



# Synthesis of haptens and selective enzyme-linked immunosorbent assay of octachlorostyrene<sup>☆</sup>



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## ABSTRACT

A sensitive, competitive indirect enzyme-linked immunosorbent assay (ELISA) was developed for the detection of octachlorostyrene (OCS), a persistent and bioaccumulative toxicant. To achieve the most sensitive antibody, several haptens with different linkers that simulated the special structure of OCS were synthesized and conjugated to carrier proteins. Polyclonal rabbit antibodies against different immunizing antigens were obtained and screened against different coating antigens. Under the optimized conditions, this indirect ELISA shows a linear detection range from 1.4 to 86.3 ng/mL, with an  $IC_{50}$  value of 4.46 ng/mL and a limit of detections (LOD) of 0.1 ng/mL. Twelve kinds of compounds were tested for calculating cross-reactivities, and almost all of them showed little cross-reactivity (< 5%). Water and sera samples spiked with OCS were analyzed by ELISA and the achieved recoveries were satisfied with a mean recovery of 92%. This immunoassay can be used as a rapid and convenient tool to monitoring OCS in environmental samples.

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## 1. Introduction

Octachlorostyrene (OCS), a toxic halogenated aromatic compound, belongs to the primary level I priority of the persistent and bioaccumulative toxicants (PBTs) categorized by the US Environmental Protection Agency (EPA). OCS is not commercially manufactured but to be an inadvertent byproduct of processes that combine carbon and chlorine at high temperature [1]. Since firstly being found in dead cormorants [2], OCS has been occasionally detected in environmental samples such as sediments, fish, marine mammals and even in human being [3–8].

Concerns over the occurrence of OCS in the environment are probably caused by two main factors: its persistence and its high bioaccumulation. Judging from its special chemical structure, it is not difficult to see that OCS is a fully chlorinated aromatic compound and is likely to be a highly persistent substance. The half-life time of OCS in liver of the artificially raised rainbow trout was nearly twice longer as that of hexachlorobenzene, and the elimination half-life time of OCS in yellow eel was 1.7 and 2.3 fold, respectively, longer as that of hexachlorobenzene

and pentachlorobenzene under natural conditions [4,8]. Moreover, the very low water solubility and the bioconcentration factor ranging from 8100 to 1,400,000 implied that OCS could bioaccumulate easily in aquatic food webs and would have an influence on aquatic organisms [9,10]. In 1984, Kamlnsky et. al [3] conducted a study about the sources of OCS in Lake Ontario and analyzed sediments of different places. OCS was detected in 8 of the 11 cores, and its concentration ranged from 20 ng/g to 140 ng/g (dry weight). By an investigation of fish and fish oils of the North Atlantic [7], OCS and its homologs were quantified. OCS and (E)- $\beta$ ,2,3,4,5,6-hexachlorostyrene were dominating in fish samples, with concentration of 0.49–24.0 ng/g fat and 0.65–41.0 ng/g fat, respectively. In 1992, OCS was detected in 7% of the breast milk samples collected from 497 Canadian women, and the mean concentration of OCS in all samples was 0.05 ng/g breast milk [5]. Seldén [6] conducted an analysis about the plasma of Sweden workers who had the past experience of degassing with hexachloroethane in an aluminum foundry. The mean concentrations of OCS in their plasma was found at 54.6 ng/g (lipid), nearly 78 folds higher than that in the control group and the OCS concentration increased with the duration of exposure to hexachloroethane. Besides the urinary porphyrin excretion of these workers was about 2 fold higher than that in the control group, which probably indicated the toxic effects of OCS on porphyrin metabolism of human [11].

Regarding above researches, a routine assay for screening and monitoring OCS in environmental or biological samples is of increasing significance and necessity. Current analytical methods

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for detection of OCS are mainly based on gas liquid chromatography (GC), GC–MS, or high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS) [2,4–8]. No immunoassay method was reported for the detection of OCS for the reason of probably shorting of the antibody. Comparing with these instrumental methods, immunoassay methods are well-known for their selectivity, detectability, reliability, analysis speed, less pretreatment and low cost. Besides, they offer an easy way to simultaneously detect a single analyte of interest in many samples. So immunoassay methods have been widely used in the field of clinical diagnostics, environmental monitoring, food quality, agriculture and testing of personnel exposed to toxic chemicals [12–15].

The aim of this study was to develop an ELISA for analysis of OCS based on polyclonal antibodies. Thus, the synthesis of new haptens to elicit antibodies, the characterization of antibodies and the analysis of OCS in water and sera samples are described. To our knowledge, this is the first paper reporting the method for detection of OCS in samples by immunoassay method.

## 2. Materials and methods

### 2.1. Chemicals, apparatus, buffers and solutions

#### 2.1.1. Chemicals and apparatus

Bovine serum albumin (BSA), ovalbumin (OVA), Goat anti-rabbit IgG peroxidase conjugate, Tween 20, 3,3',5,5'-tetramethylbenzidine (TMB), incomplete Freund's adjuvant and complete Freund's adjuvant were purchased from Sigma-Aldrich Company. Hexachlorobenzene, octachlorostyrene (10 ng/ $\mu$ L), pentachloronitrobenzene and  $\text{Pd}(\text{PPh}_3)_4$  were purchased from J&K Company. Propargyl alcohol and 3-mercaptopropanoic acid was obtained

from Alfa Aesar Company. Flash column chromatography was performed on 300–400 mesh silica gel.

#### 2.1.2. Buffers and solutions

The buffers used in this study were as follows: (1) phosphate-buffered saline (PBS, pH 7.4): 138 mM NaCl, 1.5 mM  $\text{KH}_2\text{PO}_4$ , 7 mM  $\text{Na}_2\text{HPO}_4$  and 2.7 mM KCl; (2) washing buffer (PBST): a PBS solution containing 0.05% (v/v) of Tween 20; (3) coating buffer (0.05 M carbonate buffer): 15 mM  $\text{Na}_2\text{CO}_3$  and 35 mM  $\text{NaHCO}_3$ , pH 9.6; (4) blocking buffer: PBS mixed with 1% of OVA and 0.05% (v/v) Tween 20; (5) substrate buffer ( $\text{TMB} + \text{H}_2\text{O}_2$ ): 400 mL of 0.6% TMB–DMSO mixed with 100 mL of 1%  $\text{H}_2\text{O}_2$  in citrate–acetate buffer (pH 5.5); (6) enzymatic stopping solution: 2.0 M  $\text{H}_2\text{SO}_4$ ; and (7) OCS standard solution: the octachlorostyrene dissolved with  $\text{CH}_3\text{CN}$  of different volumes to get the standard solutions at different concentrations.

