Development of a Solid-Phase Extraction Coupling Chemiluminescent Enzyme Immunoassay for Determination of Organophosphorus Pesticides in Environmental Water Samples

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Development of a Solid-Phase Extraction Coupling Chemiluminescent Enzyme Immunoassay for Determination of Organophosphorus Pesticides in Environmental Water Samples

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Supporting Information

ABSTRACT: Solid-phase extraction (SPE) and direct competitive chemiluminescence enzyme immunoassay (dcCL-EIA) were combined for the detection of organophosphorus pesticides (OPs) in environmental water samples. dcCL-EIA based on horseradish peroxidase labeled with a broad-specificity monoclonal antibody against OPs was developed, and the effects of several physicochemical parameters on dcCL-EIA performance were studied. SPE was used for the pretreatment of water samples to remove interfering substances and to concentrate the OP analytes. The coupling of SPE and dcCL-EIA can detect seven OPs (parathion, coumaphos, phoxim, quinalphos, triazophos, dichlofenthion, and azinphos-ethyl) with the limit of quantitation below 0.1 ng/mL. The recoveries of OPs from spiked water samples ranged from 62.5% to 131.7% by SPE−dcCL-EIA and 69.5% to 112.3% by SPE−HPLC−MS/MS. The screening of OP residues in real-world environmental water samples by the developed SPE−dcCL-EIA and their confirmatory analysis using SPE−HPLC−MS/MS demonstrated that the assay is ideally suited as a monitoring method for OP residues prior to chromatographic analysis.

KEYWORDS: *chemiluminescent, immunoassay, solid-phase extraction, organophosphorus pesticides, environmental water*

INTRODUCTION

Over the past few decades, there has been an increased use of pesticides in agriculture, industry, and residential settings all over the world. Their contamination in environmental water is considered to be widespread throughout the world, especially in the developing countries.[1] For instance, the output of pesticides in China reached approximately 1.74 million tons of 300 different types of pesticides in 2008, which has made China become the largest producer and user of pesticides in the world.[2] At present, there is an increasing effort in research into the environmental monitoring of pesticides that may pose a risk to human health and ecosystem dynamics.[3] Environmental monitoring studies of pesticides can show how their influx to humans from environmental media. Since the European Union Directives (1980 and 1998) have limited the maximal level for any individual pesticide to 0.1 ng/mL and for the total of all pesticides to 0.5 ng/mL in drinking water,[4,5] there has been a greater demand for developing analytical methods with very high sensitivity.

Immunochemical techniques can satisfy analytical demands for high sample loads requiring high sensitivity.[6] Their sensitivity is comparable or even superior to instrumental methods such as gas chromatography (GC), high-performance liquid chromatography (HPLC), and mass spectrometry (MS). They also exhibit some unique characteristics such as being rapid, simple, and cost-effective as well as high-throughput methods. Among the immunochemical techniques, enzyme-linked immunosorbent assay (ELISA) is the most extensively used method in pesticide residue monitoring,[7] and most ELISAs for pesticides are developed to recognize a single target with high specificity. However, the development of ELISAs that could be used for class-specific monitoring of pesticides is an attractive topic for the purpose of high-throughput screening.[8] Research on the development of ELISAs for a class of pesticides, such as triazine herbicides,[9] sulfonylurea herbicides,[10] pyrethroid insecticides[11] and organophosphorus pesticides,[12] was recently reported. These methods can identify more than one target and detect positive samples from hundreds of real samples in one simple test.

Traditional ELISA typically involves chromogenic reporters and substrates that produce some kind of observable color change to indicate the presence of antigen or analyte. As an alternative, chemiluminescence enzyme immunoassay (CL-EIA), in which enzyme labels are detected by chemiluminescent (CL) substrates, such as the luminol/peroxide/enhancer system for horseradish peroxidase (HRP) or dioxetane-based substrates for alkaline phosphatase (AP), represents one of the

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most sensitive detection systems in immunoassay development.[8,13,14] The CL-EIA can offer the possibility of improving the sensitivity of immunoassays by 2−3 orders of magnitude compared to conventional colorimetric detection.[15] Further, CL-EIA also provides a higher dynamic range of linearity, smaller sample volumes, and rapidity of the assay.[13,15] These advantages demonstrate the potential of CL-EIAs being employed for the monitoring of pesticide residues in environmental samples. Until now, only several CL-EIAs for pesticides, such as DDT and its metabolites,[15] metolcarb and carbaryl,[16] carbofuran, carbaryl, and methiocarb,[17] have been developed.

