumably formed from bacterial metabolism in sewage plants, has also been reported in environmental samples[3].

Current reported triclosan analytical methods require GC-MS or LC-MS instrumental analysis[4-6]. While these methods are capable of multiple congener analyses, samples require extensive purification. The sophisticated instrumentation requires dedicated laboratory resources and are not amenable to economical high throughput analysis. For environmental measurements of triclosan, an alternative method is needed to analyze samples in a timely manner because triclosan is labile.

Magnetic particle based enzyme immunoassays have been described previously and have been applied widely to measure pesticides, PBDEs, and other environmental contaminants in a variety of matrices (7-9). Here we report the development of a triclosan magnetic particle enzyme immunoassay which is field portable, user friendly, sensitive, capable of high throughput, and cost effective.

Materials and Methods

Materials. N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDAC) and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Supermagnetic particles (1 µm) were obtained from Seradyn (Indianapolis, IN). Triclosan reference standard was obtained from U.S. Pharmacopeia, Rockville, MD. Methyl triclosan was obtained from Wellington Laboratories Inc, Ontario, Canada. The analytical standards for PBDEs, bromobiphenyls, T3, T4 were obtained from either Chem Service, West Chester, PA or AccuStandard, New Haven, CT.

Hapten synthesis and antibody generation. Ethyl 6-bromohexanoate (100 µL, 0.55 mmol) was added to a solution of triclosan (145 mg, 0.5 mmol) in 30 mL of acetone. Anhydrous potassium carbonate (83 mg, 0.6 mmol) and potassium iodide (8 mg, 0.05mmol) were added and the mixture was refluxed for 48 hrs. The suspension was filtered and the acetone evaporated giving ethyl 6-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)hexanoate MW= 430

(positive mode electrospray = 431 m/z). The ethyl 6-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)hexanoate was then dissolved in 6.7 mL of ethanol and 3.3 mL of 1 N sodium hydroxide and refluxed overnight. After acidifying the solution with 2.5 N HCl to pH < 2, the product was extracted with ethyl acetate (10 mL x 5) and the ethyl acetate removed by evaporation. The 6-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)hexanoic acid (triclosanhexanoic acid) was isolated by applying the oil to a preparative silica plate and developing the plate with hexane:dichloromethane (3:1). A total of 146 mg oil (73% yield, positive mode electrospray = 403 m/z) was recovered. 12 mg of triclosanhexanoic acid (0.03 mmol) was dissolved in 300 μL of DMF and 0.08 mmol of NHS (9 mg) and 0.08 mmol (16 mg) of N,N'-dicyclohexyl carbodiimide was added to the solution and mixed overnight at room temperature. After removing the precipitated urea, the supernatant was added dropwise to 20 mg of KLH (in 5 mL of 100 mM borax buffer, pH 9). Four rabbits were immunized with 200 μg of the KLH-triclosanhexanoic acid conjugate every 3 weeks. The 9th boost serum from rabbit-131 was utilized for immunoassay development because of its sensitivity towards triclosan.

Tracer synthesis and magnetic particle activation. The conjugation procedure for triclosanhexanoic acid to horse radish peroxidase is similar to antigen generation except EDAC was used instead of N,N'-dicyclohexyl carbodiimide. Similarly, anti-triclosan antibodies were coupled to magnetic particles using NHS/EDAC activation in the presence of 50 mM of 2-(*N*-(morpholino)ethane sulfonic acid buffer.

Enzyme immunoassay (ELISA) for water samples. Water samples (or other samples) were collected in glass vials, immediately upon collection methanol was added to a final concentration of 25% (v/v). The sample to be tested, and antibody activated paramagnetic particles were added to a disposable glass tube and incubated at room temperature for 30 min, followed by the addition of triclosan-HRP and another 30 min incubation at room temperature. A magnetic field is applied to hold the paramagnetic particles within the tube while the unbound reagents are decanted. The particles were then washed with water. To the washed particles, hydrogen peroxide and the chromagen (3,3',5,5'-tetramethylbenzidine) were added and incubated at room temperature for 20 min. The reaction was stopped by the addition of 500 μL of 0.5% sulfuric acid. The results were read at 450 nm within 15 min of the addition of sulfuric acid. In each assay, a standard curve of 0, 25, 100, and 1000 ppt was measured and the unknown sample concentrations were determined from the standard curve.

Sample collections. Water samples were collected from different purification stages at the water and wastewater/sewage treatment plants in Fargo, North Dakota. The water was centrifuged at 2000 g for 10 minutes and the supernatant collected, methanol added to a final concentration of 25%, and stored at 4^{o}C until analyzed.

