Environmental Science & Technology

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Detection of the Antimicrobial Triclosan in Environmental Samples by Immunoassay

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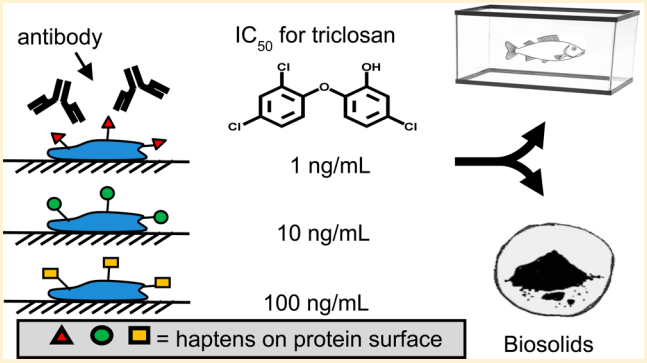
Supporting Information

ABSTRACT: A sensitive, competitive enzyme-linked immunosorbent assay (ELISA) for the detection of the antimicrobial triclosan (TCS; 2,4,4′-trichloro-2′-hydroxydiphenyl ether) was developed. Novel immunizing haptens were synthesized by derivatizing at the 4-Cl position of the TCS molecule. Compounds derived from substitutions at 4′-Cl and that replaced the 2′-OH with a Cl atom were designed as unique coating antigen haptens. Polyclonal rabbit antisera were screened against the coating antigen library to identify combinations of immunoreagents resulting in the most sensitive assays. The most sensitive assay identified was one utilizing antiserum no. 1155 and a heterologous competitive hapten, where the 2′-OH group was substituted with a Cl atom. An IC_{50} value and the detection range for TCS in assay buffer were 1.19 and 0.21−6.71 μg/L, respectively. The assay was selective for TCS, providing low cross-reactivity (<5%) to the major metabolites of TCS and to brominated diphenyl ether-47. A second assay utilizing a competitive hapten containing Br instead of Cl substitutions was broadly selective for both brominated and chlorinated diphenylethers. Using the most sensitive assay combination, we measured TCS concentrations in water samples following dilution. Biosolid samples were analyzed following the dilution of a simple solvent extract. The immunoassay results were similar to those determined by LC−MS/MS. This immunoassay can be used as a rapid and convenient tool to screen for human and environmental exposure.



INTRODUCTION

Triclosan (TCS; 2,4,4′-trichloro-2′-hydroxydiphenyl ether) is widely used as an antimicrobial agent in household and personal care products (Figure 1). The widespread use of TCS has resulted in its presence in wastewater effluents, biosolids, and in surface receiving waters.[1−5] This results in direct exposure to aquatic animals, such as fish and snails. The land application of biosolids presents concerns about the potential for reentry of TCS into the environment and, therefore, additional exposure pathways.[6] Human and animal exposure to TCS is of great concern because it has been demonstrated to be an antagonist in both estrogen-mediated and androgen-mediated bioassays and a potent Ca^{2+} channel sensitizer and uncoupler in a ryanodine receptor-mediated bioassay and primary muscle cells, and it increases susceptibility to liver carcinogenesis.[7−10]

Current analytical methods are based on LC−MS/MS, GC/ECD, or GC/MS for the detection of TCS in wastewater and environmental samples. Analysis of water and biosolids typically includes sample preparation steps such as liquid−liquid and solid-phase extraction and, for GC analysis, derivatization of the hydroxyl group.[11−13] Although these methods are well-suited for their applications, for routine monitoring, a simple, robust, rapid method that can analyze a large number of samples is desired. Immunoassay methods can serve as a rapid screen for environmental contaminants, pesticides, and their degradation products in environmental chemistry.[14,15] These techniques are widely used in diagnostics, environmental monitoring, food quality, agriculture, and field or on-site testing of personnel exposed to toxic chemicals. We have demonstrated these routine immunoanalytical techniques using environmental and biological samples such as house dust, soil, water, urine, and blood.[14,16,17]

The objective of this study was to develop an enzyme-linked immunosorbent assay (ELISA) for the analysis of TCS as a simple monitoring tool. Our approach was 2-fold. First, novel immunizing and competitive haptens were carefully designed and synthesized. For immunizing, the design focused on haptens that most closely mimicked the target analyte for the development of selective antibodies. With competitive haptens, we designed haptens that should have relatively lower affinity