Previously, a monoclonal antibody (mAb) with broad specificity for a group of organophosphorus pesticides (OPs) was generated and used to develop a heterologous ELISA for the simultaneous determination of these OPs.[18] The aim of this study was to enhance the ELISA method to be used in the trace determination of OPs in environmental water, through the introduction and optimization of CL detection to improve the assay sensitivity. Direct competitive CL-EIA (dcCL-EIA) based on horseradish peroxidase-labeled mAb was developed, and the reaction conditions were optimized. Solid-phase extraction (SPE) was also used as a pretreatment of the water samples to remove interfering substances and to concentrate the analytes. The coupling of SPE and dcCL-EIA was used to screen OP residues in environmental water samples at trace levels, and the results were validated by high-performance liquid chromatography tandem mass spectrometry (HPLC−MS/MS).

EXPERIMENTAL SECTION

Materials and Reagents. Analytical OP standards were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Bovine serum albumin (BSA), dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), ovalbumin (OVA), 3,3′,5,5′-tetramethylbenzidine, complete and incomplete Freund's adjuvants, horseradish peroxidase (HRP), and HRP-conjugated goat anti-mouse IgG (secondary antibody) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Methanol, acetone, and Tween-20 were obtained from Tianjin Damao Chemical Reagent Co., Ltd. (Tianjin, China). Sodium metaperiodate ($NaIO_4$) and sodium borohydride ($NaBH_4$) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shenyang, China). Graphitized carbon black SPE column (250 mg/3 mL) was purchased from Shanghai ANPEL Scientific Instrument Co. Ltd. (Shanghai, China). Opaque high binding 96-well plates were purchased from Shenzhen Jinchanhua Industrial Co., Ltd. (Shenzhen, China). The SuperSignal West Pico CL substrate (luminol/enhancer, A; stable peroxide buffer, B) is from Pierce Protein Research Products (Thermo Fisher Scientific Inc., Rockford, IL, USA). Mixed cellulose ester microporous membrane was purchased from Shanghai Xingya Purification Material Factory (Shanghai, China). All other reagents were of analytical grade and were obtained from a local chemical supplier (Yunhui Trade Co., Ltd., Guangzhou, China).

Instrumentation. Plates were washed in a Multiskan MK2 microplate washer (Thermo Scientific, Hudson, NH, USA). Chemiluminescent intensity was recorded using a Wallac 1420 VICTOR3 multilable counter (PerkinElmer Company, USA). HPLC−MS/MS analysis was carried out by using the 1200 series HPLC system (Agilent Technologies, USA) equipped with the Agilent 6410 Triple Quad LC−MS system (Agilent Technologies, USA).

Buffers. The following buffers were used in this study: acetate buffer (0.2 mol/L, pH 5.6); carbonate buffer (50 mmol/L, pH 9.6); phosphate buffer (20 mmol/L, pH 7.0); phosphate buffered saline (PBS_1, 10 mmol/L, pH 7.4; PBS_2, 20 mmol/L, pH 6.4); PBST (PBS_1 containing 0.05% Tween-20, pH 7.4); Tris-HCl buffer (20 mmol/L, pH 7.0).

Preparation of Hapten−Protein Conjugates. Hapten−protein conjugates were synthesized by the active ester method. Hapten 1 (4-(diethoxyphosphorothioyloxy)benzoic acid) was coupled to BSA to be used as immunogen (hapten 1−BSA), and hapten 2 (4-(diethoxyphosphorothioylamino)butanoic acid) was coupled to OVA to be used as plate coating antigen (hapten 2−OVA). Briefly, 12 μmol of hapten, 14.4 μmol of NHS and 14.4 μmol of DCC were dissolved in 1000 μL of DMF. The mixture was stirred gently at 4 °C overnight and then centrifuged at 10956*g* for 5 min. The supernatant (900 μL) was added in drops to BSA (136 mg) or OVA (90 mg) in 9 mL of PBS (pH 7.4). The conjugation mixture was stirred at 4 °C for 12 h and then purified by gel filtration on Sephadex G-25. The eluted conjugates were dialyzed against water and then freeze-dried before storage at 4 °C for further use.

Preparation of MAbs. The production of MAbs was carried out as previously described.[18] Briefly, BALB/c female mice (6−8 weeks old, supplied by the Guangdong Medical Laboratory Animal Center) were immunized with hapten 1−BSA. One week after the last injection, mice were tail-bled, and titers of antisera were determined by indirect ELISA. The mice selected to be spleen donors for hybridoma production received a final intraperitoneal injection of 100 μg of conjugate (without adjuvant). Three days later, the mice were sacrificed for cell fusion. The hybridoma cells were acquired by fusion of the spleen cells isolated from the selected mice with SP2/0 murine myeloma cells as described by Kane and Banks.[19] Eight to ten days after cell fusion, when the hybridoma cells were grown to approximately 30−40% confluence in the well, culture supernatants were collected and screened using an indirect ELISA for the presence of antihapten antibodies. Selected hybridomas were cloned by limiting dilution, and stable antibody-producing clones were expanded. Selected clones were used for antibody production by ascites growth. Ascites fluids were collected and purified using a protein-G column, and were used in the following ELISA.