Results and Discussion

Precision and recovery. Four water samples (municipal, well, pond, and creek obtained from Warminster, PA) were spiked with different levels of triclosan, diluted to 25% methanol (v/v) and assayed with ELISA. The results are shown in table 1. With all levels of spiking the recoveries were 80% or greater and the % CV was less than 10%. This demonstrates that the ELISA can accurately and precisely measure triclosan in water samples.

Table 1. Recovery and precision of triclosan from spiked water.

Spiked (ppt)	n	Found	% recovery	% CV
62.5	4	52.2 ± 4.8	83.4	9.2
125	4	109.1 ± 10.8	87.3	9.9
250	4	252.2 ± 9.1	101	3.6
500	4	565.6 ± 28.4	113.1	5.0

Sensitivity and specificity. The cross-reactivity of the triclosan antibody to various structurally related compounds was expressed as the limit of detection (LOD), which is estimated at 90% B/B_0 or as the dose required to displace 50% of triclosan (50% B/B_0). This shows that the ELISA was able to detect triclosan at 20 ppt and its metabolite, methyl-triclosan at 15 ppt. The antibody was able to recognize some low substitution polybrominated biphenyl ethers; BDE-47 in particular, equally well. The

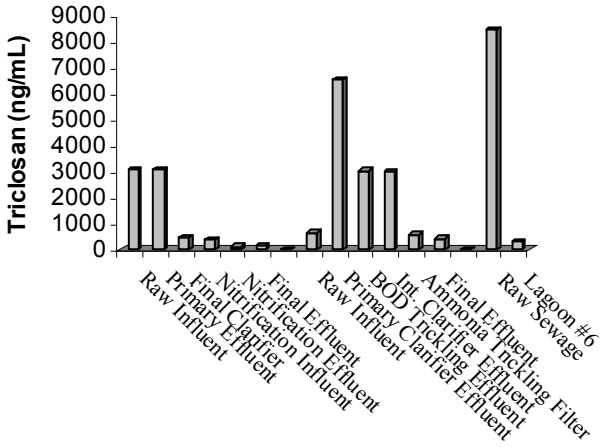
antibody lost the ability to recognize analytes when the ether linkage was eliminated, such as with the bromobiphenyl compounds.

Table 2. Sensitivity and cross-reactivity of triclosan antibody.

Compound	90% B/B_0 (ppb)	50% B/B_0 (ppb)
Triclosan	0.020	0.25
Methyl-Triclosan	0.015	0.08
PBDE-28	0.034	0.61
PBDE-47	0.020	0.39
PBDE-49	5.2	17.8
PBDE-99	2.15	15.0
4'-OH-BDE-47	0.13	7.8
5-OH-BDE-47	0.15	5.6
6-OH-BDE-47	0.66	10.2
2,4,5-tribromobiphenyl	> 100	>100
2,4',5-tribromobiphenyl	54	9,100
2,3,7,8-TCDD	> 100	>100
T3	0.94	40
T4	340	700

Triclosan levels (ppt) from water and wastewater treatment plants. Effluent samples obtained from drinking water treatment plants demonstrated triclosan concentrations below the limit of detection (20 ppt). The wastewater/sewage treatment plants showed higher concentrations of triclosan but, in general, the levels decreased following processing (Figure 1). The further downstream in the purification process, the lower the triclosan level. The three different sites employed three different treatment processes, namely activated sludge, BOD trickling, and lagoon. Overall, the treatment system removed more than 90% of the triclosan from the primary effluent to the final effluent. Our results (144 - 415 ppt this study) are similar to previous reports for wastewater treatment from other states and countries where effluent concentrations ranged from 42-213 ppt (Switzerland[10]), 70-650 ppt (Switzerland[3]), or 240-410 ppt (Ohio, USA[11]). Clearly, there are still measurable concentrations of triclosan discharged into the environment. Confirmation of triclosan levels with LC-MS/MS is planned.

Figure 1. Triclosan concentrations (ng/mL) from three different wastewater treatment plants



In conclusion, the ELISA gave good recoveries of triclosan from spiked water samples and showed small relative variance. The high cross-reactivity with methyl triclosan can be considered beneficial since this compound is

apparently a biological metabolite of triclosan produced in the processing of sewage. Rather surprising is the cross-reactivity with BDEs, which indicates the antibody can accommodate the more bulky bromine analogs.
The assay is rapid, and it can analyze up to fifty samples in about 2 hrs without any sample cleanup. Applications of the triclosan ELISA to water or wastewater/sewage treatment plants demonstrate that this assay can serve as a timely, on-site, and cost-effective monitoring method.

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Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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