Received: November 12, 2015
Revised: February 17, 2016
Accepted: March 2, 2016
Published: March 3, 2016

DOI: 10.1021/acs.est.5b05357
Environ. Sci. Technol. 2016, 50, 3754−3761

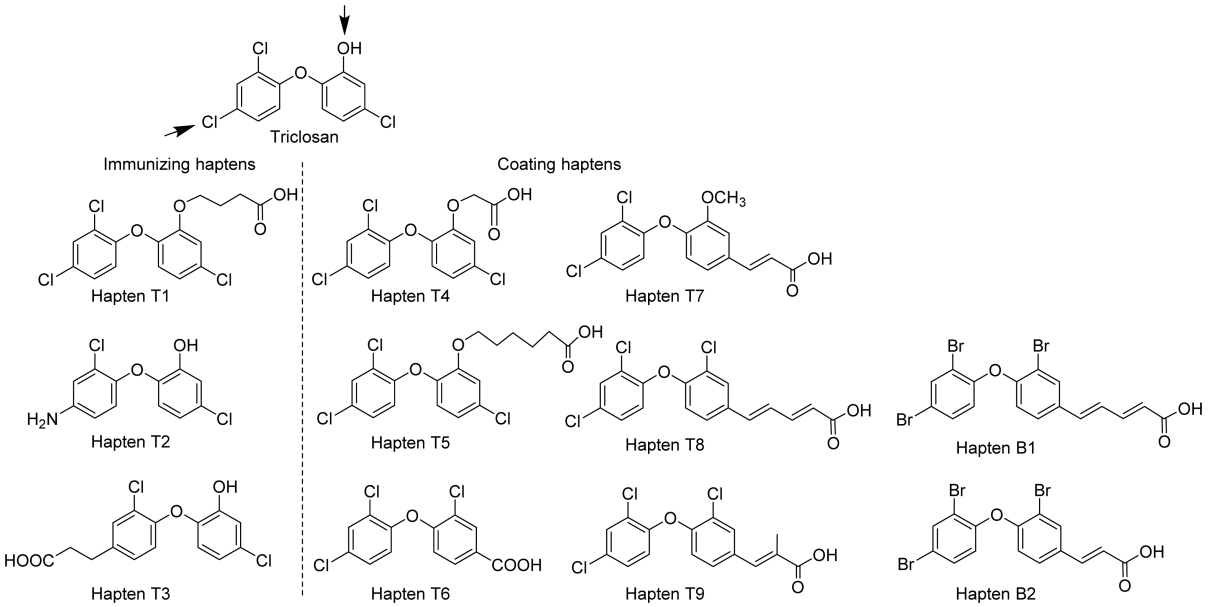


Figure 1. Structures of immunizing and coating haptens. Arrows on the TCS structure indicate sites where the functional group was introduced. Haptens B1 and B2 were previously used for the development of a BDE-47 immunoassay.[14]

compared to the target analyte to improve the sensitivity of the assay. Second, we took advantage of the strengths of using the hapten−protein-coated format. Advantages of this format include using rabbit antibody sparingly compared to the antibody-coated format that generally used 10 to 100 times more antibody reagent. Hapten, which can be costly to synthesize, is also used more sparingly in this format because hapten−protein conjugates as coating antigens are generally more stable than hapten−enzyme conjugates that require the preservation of the enzymatic activity to be functional.

Immunoassays for TCS detection have been developed previously.[18,19] However, the formats in which these have been developed require a relatively large amount of primary antibody reagent because the solid support (e.g., magnetic beads or microtiter plate) is coated with primary antibody. Because polyclonal antibody reagents are typically only generated once from a single animal, their supply is limited, thus not permitting its use for analyzing large sample sets without the potential need to reoptimize another aliquot of reagent. An ELISA format in which a labeled secondary antibody is used would permit a more sparing use of primary antibody while using a hapten−protein conjugate as the immobilized coating antigen, thereby extending the usefulness of the assay.

This study reports the preparation of novel haptens, characterization of newly generated antibodies, immunoassay optimization, and validation. This is the first report on the analysis of biosolids by immunoassay. Furthermore, the reagents generated may be further applied to immunosensor-based assays and used in the generation of antibodies from new sources, such as camelid-derived VHH nanobodies, which are ongoing projects in this laboratory.