### 2.2. Instrumentation

All melting points were determined with a Taike XT-4 micro-melting-point apparatus.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded on a Varian INOVA-400 instrument using tetramethylsilane (TMS) as internal standard. Mass spectra were obtained by SHIMADZU GC-17 QP-5000 or ThermoFinnigan MAT95XP. Ultraviolet–visible (UV–vis) spectra were obtained by UV-2100 spectrophotometer (LabTech). Polystyrene microtiter plates (96-well) were purchased from Jet Biofil Company. Absorbances were measured in a microtiter plate reader (ELx800, BioTek Company Limited). Immunoassay competitive curves were mathematically analyzed by the software of Origin 8.5. GC analysis was conducted by GC-2010 with a RTX-5 column (SHIMADZU).

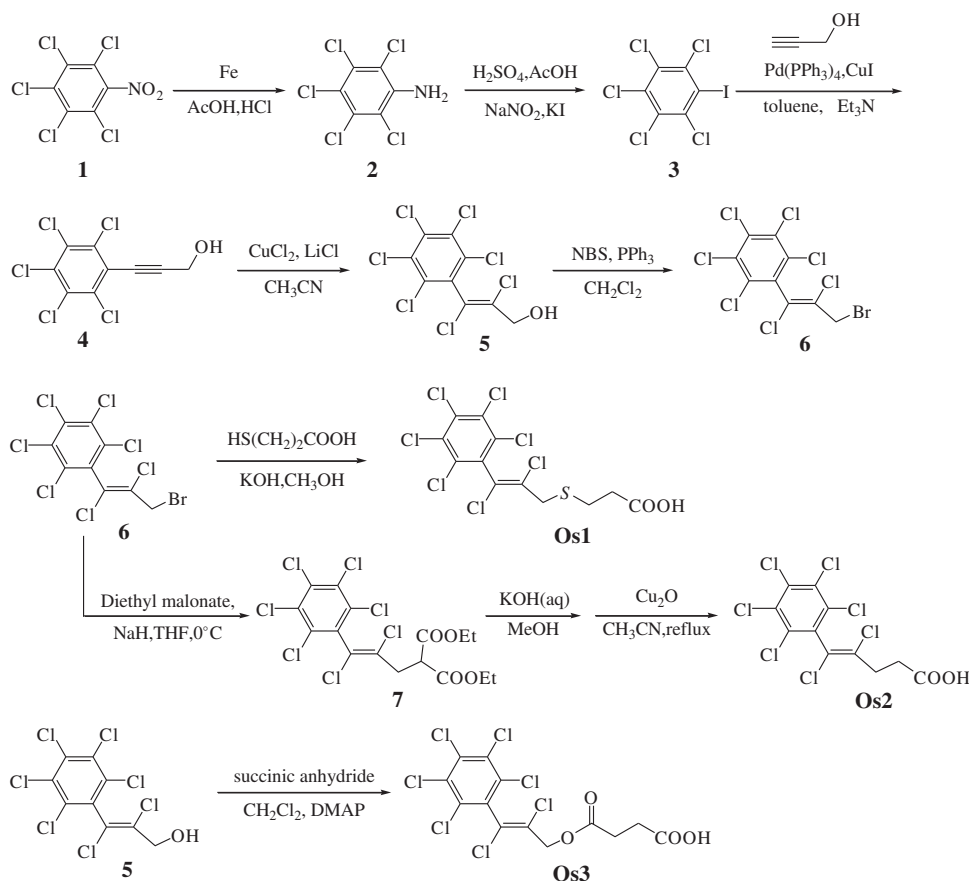


Fig. 1. Synthetic routes of three haptens of octachlorostyrene.

### 2.3. Hapten synthesis

In order to synthesize the immunogen and coating antigen, three haptens were designed and synthesized (Fig. 1) and the spectroscopic data of these compounds are provided (Table 1).

#### 2.3.1. Pentachloroaniline (**2**)

Iron powder (9.77 g, 174.5 mmol) and a mixture of acid [20 mL, V (acetic acid): V (conc. HCl)=9: 1] were added to a solution of pentachloronitrobenzene (10.3 g, 34.88 mmol) in AcOEt (25 mL). The reaction mixture was refluxed for 2 h and basified to pH 10 with NaOH. Then the insoluble solids were filtered off, washed with AcOEt. And the combined organic filtrates were washed with brine and dried (anhydrous Na<sub>2</sub>SO<sub>4</sub>). After evaporation of solvent, the pentachloroaniline was obtained as a white solid (8.95 g, yield: 97%).

#### 2.3.2. Pentachloriodobenzene (**3**)

NaNO<sub>2</sub> (2.49 g, 36.09 mmol) was added carefully to conc. Sulfuric acid (20 mL) at 0 °C, and then the mixture was added slowly to a suspended solution of pentachloroaniline (8.70 g, 32.79 mmol) in acetic acid (100 mL). The resulting solution was stirred at room temperature for another 1.5 h and followed by addition of KI solution (10.88 g, 65.54 mmol). The reaction solution was warmed up to 60 °C and stirred for 12 h. A yellow precipitate was formed, and then this solid was filtered, washed with water, dried in vacuo. The crude product was obtained as a pale yellow solid and the crude product was purified by column chromatography (petroleum ether) to yield pentachloriodobenzene as a white solid (9.87 g, yield: 80%).

#### 2.3.3. 3-(Pentachlorophenyl)prop-2-yn-1-ol (**4**)

Under an atmosphere of nitrogen and at 80–85 °C, Et<sub>3</sub>N (6 mL) and toluene (30 mL) were added to a mixture of pentachloriodobenzene (2.60 g, 6.91 mmol), Pd(PPh<sub>3</sub>)<sub>4</sub> (201 mg, 0.17 mmol), and CuI (38 mg, 0.20 mmol). Then a solution of propargyl alcohol (1.16 g, 20.71 mmol) in toluene (5 mL) was added dropwisely to the reaction mixture during 3 h. After addition, the reaction mixture was stirred for another 12 h at this temperature. Then the reaction mixture was filtered, concentrated in vacuo, and the residue was purified by column chromatography (petroleum

ether–AcOEt, 30:1 v/v) to give 3-(pentachlorophenyl)prop-2-yn-1-ol as a white solid (1.73 g, yield: 82%).