Preparation of HRP-Labeled mAb (mAb-HRP). The mAb-HRP tracer was prepared using a modified $NaIO_4$ method described by Tsang et al.[20] Briefly, 5 mg of HRP (M_w 40000) was dissolved in 500 μL of acetate buffer, and 100 μL of $NaIO_4$ (0.1 mol/L) was then added. The mixture solution was stirred with a magnetic stirrer at 100 rpm for 30 min at 4 °C, and 500 μL of glycol (2.5%, w/v) was added in drops. After reaction for 30 min at room temperature, 5 mg of mAb (M_w 162000) dissolved in PBS_1 was added in drops and stirred at 100 rpm for 30 min at 4 °C. The mixture solution was dialyzed against carbonate buffer overnight, and 100 μL of $NaBH_4$ (5 mg/mL) was added and stirred at 100 rpm for 2 h at 4 °C. The mixture was dialyzed against PBS_1 overnight and then purified by ammonium sulfate precipitation. The precipitate was collected and dissolved in PBS_1 to obtain mAb-HRP.

Procedure of dcCL-EIA. The plates were coated with hapten 2−OVA (80 ng/mL, 100 μL/well) in carbonate buffer overnight at 4 °C. The wells were washed 5 times with PBST and blocked with 200 μL/well of 5% skim milk in PBST for 3 h at 37 °C. After washing 2 times with PBST, the plates were dried at 37 °C overnight. Individual OP standard (prepared in PBS_2 by serial dilutions from a stock solution in methanol, the final methanol concentration was kept for 5% for each standard) or samples (50 μL/well) were added to the wells followed by addition of the PBS_2 diluted (1/8,000) mAb-HRP (50 μL/well). The wells were incubated with gentle shaking for 50 min at room temperature. After washing 5 times with PBST, the CL substrate (50 μL of A and 50 μL of B) was added. The plate was shaken gently for 5 min, and the CL intensity was recorded (expressed as relative light units, RLU). Competitive curves were obtained by plotting absorbance against the logarithm of analyte concentration. The sigmoid curves were generated by using Originpro 7.5 software (OriginLab Corp., Northampton, MA, USA).

Sample Preparation. River water samples were collected from the Zhujiang River, the largest drinking water source for the city of Guangzhou, China. Lake water samples were collected from Poyang Lake on the campus of South China Agricultural University (Guangzhou, China). Wastewater samples were collected from the living area of South China Agricultural University. The pH value of the water samples was measured. All water samples were filtered over a mixed cellulose ester microporous membrane to remove particles

larger than 0.45 μm, and the samples were then stored at 4 °C until further use. For SPE pretreatment, 100 mL of water sample was passed through the SPE column (preconditioned with 5 mL of dichloromethane:methanol (4:1), 1 mL of methanol, and 10 mL of 2% acetic acid solution) at 5 mL/min. The column was dried under vacuum and washed with 1 mL of methanol and 2 × 3.5 mL of dichloromethane:methanol (4:1). The eluant was evaporated under vacuum at 40 °C. The residue was dissolved with 1.0 mL of methanol for HPLC−MS/MS analysis or dissolved with 5.0 mL of PBS_2 containing 5% methanol for dcCL-EIA analysis.

Evaluation of Recovery. Diluted OP analytical standard solutions in methanol (1 μg/mL) were used for spiking water samples up to the final concentrations of 0.1 or 1 ng/mL. The water samples were then treated with SPE pretreatment as described above. The final solution was used for both dcCL-EIA analysis and HPLC−MS/MS analysis. The recovery (%) was calculated as follows: recovery (%) = [(quantity measured)/(quantity spiked)] × 100.