EXPERIMENTAL SECTION

Chemicals and Instruments. All hapten coupling reagents (sulfo-*N*-hydroxysuccinimide, *N*-hydroxysuccinimide, 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide, dicyclohexylcarbodiimide, isobutyl chloroformate, tri-*n*-butylamine), bovine serum albumin (BSA), thyroglobulin (Tg), goat antirabbit IgG peroxidase conjugate (GAR-HRP), Tween 20, and 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). ELISA was performed on 96-well microtiter plates (Nunc MaxiSorp, Roskilde, Denmark) and read spectrophotometrically with a microplate reader (Molecular Devices, Sunnyvale, CA) in dual-wavelength mode (450 and 650 nm).

Hapten Synthesis. Due to their molecular size, TCS haptens require conjugation to carrier proteins to be immunogenic. Thus, TCS haptens containing a carboxylic group or an amino group were designed and synthesized (Figure 1). The $-NH_2$ or −COOH linker was introduced at the 4-Cl (hapten **T2** or **T3**) position in the TCS molecule to keep the −OH group free and most distal to the point of attachment to the protein. To retain the −OH group, we protected it during synthesis of the haptens and then deprotected it using boron tribromide. When coupled to the carrier protein, these haptens fully presented the majority of the TCS molecule for antibody recognition resulting in an antibody selective for TCS.

Hapten precursors described in the Supporting Information were initially synthesized with a nitroaromatic (**4**; Scheme 2) or aldehyde (**7**, Scheme 3) analog prepared from commercially available starting materials via nucleophilic aromatic substitution. Compound **4** was hydrogenated to provide the aniline (**5**, Scheme 2), followed by boron tribromide-mediated cleavage of the methyl ester to obtain the immunizing hapten **T2** that was conjugated to protein by the diazotization method.

An unsaturated spacer was introduced into methylated dichlorophenoxybenzaldehyde (**7**) by enylation with phosphonoacetate or phosphonocrotonate using lithium hydroxide and molecular sieve by the Wittig or Horner−Wadsworth−Emmons reaction[20] to synthesize the intermediates **8**, **18**, or **20** (Schemes 3, 8, or 9, respectively). To produce hapten **T3**, we converted the unsaturated spacer in intermediate **8** to a saturated spacer (**9**) by using Pd/C under H_2 atmosphere. Cleavage of the aryl ether ($Ar-OCH_3$) and the ester by boron tribromide provided a strategy for the unmasking of both the ArOH and $ArCO_2H$ functional groups.[21] For coating hapten **T7**, compound **8** was not converted to a saturated spacer. A

DOI: 10.1021/acs.est.5b05357
Environ. Sci. Technol. 2016, 50, 3754−3761

similar strategy was used for coating haptens **T8** and **T9** (Figure 1) using compounds **18** and **20** with unsaturated linkers. All four haptens were coupled to proteins using the *N*-hydroxysuccinimide method.[22]

The haptens **T1** and **T5** described in Schemes 1 and 5 were alkylated to obtain different lengths of a single-bonded hydrocarbon linker at the 2′-OH position of the TCS molecule. Ethyl bromobutyrate or ethyl bromohexanoate was used for the carbon linker attachment on the TCS structure. More detailed synthetic procedures are provided in the Supporting Information.

Preparation of Immunogen and Coating Antigens. For haptens containing a −COOH group, conjugation to proteins was made using the *N*-hydroxysuccinimide (NHS),[22] sulfo-NHS carbodiimide,[22] or mixed anhydride method.[23] Haptens containing an $-NH_2$ group were conjugated by a diazotization method.[22] Haptens **T1**, **T2**, and **T3** (Figure 1) conjugated to Tg were used as immunogens. Haptens **T1**−**T9** were conjugated to bovine serum albumin (BSA) for use as coating antigens. Further hapten−protein conjugation details can be found in the Supporting Information.

Immunization and Antiserum Preparation. The immunization was performed according to the procedure reported previously.[24] For each immunogen, three female New Zealand white rabbits were immunized (rabbits 1288, 1289, and 1290 against hapten **T1**−Tg, rabbits 1154, 1155, and 1156 against hapten **T2**−Tg, and rabbits 1157, 1158, and 1159 against hapten **T3**−Tg). After seven boosts, a final serum was collected about 5 months following the first immunization. Antiserum was obtained by centrifugation, stored at −20 °C, and used without purification.