#### 2.3.4. (E)-2,3-Dichloro-3-(pentachlorophenyl)prop-2-en-1-ol (**5**)

Anhydrous CuCl<sub>2</sub> (14.22 g, 105.8 mmol) and LiCl (4.49 g, 105.8 mmol) were added to a solution of **4** (1.61 g, 5.29 mmol) in acetonitrile (35 mL) in on portion, and the mixture was refluxed for 24 h. Anhydrous CuCl<sub>2</sub> (14.22 g, 105.8 mmol) and LiCl (4.49 g, 105.8 mmol) were added again to the mixture which was refluxed for another 24 h. Then the organic solvent was evaporated and CH<sub>2</sub>Cl<sub>2</sub> was added. The insoluble salts were filtered off and the filtrate was washed with saturated NH<sub>4</sub>Cl, dried (Na<sub>2</sub>SO<sub>4</sub>). The solvent was filtered and evaporated, and the residue was purified by column chromatography (petroleum ether–AcOEt, 30:1 v/v) to give (E)-2,3-dichloro-3-(pentachlorophenyl)prop-2-en-1-ol as a white solid (1.58 g, yield: 80%).

#### 2.3.4. (E)-1,2-Dichloro-1-(pentachlorophenyl)-3-bromo-1-propylene (**6**)

**5** (590 mg, 1.57 mmol) and triphenylphosphine (700 mg, 2.67 mmol) were dissolved by CH<sub>2</sub>Cl<sub>2</sub> (14 mL), and NBS (420 mg, 2.36 mmol) was added in portions during 15 min at 0 °C. After addition, the reaction mixture was stirred for 30 min at 0 °C, and then warmed up to room temperature and stirred for 1 h. Then the organic solvent was washed with water, dried (Na<sub>2</sub>SO<sub>4</sub>), filtered and evaporated. The residue was purified by column chromatography (petroleum ether) to give (E)-1,2-dichloro-1-(pentachlorophenyl)-3-bromo-1-propylene as a white solid (633 mg, yield: 92%).

#### 2.3.5. 3-((E)-2,3-dichloro-3-(pentachlorophenyl)allylthio)propanoic acid (hapten **Os1**)

Under an atmosphere of nitrogen and at 0 °C, a solution of KOH (39 mg, 0.69 mmol) in methanol (3 mL) was added dropwisely to 3-mercaptopropanoic acid (37 mg, 0.37 mmol) in methanol (1.5 mL). Then the solution of **6** (151 mg, 0.34 mmol) in methanol (3 mL) was added dropwisely to the mixture. After being stirred at 50 °C for 2 h, the mixture was concentrated in vacuo, acidified with diluted HCl, and extracted with AcOEt. The solvent was dried (Na<sub>2</sub>SO<sub>4</sub>), filtered and evaporated. The residue was purified by