HPLC−MS/MS Analysis. HPLC−MS/MS for simultaneous determination of coumaphos, parathion, phoxim, quinalphos, triazophos, dichlofenthion, and azinphos-ethyl was performed according to the Chinese National Standard methods (GB/T 23214-2008).[21] The 1200 series HPLC system was used for separation of the studied pesticides on a Hypersil BDS C8 column (100 mm × 2.1 mm i.d., 2.4 μm particle size). Mobile phase A consisted of 0.2% acetic acid and 10 mmol/L ammonium acetate in water, mobile phase B consisted of 0.2% acetic acid in acetonitrile, and they were used in the following gradient profile: 0 min, 55% A and 45% B; 8 min, 10% A and 90% B; then 8.1−14 min, 55% A and 45% B. The flow rate of the mobile phase was 0.2 mL/min, and an aliquot of 10 μL of each sample was injected into the HPLC system. The mass spectra were obtained with an Agilent 6410 Triple Quad mass spectrometer using the electrospray ionization technique. All pesticides were analyzed in the positive ionization mode.

dcCL-EIA Screening and HPLC−MS/MS Analysis of Real Water Samples. Two river water samples (samples 1 and 2) were collected from the Zhujiang River, and a further eight river water samples (sample 3 to 10) were collected from small rivers (no names) in Guangzhou City. Eight lake water samples (samples 11 to 18) were collected from small lakes in Guangzhou City. Five wastewater samples (samples 19 to 23) were collected from South China Agricultural University and nearby living areas. After filtration, the water samples were treated with SPE and determined by both dcCL-EIA and HPLC−MS/MS. For dcCL-EIA analysis, the average absorbance of each sample was recorded and then used to calculate the percent inhibition using the following equation: I (%) = $[(A_0 - A_x)/A_0] \times 100$, where A_0 is the absorbance of the control (PBS_2) and A_x is the absorbance of the blind samples at 450 nm. The samples that demonstrated an inhibition lower than 15% were regarded as negative samples, and samples with a percent inhibition higher than 15% were considered positive.

RESULTS AND DISCUSSION

Optimization of dcCL-EIA. With the aim to improve dcCL-EIA performance, the influence of several physicochemical parameters including coating concentration, antibody dilution, ionic strength, pH, Tween-20 concentrations, and assay time on dcCL-EIA was carefully examined. For each condition, the standard curves for parathion were established (n = 3) and the maximum RLU value of the inhibition curve (RLU_{max}) as well as the concentration of analyte that produces a 50% decrease in RLU_{max} (IC_{50}) were obtained from the standard curves. The RLU_{max}/IC_{50} ratio was used to estimate the effect of a certain factor on the dcCL-EIA performance, a higher ratio indicating a higher sensitivity response to the condition being tested.[15,22]

For coating concentration and antibody dilution, a preliminary checkerboard titration was applied to choose several pairings of coating concentration and antibody dilution, under which the RLU was around 200 000. The result indicated that the pairing of coating concentration (80 ng/mL) and antibody dilution (1:8000) was optimal to obtain the highest sensitivity.

For working buffer, three buffer systems (PB, PBS, and Tris-HCl, 20 mmol/L, pH 7.0) were used. Although the IC_{50} values for parathion are similar using PBS (0.11 ng/mL) and PB (0.14 ng/mL), the higher RLU_{max} was obtained when PBS was used. This indicated that the salt concentration of this buffer was advantageous for antibody−antigen interaction. The buffer Tris-HCl was deemed less suitable for antigen−antibody interaction as lower RLU_{max} and higher IC_{50} values (0.42 ng/mL) were obtained. The further study of pH, ionic strength, and Tween-20 concentration effects indicated that a pH value of 6.4 and an ionic strength of 20 mmol/L were most suitable for the antigen−antibody interaction, and the presence of Tween-20 was detrimental to the assay sensitivity. These results were identical to that of a previous study on the development of ELISA for OPs.[23]

For inhibition assay time, by increasing the incubation time, the RLU_{max} increased gradually. The RLU_{max}/IC_{50} ratio reached a peak value and then stabilized after an incubation period of 50 min, which indicated that 50 min incubation was sufficient to reach equilibrium of the antibody−antigen interaction. Unlike the colorimetric assay, which requires a 10−30 min incubation step for color development in addition to the enzyme activity reducing step, the CL signal can be measured immediately after the addition of substrate in dcCL-EIA. The CL intensity was reported to reach equilibrium at 2−3 min[15] or 5−10 min[24] after the addition of CL substrate. In this study, the kinetics of CL reaction with the presence of mAb-HRP and absence of analyte (control) and without the presence of mAb-HRP (background) after the addition of CL substrate was studied. The results indicated that the CL intensity reached a peak value immediately at 1 min and formed a plateau at 1−7 min. To eliminate the time-dependent drift caused by the time required for pipetting samples or standards into 96-well plates and make the sufficient reaction between luminol/peroxide/enhancer and HRP, it was decided to oscillate the microtiter plates for 5 min prior to the determination of RLU. Compared with the colorimetric assay, the time for detection of signal can be reduced at least 5−25 min in the dcCL-EIA, which made it more useful for the purpose of rapid analysis. The results also showed that the dcCL-EIA had a low background of detection (the average CL intensity was below 1200 in the absence of mAb-HRP).