Immunoassay. The ELISA was performed according to the procedure described previously.[24] In this format, the hapten−protein conjugate is coated to the well of the microtiter plate. After incubation with analyte and primary antibody, unbound primary antibody is washed away. The remaining primary antibody is detected using a secondary antibody that is conjugated to an enzyme. The IC_{50} value, an expression of the sensitivity of the immunoassay, and the limit of detection (LOD), defined as the IC_{10} value, were obtained from a four-parameter logistic equation. Borosilicate glass tubes were used to prepare standard and sample solutions.

Cross-reactivity. The cross-reactivity studies were evaluated using compounds that are structurally similar to TCS. The cross-reactivity was calculated as the (IC_{50} of the target analyte/IC_{50} of the tested compound) × 100.

Preparation of Water Samples. Water samples used for TCS analysis were taken from a fish exposure study representative of U.S. Environmental Protection Agency (USEPA) standard exposure procedures.[25] Water samples used in the fish exposure study were prepared using a single batch of deionized water adjusted to USEPA moderately hard standards (pH 7.4−7.8; hardness 80−100 mg/L; alkalinity 57−64 mg/L).[26] Aliquots of the adjusted water were added to beakers and then spiked with 100 μL of concentrated stock solutions of TCS in methanol to obtain TCS concentrations of 0−300 μg/L with a final concentration of 0.01% methanol. Aliquots from each beaker were taken for analysis prior to the addition of fish. For analysis, water samples were diluted 5−25 times with 10% methanol in phosphate-buffered saline (PBS) buffer to bring the absorbance values within the linear range for immunoassay. For LC−MS/MS analysis, water samples were extracted in triplicate by solid-phase extraction (SPE) as detailed in the Supporting Information.

Analysis of Biosolid Samples. Biosolid samples were collected from a regional wastewater-treatment plant that processes about 140 million gallons of wastewater daily. At the time of sample collection, this facility diverted about 20% of the total sewage sludge to the production of biosolids for use as fertilizer, while the remainder was disposed into unlined dedicated land disposal areas. Samples were obtained from the dedicated land disposal area and were not further characterized. The samples were prepared according to the method of Ogunyoku and Young.[27] Briefly, samples were dried at 70 °C for 24 h and homogenized with a mortar and pestle. For analysis by immunoassay, 15 mL of a mixture of methanol and acetone (1:1, v/v) were added to 1 g of dried sample. The mixture was shaken for 24 h at 210 rpm at 60 °C and centrifuged for 30 min at 4000 rpm. The extract was further diluted 375−3000 times with 10% methanol in PBS buffer prior to the immunoassay. For LC−-MS/MS analysis, the dried biosolid samples (1.0 g) were extracted with methanol using a reflux apparatus. The extract was purified by SPE prior to using the LC−MS/MS analysis method of Ogunyoku and Young.[27] (see the Supporting Information for details).

RESULTS AND DISCUSSION

Hapten Synthesis. Novel immunizing haptens that mimic the whole TCS molecule were designed and synthesized (Figure 1). Other researchers have designed haptens that linked the TCS molecule to carrier proteins through the 2′-OH group.[28] Our design focused on utilizing linkers that allowed the 2′-OH group to remain free, as it is in the parent compound, because the 2′-OH is one element that distinguishes TCS from other diphenyl ethers. Another study explored using haptens that mimic only part of the TCS molecule but found that titers were low to these fragmentary haptens.[19] Although we and others have utilized this fragmentary strategy successfully for polybrominated diphenyl ethers and polychlorinated biphenyls,[14,29] for this study we focused on haptens that mimic the whole molecule for immunization.

In a competitive ELISA, reducing the apparent affinity of the antibody for the coating antigen relative to its affinity for TCS usually results in more sensitive assays. Our coating hapten design utilized several strategies for achieving this goal. For example, haptens **T7**, **T8**, and **T9** contain linkers that are unsaturated, and immunizing haptens contained linkers that were saturated. All coating haptens contained substitutions at the 2′-OH position, while immunizing haptens retained the 2′-OH. Linkers were attached at the 2′- or 4′- positions in coating haptens but were in the 4- position for immunizing haptens. Finally, haptens where the 2′-OH was substituted with Cl or Br were also synthesized. Such changes in linker composition, linker location, and substitutions at key positions provide a library of coating antigens that can greatly improve the chances of developing a highly sensitive and selective immunoassay.[30−32] The structural characterization of all haptens is provided in the Supporting Information.