**Table 1**  
Spectroscopic Data of Compounds.

Compound	Spectroscopic data
<b>2</b>	<sup>1</sup> H NMR (400 MHz, CDCl <sub>3</sub> ) δ 4.76 (s, 2H, NH <sub>2</sub> ). <sup>13</sup> C NMR (100 MHz, CDCl <sub>3</sub> ) δ 116.84 (C), 120.90 (C), 131.15 (C), 140.57 (C); MS (EI) <i>m/z</i> (%) 262.8 (M <sup>+</sup> , 66), 264.8 (M <sup>+</sup> +2, 100), 266.8 (M <sup>+</sup> +4, 67), 268.8 (M <sup>+</sup> +6, 20).
<b>3</b>	<sup>13</sup> C NMR (100 MHz, CDCl <sub>3</sub> ) δ 103.11 (C), 130.54 (C), 134.01 (C), 138.00 (C); MS (EI) <i>m/z</i> (%) 376.0 (M <sup>+</sup> +2, 100), 378.0 (M <sup>+</sup> +4, 64), 380.0 (M <sup>+</sup> +6, 20), 382.0 (M <sup>+</sup> +8, 3).
<b>4</b>	<sup>1</sup> H NMR (400 MHz, CDCl <sub>3</sub> ) δ 1.82 (t, 1H, <i>J</i> =6.40 Hz, OH), 4.62 (d, 2H, <i>J</i> =6.00 Hz, OCH <sub>2</sub> ); <sup>13</sup> C NMR (100 MHz, CDCl <sub>3</sub> ) δ 51.67 (CH <sub>2</sub> ), 79.39 (C≡C), 100.01 (C≡C), 123.33 (C), 131.89 (C), 133.84 (C), 134.76 (C); MS (EI) <i>m/z</i> (%) 301.9 (M <sup>+</sup> , 21), 303.9 (M <sup>+</sup> +2, 31), 305.9 (M <sup>+</sup> +4, 20), 307.9 (M <sup>+</sup> +6, 6), 309.9 (M <sup>+</sup> +8, 2).
<b>5</b>	<sup>1</sup> H NMR (400 MHz, CDCl <sub>3</sub> ) δ 2.13 (t, 1H, <i>J</i> =6.80 Hz, OH), 4.66 (d, 2H, <i>J</i> =6.40 Hz, OCH <sub>2</sub> ); <sup>13</sup> C NMR (100 MHz, CDCl <sub>3</sub> ) δ 62.41 (CH <sub>2</sub> ), 122.90 (C), 132.20 (C), 132.46 (C), 134.77 (C), 135.16 (C), 135.30 (C); MS (EI) <i>m/z</i> (%) 371.8 (M <sup>+</sup> , 14), 373.8 (M <sup>+</sup> +2, 29), 375.8 (M <sup>+</sup> +4, 28), 377.8 (M <sup>+</sup> +6, 16), 379.8 (M <sup>+</sup> +8, 5).
<b>6</b>	<sup>1</sup> H NMR (400 MHz, CDCl <sub>3</sub> ) δ 4.46 (s, 2H, CH <sub>2</sub> Br); <sup>13</sup> C NMR (100 MHz, CDCl <sub>3</sub> ) δ 30.34 (CH <sub>2</sub> ), 125.49 (C), 132.03 (C), 132.23 (C), 132.52 (C), 134.42 (C), 135.36 (C); MS (EI) <i>m/z</i> (%) 433.8 (M <sup>+</sup> , 7), 435.8 (M <sup>+</sup> +2, 22), 437.7 (M <sup>+</sup> +4, 29), 439.7 (M <sup>+</sup> +6, 21), 441.7 (M <sup>+</sup> +8, 10), 443.7 (M <sup>+</sup> +10, 3), 445.7 (M <sup>+</sup> +12, 1).
<b>Os1</b>	<sup>1</sup> H NMR (400 MHz, CDCl <sub>3</sub> ) δ 2.79 (t, 2H, <i>J</i> =7.20 Hz, CH <sub>2</sub> CO), 2.99 (t, 2H, <i>J</i> =7.20 Hz, SCH <sub>2</sub> ), 3.82 (s, 2H, OCH <sub>2</sub> ); <sup>13</sup> C NMR (100 MHz, DMSO- <i>d</i> <sub>6</sub> ) δ 26.59 (CH <sub>2</sub> ), 34.60 (CH <sub>2</sub> ), 35.31 (CH <sub>2</sub> ), 122.23 (C), 132.05 (C), 132.33 (C), 135.03 (C), 135.28 (C), 135.41 (C), 173.21 (C=O). MS (EI) <i>m/z</i> (%) 459.8 (M <sup>+</sup> , 3), 461.8 (M <sup>+</sup> +2, 7), 463.8 (M <sup>+</sup> +4, 7), 465.8 (M <sup>+</sup> +6, 4), 467.8 (M <sup>+</sup> +8, 2).
<b>7</b>	<sup>1</sup> H NMR (400 MHz, CDCl <sub>3</sub> ) δ 1.30 (t, 6H, <i>J</i> =6.80 Hz, CH <sub>3</sub> ), 3.37 (d, 2H, <i>J</i> =7.60 Hz, CH <sub>2</sub> C(Cl)=), 3.85 (t, 1H, <i>J</i> =7.60 Hz, CH), 4.18–4.30 (m, 4H, OCH <sub>2</sub> ). <sup>13</sup> C NMR (100 MHz, CDCl <sub>3</sub> ) δ 14.09 (CH <sub>3</sub> ), 30.08 (CH <sub>2</sub> ), 49.51 (CH), 62.09 (CH <sub>2</sub> ), 123.77 (C), 132.22 (C), 132.37 (C), 133.00 (C), 134.95 (C), 135.28 (C), 167.90 (C=O); MS (EI) <i>m/z</i> (%) 513.9 (M <sup>+</sup> , 31), 515.9 (M <sup>+</sup> +2, 68), 517.9 (M <sup>+</sup> +4, 63), 519.9 (M <sup>+</sup> +6, 35), 521.9 (M <sup>+</sup> +8, 14), 523.9 (M <sup>+</sup> +10, 3).
<b>Os2</b>	<sup>1</sup> H NMR (400 MHz, CDCl <sub>3</sub> ) δ 2.78 (t, 2H, <i>J</i> =8.00 Hz, CH <sub>2</sub> CO), 3.09 (t, 2H, <i>J</i> =8.00 Hz, CH <sub>2</sub> C(Cl)=); <sup>13</sup> C NMR (100 MHz, CDCl <sub>3</sub> ) δ 30.26 (CH <sub>2</sub> ), 30.69 (CH <sub>2</sub> ), 122.49 (C), 132.30 (C), 132.33 (C), 134.65 (C), 134.89 (C), 135.25 (C), 177.15 (C=O); MS (EI) <i>m/z</i> (%) 413.7 (M <sup>+</sup> , 43), 415.7 (M <sup>+</sup> +2, 69), 417.7 (M <sup>+</sup> +4, 63), 419.7 (M <sup>+</sup> +6, 32).
<b>Os3</b>	<sup>1</sup> H NMR (400 MHz, acetone- <i>d</i> <sub>6</sub> ) δ 2.65–2.69 (m, 2H), 2.72–2.75 (m, 2H), 5.18 (s, 2H); <sup>13</sup> C NMR (100 MHz, acetone- <i>d</i> <sub>6</sub> ) δ 28.99 (CH <sub>2</sub> ), 29.27 (CH <sub>2</sub> ), 63.07 (CH <sub>2</sub> ), 125.60 (C), 132.71 (C), 132.84 (C), 133.12 (C), 135.80 (C), 135.86 (C), 172.15 (C=O), 173.35 (C=O); MS (EI) <i>m/z</i> (%) 472.0 (M <sup>+</sup> , 1), 474.0 (M <sup>+</sup> +2, 2), 476.0 (M <sup>+</sup> +4, 2), 478.0 (M <sup>+</sup> +6, 1).

column chromatography (petroleum ether–AcOEt, 8:1 v/v) to give hapten **Os1** as a white solid (155 mg, yield: 97%).

### 2.3.6. Diethyl 2-((E)-2,3-dichloro-3-(pentachlorophenyl)allyl) malonate (**7**)

Under an atmosphere of nitrogen and at 0 °C, NaH (60%, 33 mg, 0.83 mmol) was added portionwisely to a solution of diethyl malonate (135 mg, 0.84 mmol) in DMF (3 mL). After addition, the mixture was stirred for 15 min, and then a solution of **6** (128 mg, 0.29 mmol) in DMF (3 mL) was added dropwisely to this mixture. After being stirred at 30 °C for 1 h, the mixture was diluted with water and extracted with CH<sub>2</sub>Cl<sub>2</sub>. The organic solvent was washed with water and brine, dried (Na<sub>2</sub>SO<sub>4</sub>), filtered and evaporated. The residue was purified by column chromatography (petroleum ether–AcOEt, 10:1 v/v) to give diethyl 2-((E)-2,3-dichloro-3-(pentachlorophenyl)allyl) -malonate as a white solid (150 mg, yield: 99%).

### 2.3.7. (E)-4,5-Dichloro-5-(pentachlorophenyl)pent-4-enoic acid (**Os2**)

Aqueous KOH (1.0 M, 5 mL) was added to a solution of **7** (126 mg, 0.244 mmol) in methanol (5 mL). After being stirred at 60 °C for 18 h, the reaction mixture was concentrated in vacuo, acidified with diluted HCl and extracted with AcOEt. The organic solvent was dried (Na<sub>2</sub>SO<sub>4</sub>) and filtered. After evaporation of solvent, the crude product 2-((E)-2,3-dichloro-3-(pentachlorophenyl)allyl)malonic acid (90 mg) was obtained, which was used in the next reaction without further purification. Under an atmosphere of nitrogen, Cu<sub>2</sub>O (20 mg, 0.14 mmol) was added to a solution of 2-((E)-2,3-dichloro-3-(pentachlorophenyl)allyl)malonic acid (90 mg, 0.20 mmol) in acetonitrile (2 mL), and the reaction mixture was stirred at 80 °C for 14 h. Then the mixture was concentrated in vacuo, and the residue was purified by column chromatography (petroleum ether–AcOEt, 1:2 v/v) to give hapten **Os2** as a white solid (71 mg, yield of two steps: 59%).