Antibody Characterization. The titer of the antisera collected after each boost was determined by the homologous indirect ELISA. All of the antisera showed relatively high constant titers after the fifth immunization and no significant affinity for BSA alone. All nine antisera were then screened for inhibition against all coating antigens at two concentrations (5

DOI: 10.1021/acs.est.5b05357
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Table 1. Characteristics of the ELISA Using Various Combinations of Antiserum and Coating Antigen

format	immunogen	antiserum no.	coating antigen					A/D
				A_{max} [a]	slope	IC_{50} (μg/L)	A_{min}	
homologous	T1-Thy	1288	T1-BSA	1.03	1.00	788	0.17	6
heterologous	T1-Thy	1288	B1-BSA[b]	0.78	0.94	49.1	0.06	14
heterologous	T3-Thy	1158	T2-BSA	1.11	1.45	1240	0.31	4
heterologous	T3-Thy	1158	B1-BSA[b]	1.26	1.58	488	0.04	33
heterologous	T3-Thy	1158	B2-BSA[b]	1.11	1.45	1240	0.31	
homologous	T2-Thy	1155	T2-BSA	0.26	0.40	1950	−0.02	16
heterologous	T2-Thy	1155	T3-BSA	0.75	0.24	1590	−0.24	3
heterologous	T2-Thy	1155	B1-BSA[b]	0.72	0.71	15.4	0.23	3
heterologous	T2-Thy	1155	B2-BSA[b]	0.92	0.68	20.7	0.28	3
heterologous	T2-Thy	1155	T6-BSA	0.34	0.69	3.8	0.05	6
heterologous	T2-Thy	1155	T8-BSA	0.86	0.60	1.5	0.07	13
heterologous	T2-Thy	1155	T7-BSA	0.69	0.21	8010	−0.01	62
heterologous	T2-Thy	1155	T9-BSA	0.89	0.86	2.0	0.07	12
homologous	T2-Thy	1156	T2-BSA	0.16	0.98	8	0.05	3
heterologous	T2-Thy	1156	T3-BSA	0.36	1.06	24	0.06	6
heterologous	T2-Thy	1156	B1-BSA[b]	0.12	0.54	327	0.02	6

[a]Parameters calculated from a four-parameter logistic fit of a calibration curve. [b]From Ahn et al.[14]

and 500 μg/L) of TCS. Antisera/coating antigen combinations that showed over 50% inhibition at 500 μg/L and over 20% inhibition at 5 μg/L were rescreened at 10 concentrations of TCS ranging from 0.003 to 5000 μg/L.

All antisera−coating antigen combinations that were homologous assays had IC_{50} values above 750 μg/L or, in the case of antiserum no. 1156, exhibited low binding, as demonstrated by the low A_{max}. Among the heterologous antisera−coating antigen assays, those utilizing antisera against hapten **T1** (no. 1288) or **T3** (no. 1158) had higher IC_{50} values, while antiserum against hapten **T2** (no. 1155 and no. 1156) had mixed results depending upon the coating antigen used (Table 1). The assay demonstrating the lowest IC_{50} used antiserum no. 1155 that was raised against immunizing hapten **T2** and coating antigen **T8**-BSA. Similar to the results from Brun and colleagues,[19] the best immunogen identified was one that contained a nitrogen as the first atom on the linking arm.

Haptens **B1** and **B2** that replace chlorine substituents of TCS hapten **T8** by bromine atoms were synthesized to develop a BDE immunoassay (Figure 1).[14] These haptens were screened to introduce additional heterology in the coating antigen. A similar strategy was used for an earlier TCS assay[28] and for an assay for deltamethrin.[33] For antiserum no. 1288 (raised against **T1**-Tg) and antiserum no. 1158 (raised against **T3**-Tg), the use of **B1**-BSA resulted in improved sensitivity compared to the best TCS-based coating antigen for each antibody. For antiserum no. 1155, both **B1**- and **B2**-BSA resulted in sensitive assays but were not superior to assays utilizing TCS haptens **T8** and **T9**. Among the combinations of **B1**-BSA−antiserum no. 1288, **B1**-BSA−antiserum no. 1158, and **B1**-BSA−antiserum no. 1155, the latter was selected as one of the assays used in further assay development because it had the lowest overall IC_{50} among the assays utilizing brominated coating antigens (Table 1).

Unique coating haptens in which the hydroxyl group was replaced by a Cl atom (haptens **T6**, **T8**, and **T9**) for the heterologous competition format resulted in remarkably increased assay sensitivity. Although the antibody (no. 1155) bound these coating haptens with moderate affinity, the affinity for free TCS was greater, allowing the displacement of the antibody by low concentrations of TCS resulting in a sensitive assay. Conversely, the assay that used hapten **T7** that contained a $-OCH_3$ group in place of the −OH was not sensitive, indicating that the affinity of the antibody for the $-OCH_3$ group was greater than for the −OH. In conclusion, the coating antigen, hapten **T9**-BSA (where the hydroxyl group was replaced by a Cl atom and a medium-length rigid carbon linker was included), along with antibody no. 1155, was selected for further assay development due to its high maximum signal, high signal-to-noise ratio, steep slope, and low IC_{50} value.

Optimization. Utilizing immunizing haptens **T1** and **T2** resulted in reasonable antibody affinity to the target analyte in a competitive assay format and rabbit antiserum no. 1155 generated against **T2** provided the lowest IC_{50} (Table 1). Hapten **T9**-BSA and antiserum no. 1155 was found to be the best combination and was used for these optimization studies. Methanol (MeOH) is generally used as a cosolvent in the assay buffer (PBS) to ensure the solubility of lipophilic analytes. As seen in Table 2, because lower amounts of MeOH provided better sensitivity, 10% MeOH was selected for the further assay development. The IC_{50} was not significantly affected by pH values ranging from 5.5 to 8.5 in the buffer, but the maximum

Table 2. Effect of pH or Solvent on the Assay Sensitivity

	parameters derived from the four-parameter curve fit[a]				
	A_{max}	slope	IC_{50} (μg/L)	A_{min}	A/D
solvent effect					
10% MeOH	0.896	1.07	63.0	0.132	7
20% MeOH	0.985	0.89	67.5	0.130	8
40% MeOH	0.997	0.94	120	0.135	7
pH effect					
pH 5.5	1.10	1.12	2.1	0.088	13
pH 7.4	1.11	1.02	1.6	0.103	11
pH 8.5	0.940	0.81	1.2	0.059	16
pH 10.7	0.789	0.83	1.6	0.076	10

[a]A_{max} is the absorbance observed in the absence of analyte, IC_{50} is the calculated valued described as the concentration resulting in a 50% decrease in maximum signal, and A_{min} is the absorbance observed at maximum inhibition of signal. Hapten B1-BSA and antiserum no. 1288 were used for the analysis of methanol effect.

DOI: 10.1021/acs.est.5b05357
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Coating antigen	Immunogen	Antibody	Assay buffer	Secondary antibody
1 μg/mL (T9-BSA)	T2-Thy	Antiserum #1155 (1/5,000 in PBST)	10% MeOH-PBS (pH 8.4)	1/3,000 in 0.2 % BSA in PBS

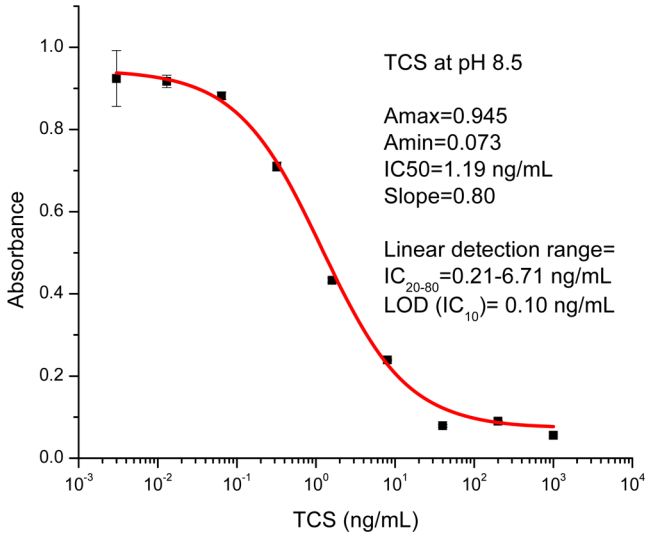
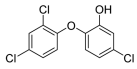
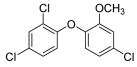
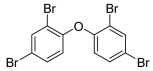
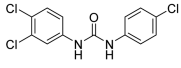
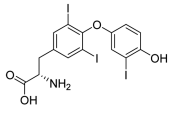
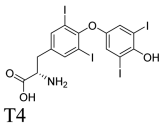


Figure 2. Optimized conditions and standard curve for TCS immunoassay.