### 2.3.8. Mono-2-(E)-2,3-dichloro-3-(pentachlorophenyl)allyl succinate (hapten **Os3**)

A mixture of **5** (150 mg, 0.40 mmol), succinic anhydride (44 mg, 0.44 mmol) and DMAP (49 mg, 0.40 mmol) were dissolved by dichloromethane (5 mL) and stirred overnight at room temperature. Then the reaction mixture was concentrated and purified by column chromatography (dichloromethane, then AcOEt) to give hapten **Os3** as a white solid (119 mg, yield: 63%).

## 2.4. Preparation of immunizing antigens and coating antigens

Hapten **Os1**, **Os2** and **Os3** were conjugated to BSA for preparing immunizing antigens and OVA for preparing coating antigens. In all cases, these conjugations were finished by the N-Hydroxysuccinimide (NHS) active ester method. Each hapten (20 µmol) was dissolved in dry DMF (0.32 mL) in which NHS (30 µmmol) and dicyclohexylcarbodiimide (DCC, 30 µmol) was then added. After the mixture was stirred overnight at room temperature, the precipitated dicyclohexylurea was removed by centrifugation. Then the active ester (about 0.30 mL) was added slowly to the solution of BSA (53.6 mg) in 0.05 M PBS (8 mL, pH 8) with vigorous stirring at 8 °C. After the reaction mixture was stirred gently at 4 °C for 18 h to complete the conjugation, the mixture was dialyzed (4 °C, 0.01 M PBS buffer at pH 7.4) for 72 h with buffer changed every 12 h. Then the conjugates were lyophilized and stored at –20 °C. Conjugate formation was confirmed by UV spectrophotometer, and the molar ratios were approximately 1/15 to 1/22.

## 2.5. Immunization of rabbits

Two male New Zealand white rabbits were immunized for each immunogen (rabbits 101/102 for immunogen BSA–**Os1**, rabbits 103/104 for immunogen BSA–**Os2**, rabbits 105/106 for immunogen BSA–**Os3**). The immunization procedures were followed the protocol described previously [16,17]. Briefly, each immunogen solution (0.2 mg in 0.5 mL PBS) was suspended in 0.5 mL Freund's complete adjuvant and injected hypodermically into a New Zealand white rabbit. About 21 days latter, the rabbit was boosted at 14-day intervals with the same immunogen solution suspended in 0.5 mL Freund's incomplete adjuvant. After 3 months, if the binding of serial dilutions of the antisera was acceptable, the final blood was collected and the antisera were obtained by centrifugation, stored at –20 °C and used without purification.

## 2.6. Sera evaluation

The library of coating antigens was screened against the various rabbit antisera, and the sensitivity and specificity of antisera were tested analogously to the method reported [18,19]. Serial dilutions of each antisera (1:2000 to 1:1,28,000 in PBST, 50 µL/well) were added to the microtiter plates which was coated with three kinds of coating antigens at different concentrations (0.01–100 µg/mL in coating buffer, 100 µL/well). The plates was incubated overnight at 4 °C and blocked with blocking buffer. Then the plate was washed with PBST for 4 times. A solution of anti-IgG–HRP (1/4000 in PBST) was added to the wells (100 µL/well), and the mixture was incubated at 37 °C for 1 h. Then the plate was washed for five times. Substrate buffer was added and color development was stopped by 2.0 M H<sub>2</sub>SO<sub>4</sub> (50 µL/well). The absorbance was measured at 450 nm. Optimal concentrations for the coated antigens and dilutions of antisera were chosen to produce absorbances around 1.0 unit.

## 2.7. Indirect competitive ELISA procedure

OVA–**Os1** was used as a coating antigen and antisera 104 was chosen for further study, and the competitive ELISA was performed as following. Polystyrene 96-place microwell plates were treated with the coating buffer (10 µg/mL, 100 µL/well) and incubated at 37 °C for 1 h and at 4 °C for 12 h. The plates were then washed for three times and the unbound sites were blocked at 37 °C for 1 h. After another wash step, OCS standard solution at different concentrations (0.001, 0.01, 0.1, 1, 10, 100, and 1000 ng/mL) or sample solution (50 µL/well) in PBS and antisera (50 µL/well, diluted 1/1,28,000 in PBST) were added to wells. Then the wells were incubated 37 °C for 1 h and washed. 100 µL/well of goat anti-rabbit peroxidase-conjugated IgG (diluted at 1:4000) was added. After incubation for 1 h at 37 °C, the plates were washed again for five times. Then, substrate buffer was added and color development was stopped by 2.0 M H<sub>2</sub>SO<sub>4</sub> (50 µL/well). The absorbance was measured at 450 nm.

The standard experiment was performed in quadruplicate and repeated five times. Competitive curves were obtained by plotting absorbance against the logarithm of analyte concentration. The IC<sub>50</sub> value, an expression of the sensitivity of immunoassay, and the limit of detection (LOD) defined as the IC<sub>10</sub> value were obtained from the absorbance (Abs)-antigen concentration data following a four-parameter logistic equation [20]:  $Abs = (A - B) / [1 + (x/C)^D] + B$ , where A is the maximal absorbance; B is the minimum absorbance; C is the concentration producing 50% of the maximal absorbance, i.e. the IC<sub>50</sub> value; and D is the slope near the midpoint inhibition of the sigmoid curve.



## 2.8. Cross-reactivity study

The cross-reactivity (CR) studies were performed by using a set of polyhalogenated aromatic compounds and polycyclic aromatic compounds as interferences. The  $IC_{50}$  value of each compound was determined by the proposed immunoassay and each concentration level was assayed in four replicates. The cross-reactivity values (CR %) were calculated according to the expression:  $CR\% = [IC_{50}(OCS) / IC_{50}(\text{cross-reactant})] \times 100\%$ .

## 2.9. Analysis of tap water, Xiang river water and sera of rabbit and human

Both of the tap water and the Xiang River water were collected in Hunan University with glass flasks (500 mL) in November 22 of 2012. The human sera samples were collected from the Hospital of Hunan University and the rabbit sera samples were from the rabbits which we raised at our laboratory. These samples were stored at 4 °C immediately after sampling and did not contain OCS by analysis with both the proposed ELISA and GC. The water samples and the sera samples were filtrated with micro filtration membranes, and then spiked with OCS standard solution to obtain the samples containing OCS at the concentrations of 2.5, 5.0, 10, 25, 50 ng/mL. Following this, 50  $\mu$ L of each sample was added directly to the plates and analyzed by the proposed ELISA as described in Section 2.7.

## 2.10. GC analysis

The water samples were filtrated by micro filtration membranes. Each 8 mL Xiang River water sample was spiked with the OCS standard solution containing 20, 80, 400 ng OCS. Then each spiked water sample was diluted by 16 mL PBS buffer and

extracted with AcOEt (8 mL  $\times$  3 times). The extracts were filtered through a short gel chromatography, and the solvent was removed by the rotary evaporator. Then the residue was redissolved 20  $\mu$ L hexane and 1  $\mu$ L of the solution was injected for GC assay equipped with a RTX-5 column (30 m  $\times$  0.25 mm, with 0.25  $\mu$ m film thickness) and an electron capture (ECD) detector. The concentration of OCS was quantified by comparison of integral areas (retention time, 18.475 min) to the standard solution of OCS. The same treatments were used to human sera samples.

## 3. Results and discussion

### 3.1. Design and synthesis of haptens

The design and synthesis of haptens are critical to the immunoassays and a suitable hapten should preserve the structure of the target compound as much as possible. To simulate the special structure, electronic properties, and hydrophobicity of OCS, we chose to introduce the spacer arm at the trans-position of vinyl chloride and preserve the other structure of OCS. Three haptens containing different linkers and a functional group (–COOH) were designed for conjugation to carrier proteins. Considering there is no material with a similar structure of OCS to derivatize directly, we have to synthesize these haptens through more than five steps from pentachloronitrobenzene. Fortunately, we succeed in synthesis of the haptens with great yields in each step and acceptable overall yields ( $\geq 30\%$ ). These synthesized haptens preserve the structure of OCS well, and moreover, the number of atoms in the linkers ranged from 2 to 5 (Fig. 2). It is worth noticing that this is the first literature described the synthesis of haptens for OCS.

### 3.2. Sera evaluation

All antisera were titrated in homologous and heterologous conjugate-coated format (Tables 2 and 3). The antisera against immunogen BSA–Os3 consistently showed a relatively low titers and a relatively high  $IC_{50}$  value, while the antisera against BSA–Os1 and BSA–Os2 had different results depending on the coating antigen used. Especially, the antisera 104 against immunogen BSA–Os2 showed the highest titers with homologous conjugates and OVA–Os3, and the lowest  $IC_{50}$  was raised when the coating antigen OVA–Os1 was used. These results led us to compare the different linkers of three haptens. Considering the lipophilicity of OCS, a possible explanation would be that a long side chain could allow the lipophilic hapten to fold back into the hydrophobic interior of the carrier protein, leading to hide its structure and decrease the affinity of the resulting antibodies [21,22]. Therefore, the immunizing haptens containing the shortest carbon linker (hapten Os2) could generate the most sensitive antibody for OCS among the synthesized haptens, while the hapten containing the

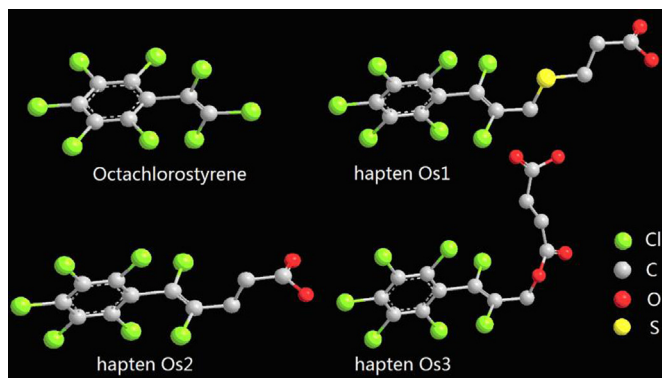


Fig. 2. Calculated lowest energy structure of OCS and three haptens by Chem3D Ultra.

Table 2  
Titration results of the different coating conjugates and sera.

Coating conjugate	Sera					
	BSA–Os1		BSA–Os2		BSA–Os3	
	Abs 101	Abs 102	Abs 103	Abs 104	Abs 105	Abs 106
OVA–Os1	1:1,28,000	1:1,28,000	1:1,28,000	<b>1:1,28,000</b>	1:8000	1:16,000
OVA–Os2	1:64,000	1:64,000	1:1,28,000	1:2,56,000	1:8000	1:8000
OVA–Os3	1:32,000	1:64,000	1:1,28,000	1:2,56,000	1:8000	1:8000

longest linker (hapten Os3) generated the poor antisera. So the combination of antisera 104 against the immunizing antigen BSA–Os2 and the coating antigen OVA–Os1 was selected for further development of immunoassay due to the lowest  $IC_{50}$  value and a high dilution.

### 3.3. Optimization of ELISA conditions

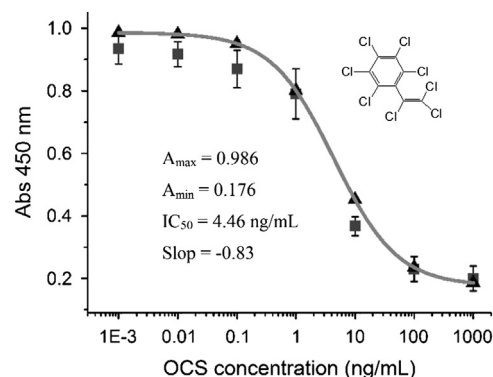
The performance ( $A_{max}$  and  $IC_{50}$ ) of ELISA is related to experimental conditions including the solution pH, surfactant concentration, and organic solvent additives which were optimized by ELISA format. The solution pH was firstly optimized in the range of pH 5.0–9.0 as the immunoreaction was pH dependent (Fig. 3A). The  $IC_{50}$  value decreased to 29.4 ng/mL and then increased to 36.5 ng/mL with pH increasing from 5 to 8. Because the immunoreaction favored a weak alkaline solution and a stable and relatively low  $IC_{50}$  value was obtained between pH 7 and 8, a PBS buffer at pH 7.4 was selected for the further experiments. Considering the probability of hindering hydrophobic interactions between analyte and antibody [17], Tween 20 was used to improve the immunoassay performance even though this assay still could work in its absence. Fig. 3B showed that low or high concentration of Tween 20 was unfavorable to the immunoassay and the lowest

$IC_{50}$  was obtained at 0.05% Tween 20 which was used in the following assays. The use of organic solvent was essential for analyzing OCS in water and sediments as the high lipophilicity of OCS. Acetonitrile, methanol and dimethylsulfoxide (DMSO) were investigated as the additives to improve the solubility of OCS in water (Fig. 3C–E). Compared with methanol and DMSO, acetonitrile had great solubility of OCS and the lowest  $IC_{50}$  value was obtained when a 15% acetonitrile solution was used.