Table 3. Cross-Reactivity (%) of Compounds Structurally Related to TCS

Compound	Combination of Cag Hapten T9/antiserum #1155	Combination of Cag B1-BSA/antiserum #1288
Triclosan 	100	100
Methyl triclosan 	3	107
BDE-47 	4	140
Triclocarban 	NI[a]	Not tested
T3 Na salt 	0.05	Not tested
T4 	0.04	Not tested

[a]NI = no inhibition at 5000 μg/L.

DOI: 10.1021/acs.est.5b05357
Environ. Sci. Technol. 2016, 50, 3754−3761

absorbance signals varied. A pH 8.5 PBS assay buffer containing 10% MeOH was selected for further experiments because the assay had the lowest IC_{50} and a good ratio of maximum to minimum signal (A/D) of 16. Because the maximum absorbance signals are generally significantly reduced by higher ionic strength (2× or 4× PBS) due to the suppression of the binding interaction of antibody to antigen, 1× PBS (0.15 M) was used for the assay.

The final assay conditions were as follows: Primary antiserum no. 1155 was diluted in PBST (PBS containing 0.05% Tween 20). The optimized ELISA used a coating antigen of **T9**-BSA at a concentration of 1 μg/mL and antiserum no. 1155 produced against hapten **T2**-Tg at a dilution of 1/5000 in PBST before the competition. The plate coated with the coating antigen was blocked with 0.5% BSA. The assay buffer was 10% MeOH in 0.15 M PBS, pH 8.5. Under these conditions, the assay had a linear range (IC_{20-80}) of 0.2−6.7 μg/L of the target and an IC_{50} value of 1.2 μg/L. The LOD in buffer was 0.1 μg/L, the IC_{10} value (Figure 2). This assay is comparable in sensitivity to the formats reported earlier that exhibited IC_{50} values for TCS of 3.85[19] and 0.25 μg/L[18]

Cross-reactivity. TCS metabolites and its structural analogs were evaluated for cross-reactivity. The assay was selective for the target analyte TCS. Cross-reactivities to methyl TCS (2,4,4′-trichloro-2′-methoxydiphenyl ether) and BDE-47 (2,2′,4,4′-tetrabromodiphenyl ether) were 3 and 4%, respectively, while the antimicrobial triclocarban and two thyroid hormones cross-reacted <1% (Table 3). These cross-reactivity patterns are very similar to the assay described by Brun et al.,[19] and the assay by Shelver[18] cross-reacted with methyl-TCS (312%) and BDE congeners (2.5−64%). It is likely that both the phenyl and hydroxyl are important for binding by the antibody that was generated against immunizing hapten **T2**, where the −OH was exposed, and explains the similar findings by Brun et al. in which the phenyl and hydroxyl are also exposed.[19] The antibody generated against hapten **T1**, where the −OH group of TCS was alkylated to $-OCH_2(CH_2)_2-$COOH, highly recognized methyl TCS and BDE-47, and showed less specificity to TCS due to the lack of H-bonding donation, which is consistent with earlier findings using a similar immunizing hapten conjugated through the hydroxyl.[18]

Analysis of Fish Exposure and Biosolid Samples. Water samples that were used to test the toxicity of TCS to fish were diluted 5-fold at low concentrations and 25-fold at high concentrations prior to the immunoassay to bring the samples into the linear range of the assay. Recoveries of 85−92% were obtained from both the immunoassay and LC−MS/MS analysis. The recoveries obtained using the immunoassay method are comparable to other studies in which fortified mineral and wastewater samples were analyzed without cleanup,[18,19] having reported recoveries ranging from 75 to 113%. Similarly, recoveries above 85% were reported for LC−MS/MS methods that utilized solid-phase extraction.[34,35] An average ratio between instrumental analysis and immunoassay results was less than 1.2 (Table 4).

Analysis of biosolid samples can be challenging because of the complexity of the samples; thus, multistep sample extraction and cleanup methods are necessary,[36] using techniques such as accelerated solvent extraction coupled to solid-phase cleanup.[37] Immunoassays are often advantageous because the antibody provides some selectivity for the analyte in the presence of matrix, eliminating the need for exhaustive cleanup before analysis.[38] Our goal was to use a simple, field-portable sample preparation method; thus, samples were extracted with methanol−acetone and shaking. For instrumental analysis, the samples were extracted using a methanol reflux method described in the Supporting Information. The LC−MS/MS extraction and analysis showed higher levels of TCS than the ELISA method. Because the sample preparation methods were not identical, it is not known whether the difference is due to extraction efficiency or a matrix effect on either or both detection methods. Nevertheless, the concentrations of TCS found in these biosolid samples were within the wide range of TCS concentrations found in other biosolids.[39] The average ratio between the results of both methods was less than 1.6 (Table 5), indicating that this immunoassay is suitable for the determination of TCS in biosolid samples as an alarm or primary screening method.

Table 4. TCS Concentrations in Water Samples by ELISA and LC−MS/MS

	TCS measured (μg/L)		
TCS added (μg/L)	immunoassay	LC−MS/MS	ratio (LC−MS/MS/ immunoassay)
0	0.38 ± 0.14	0.54 ± 0.08	1.42
3	2.36 ± 0.70	3.05 ± 0.91	1.29
30	26.9 ± 3.2	25.6 ± 1.07	0.95
100	73.6 ± 13.0	84.0 ± 1.31	1.14
300	298 ± 20.4	287.6 ± 6.45	0.96
average recovery (%)	85.3 ± 11.6	91.7 ± 8.47	1.15 ± 0.20

Samples containing spiked concentrations of 0−3 μg/L were diluted five times in the assay buffer prior to analysis; samples containing 100−300 μg/L were diluted 25 times in the assay buffer.

Table 5. TCS Concentrations in Biosolid Samples by ELISA and LC−MS/MS

	TCS concentration determined in biosolids (ng/g dry weight)		
sample	immunoassay	LC−MS/MS	ratio (LC−MS/MS/ Immunoassay)
DLD 2	966 ± 68	1720 ± 247	1.78
DLD 3	392 ± 39	521 ± 102	1.33
DLD 4	489 ± 20	826 ± 223	1.69
average			1.60 ± 0.24

Immunoassay data is the mean of triplicate extractions. LC−MS/MS data is the mean of two replicates.

In conclusion, for the development of a sensitive and selective immunoassay for TCS, a hapten preserving the 2′-OH group in a near-perfect molecular mimic of TCS aids in the selective recognition of the TCS by the resulting antibody. A unique feature of this immunoassay is the use of a novel heterologous coating hapten, where the −OH was substituted with a −Cl. This resulted in a decreased affinity of the antibody for the coating antigen and subsequently increased the ability of the analyte to compete resulting in the highest sensitivity assay. This immunoassay was more sensitive (IC_{50} value of 1.2 μg/L) compared to the TCS assay developed by Brun et al. (3.85 μg/L)[19] but less sensitive than the existing Abraxis assay kit for TCS (0.25 μg/L, http://www.abraxiskits.com). Moreover, this immunoassay utilizes less primary antibody than the previously described assays.[18,19] Furthermore, the results from this assay were in agreement with the LC−MS/MS method for testing

DOI: 10.1021/acs.est.5b05357
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environmental samples with fewer, simpler extractions steps needed and is the first report of the analysis of TCS in biosolids by immunoassay.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b05357.

Additional details include schemes and experimental details on hapten synthesis, immunization, coupling methods and an instrumental analysis of water and biosolid samples. (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was supported in part by the National Institute of Environmental Health Sciences Superfund Basic Research Program, P42 ES04699, the UC Davis Environmental Health Sciences Core Center, P30 ES023513, and the National Institute of Occupational Safety and Health Western Center for Agricultural Health and Safety, 2U54 OH007550.

■ ABBREVIATIONS

AhR	aryl hydrocarbon receptor
BDE	polybrominated diphenyl ether
BSA	bovine serum albumin
ECD	electron-capture detector
ELISA	enzyme linked immunosorbent assay
GAR-HRP	goat antirabbit IgG peroxidase conjugate
GC	gas−liquid chromatography
HPLC	high-performance liquid chromatography
LC−MS/MS	liquid chromatography tandem mass spectrometry
LOD	limit of detection
MeOH	methanol
MS	mass spectrometry
NHS	*N*-hydroxysuccinimide
PBS	phosphate buffered saline
PBST	phosphate buffered saline containing 0.05% Tween 20
SPE	solid-phase extraction
TCS	triclosan
Tg	thyroglobulin
TMB	3,3′5,5′-tetramethylbenzidine

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