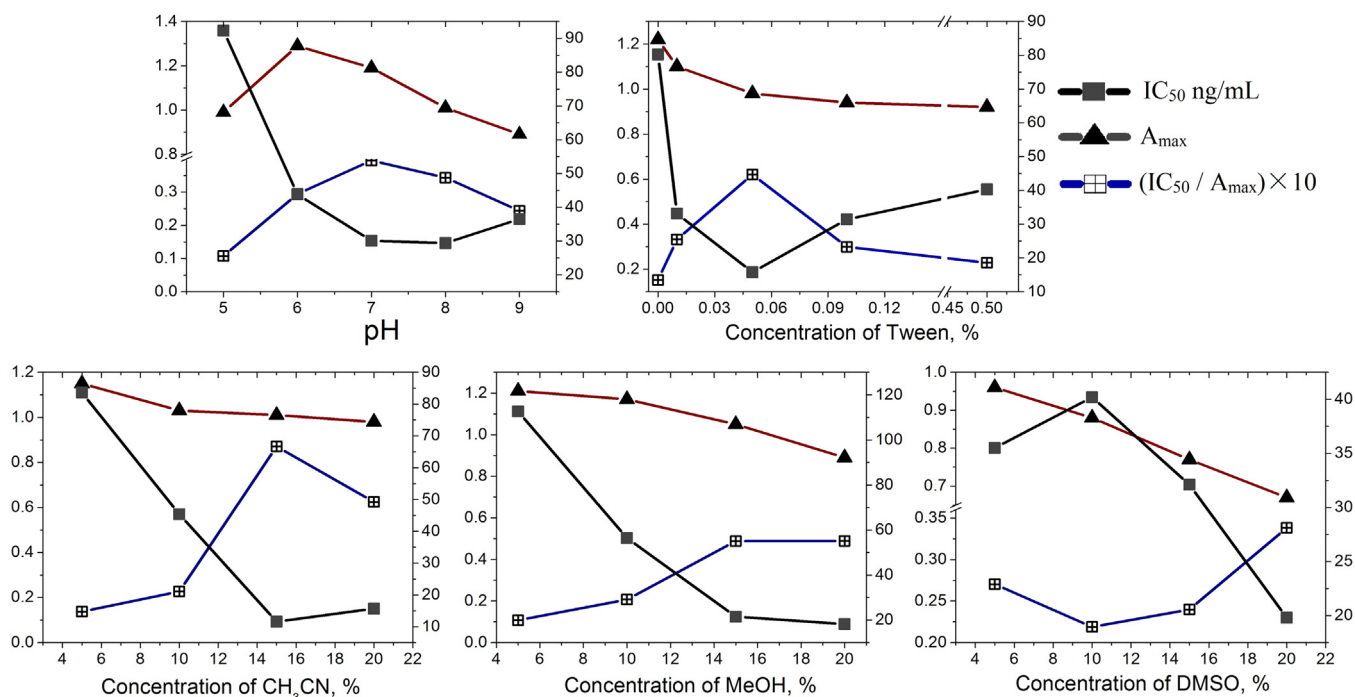
In summary, the optimal ELISA conditions used the combination of the coating antigen hapten OVA–Os1 at a concentration of 10  $\mu$ g/mL and the antisera 104 at a dilution of 1/1,28,000, together with the immunoassay buffer as 15% CH<sub>3</sub>CN in PBST. Under the optimized conditions, a competition curve is established as shown in Fig. 4. This indirect immunoassay showed a linear range ( $IC_{20-80}$ ) of 1.4–86.3 ng/mL and an  $IC_{50}$  value of 4.46 ng/mL. The low detection limit ( $IC_{10}$ ) and the high detection limit ( $IC_{90}$ ) in the buffer system were 0.1 and 100 ng/mL, respectively.

**Table 3**  
 $IC_{50}$  values for screening the best antisera-coating conjugate combination.

Antisera (antigen)	Coating conjugate	$IC_{50}$ ( $\mu$ g/L)
102 (BSA–Os1)	OVA–Os1	270
	OVA–Os2	75
	OVA–Os3	140
<b>104 (BSA–Os2)</b>	<b>OVA–Os1</b>	<b>32</b>
	OVA–Os2	96
	OVA–Os3	122
105 (BSA–Os3)	OVA–Os1	256
	OVA–Os2	300
	OVA–Os3	790



**Fig. 4.** ELISA inhibition curves for OCS determined on five different plates. The triangles represent the calculated value according to curves, and the squares represent the mean absorbance of five analyses at each concentration. Sigmoidal fit for quadruplicate  $\times n$  ( $n=5$ ,  $R^2=0.98$ ).



**Fig. 3.** Effect of pH, Tween 20 and organic solvent additives on the ELISA for OCS. The unit for  $IC_{50}$  was ng/mL, and the right Y-axis was for  $IC_{50}$ .

### 3.4. Cross-reactivity (CR) investigations

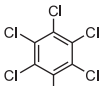
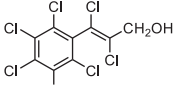
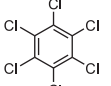
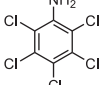
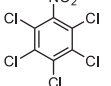
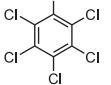
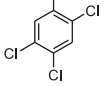
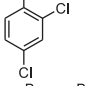
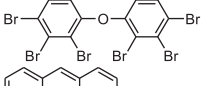
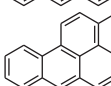
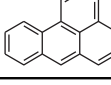

The cross-reactivities of several typical polycyclic aromatic hydrocarbons (PAHs) as well as polychloride/polybromide compounds were investigated. The choice of PAHs is because of their global environmental distribution and also as potent atmospheric pollutants, and the selection of the polychloride/polybromide compounds is for their structural similarity to OCS. The  $IC_{50}$  of these possible interferents were determined and listed in Table 4. Noticeably, all of the tested compounds show small CR values with OCS (> 4%) except for (*E*)-2,3-dichloro-3-(pentachlorophenyl) prop-2-en-1-ol (compound 5) owning a CR value of 11.1%. If we notice the high similarity in the structures between it and OCS, such a cross-reactivity value is highly acceptable. Besides, compound 5 was synthesized by us for the first time and did not exit in natural environment. So it can be concluded that the designed hapten **Os2** has succeeded in eliciting high-affinity antibodies against OCS and the immunoassay is sensitive enough for the specific detection of existing OCS in environmental samples.

### 3.5. Analysis of water and sera samples

#### 3.5.1. Recovery and precision of developed ELISA

As we know, various substances existing in complex matrixes can affect the interaction between antigen and antibody in immunoassay. Filtration with micro filtration membranes and dilution by PBS were used to reduce matrix effects. Then the recoveries of tap water, Xiang River water and sera (rabbit and human) were analyzed by the proposed ELISA method. As shown in Table 5, the rabbit and human sera samples are with the highest recovery rate ranging from 92% to 99%, while the river water samples owned the lowest recovery rate ranging from 74% to 94%. The variation of the assay was also calculated and the result (0.95%–11.4%) was acceptable. Interestingly, the recovery of the sera is almost quantitative and much better than that of water samples. This phenomenon may be due to the lipophilic property of plasma proteins [23,24]. As we know, the serum contains plasma proteins, such as  $\alpha_1$ -acid glycol-protein and albumin which are able to bind the lipophilic compounds, such as OCS.

**Table 4**  
Cross-reactivity of OCS and its related compounds.

Compound		$IC_{50}$ ( $\mu\text{g/L}$ )	CR (%)
Hexachlorobenzene		297.6	1.5
( <i>E</i> )-2,3-dichloro-3-(penta-chlorophenyl)prop-2-en-1-ol		40	11.1
Pentachloriodobenzene		486.1	0.9
Pentachlorobenzenamine		264.3	1.7
Pentachloronitrobenzene		131.6	3.4
Pentachlorophenol		1100	0.41
2,4,5-Trichlorophenol		1450	0.31
2,4-Dichlorophenol		1356	0.33
Hecabromodiphenyl ether		2000	0.22
Anthracene		1850	0.24
Fluorene		2800	0.16
Benzo(a)pyrene		4300	0.10

**Table 5**  
Recoveries of OCS in spiked samples of top water, river water and rabbit sera measured by indirect ELISA.

Concentration (ng/mL)	Measured <sup>a</sup> (ng/mL)	Recovery (%)	CV (%)
<i>Tap water</i>			
2.5	2.3 ± 0.3	92 ± 12	8.34
5	4.7 ± 0.6	94 ± 12	8.51
10	9.6 ± 0.5	96 ± 5	4.37
25	21.4 ± 3.1	86 ± 12	11.37
50	46.0 ± 2.3	92 ± 5	4.61
<i>River water</i>			
2.5	2.1 ± 0.2	84 ± 8	8.69
5	3.7 ± 0.2	74 ± 5	5.9
10	8.6 ± 0.1	86 ± 1	0.95
25	23 ± 2.8	92 ± 11	10.21
50	46.8 ± 3.4	94 ± 7	5.44
<i>Rabbit Sera</i>			
2.5	2.5 ± 0.1	99 ± 4	2.78
5	4.9 ± 0.6	98 ± 12	9.5
10	9.6 ± 1.1	96 ± 11	7.88
25	24.5 ± 3.1	98 ± 12	9.57
50	47.2 ± 3.5	94 ± 7	5.66
<i>Human Sera</i>			
2.5	2.3 ± 0.1	92 ± 4	3.48
5	4.6 ± 0.3	92 ± 6	4.9
10	9.8 ± 0.6	98 ± 6	4.21
25	23.2 ± 2.0	93 ± 8	6.18
50	48.9 ± 2.9	98 ± 6	4.06

<sup>a</sup> The analysis performed for four times ( $n=4$ ).

**Table 6**  
Comparison of recoveries in spiked samples of river water measured by indirect ELISA and GC method.

Spiked concentration (ng/mL)	Measured (ng/mL)	Recovery (%)	CV (%)
<i>ELISA</i>			
2.5	2.3 ± 0.3	92 ± 12	11.91
10	9.6 ± 0.5	96 ± 5	4.14
50	46.0 ± 2.3	92 ± 5	4.08
<i>GC method</i>			
2.5	1.87 ± 0.13	75 ± 5	5.33
10	9.62 ± 0.1	96 ± 1	0.86
50	42.60 ± 6.4	85 ± 14	12.79

Meanwhile, plasma proteins tends to adsorb on the surface of microwell plates. As a result, OCS is enriched on the surface of the microwell plates, leading to the higher recovery rates than that obtained in water samples. The river water contains complex impurities some of which may adsorb or react with OCS and form composite, leading to the lower recovery rates.

### 3.5.2. Validation of developed ELISA with GC method

The recovery rates of Xiang River water samples were compared with the determination by GC method. A good agreement between ELISA and GC data are obtained (Table 6) and the proposed ELISA gives better and more stable recovery rates than those of GC method. Besides, no pretreatment except for a simple filtration or centrifugation is needed by ELISA. Fifty human sera samples collected from the volunteers at different ages and sex were analyzed by the proposed ELISA method and GC method.

However, OCS could not be detected in all the samples by both methods. The reason of this is probable that there is few magnesium production and chlorinated solvents manufacturing in the city of Changsha and these volunteers had little chance to be exposed to octachlorostyrene.

## 4. Conclusions

In this paper, polyclonal anti-octachlorostyrene antibody was prepared, and a sensitive, competitive indirect enzyme-linked immunosorbent assay (ELISA) was developed for the detection of OCS. A linear range from 1.4 to 86.3 ng/mL together with an  $IC_{50}$  value of 4.46 ng/mL and a detection limit of 0.1 ng/mL were achieved. The developed method showed small cross-reactivity values with other compounds including ones with high similarity in structure. The test of water and serum samples confirmed that the developed immunoassay was quite suitable for fast detection of OCS in environment.

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