Tolerance of Methanol Solvent. Most OPs are hydrophobic compounds, and organic solvents are required for the extraction of OPs. The results of our previous study[23] indicated that methanol caused the least negative effects on ELISA performance and the concentration of methanol was tolerated up to 5%. In this study, the effect of methanol on the performance of dcCL-EIA was also studied since the reaction system was different between ELISA and CL-EIA. As shown in Figure 1, a gradual increase in the maximum signal (RLU_{max}) and decrease in the sensitivity (IC_{50}) of the assay were observed with increasing amounts of methanol in the assay buffer. The ratio of RLU_{max}/IC_{50} decreased gradually with the increasing amount of methanol. Since the decrease of RLU_{max}/IC_{50} was not significant when the amount of methanol was below 5%, the maximum tolerance of 5% methanol concentration was selected. The results indicated that the tolerance of methanol for ELISA and CL-EIA was similar.

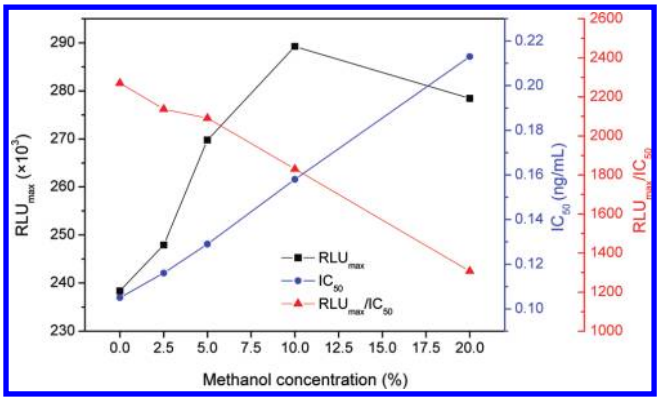

Figure 1. Effect of methanol concentration on dcCL-EIA performance.

Assessment of dcCL-EIA Screening Method. Based on the optimized reaction conditions, dose−response curves for 31 OPs in assay buffer (PBS_2 containing 5% methanol) were constructed. Compared to the results of indirect competitive ELISA,[22] the sensitivities for most OPs were improved 5- to 10-fold. The dcCL-IEA showed high CRs (>200%, CR of immunizing hapten (hapten 1) was defined as 100%) for seven OPs (coumaphos, parathion, phoxim, quinalphos, triazophos, dichlofenthion, and azinphos-ethyl). The limit of detection (LOD) is the smallest concentration of the analyte that produces a signal which can be significantly distinguished from zero for a given sample matrix with a stated degree of confidence. There is a general consensus in favor of selecting the dose which inhibits 10% of the binding of the antibody with the enzyme tracer at 90% B/B_0 (IC_{10}).[25] In this study, the LOD was defined as the concentration of analyte that produces 15% of the RLU_{max} (IC_{15}), which can improve the reliability of the assay in comparison with using IC_{10}. However, only the LOD (defined as IC_{15}) for parathion (0.02 ng/mL) and coumaphos (0.01 ng/mL) can fulfill the maximal residue level set by the European Union Directives (0.1 ng/mL for any individual pesticide in drinking water). Since the developed dcCL-IEA only show high sensitivity to parathion and coumaphos, the advantages of a broad-specificity immunoassay cannot be reflected. Therefore, sample pretreatment was further studied in this work.

Evaluation of Matrix Effects and Development of SPE−dcCL-EIA. Evaluation of matrix effects is of great importance when developing an immunoassay method because the interaction of antigen and antibody is greatly affected by effects existing in real water samples such as pH, ionic strength, organic content, and so on. The effect of water matrixes on dcCL-EIA performance was studied by constructing dose−response curves with river, lake, and groundwater samples. As shown in Figure 2, the RLU_{max} decreased while the IC_{50} value increased in the curves obtained from three water samples, which indicated significant matrix effects on dcCL-EIA assay. To determine the main factors that caused the matrix effects on dcCL-EIA, the river, lake, and groundwater samples were all diluted with 2× PBS_2 (40 mmol/L, pH 6.4) one time (water/PBS_2 = 1:1, v/v) and used to construct dose−response curves for parathion. Almost no shift of standard curves prepared in the treated water matrix was observed in comparison with the standard curve prepared in PBS_2 (data not shown). The results indicated that the ionic strength and pH value of the media mainly influenced assay performance. Although the dilution with 2× PBS_2 can eliminate the matrix effects of water samples,

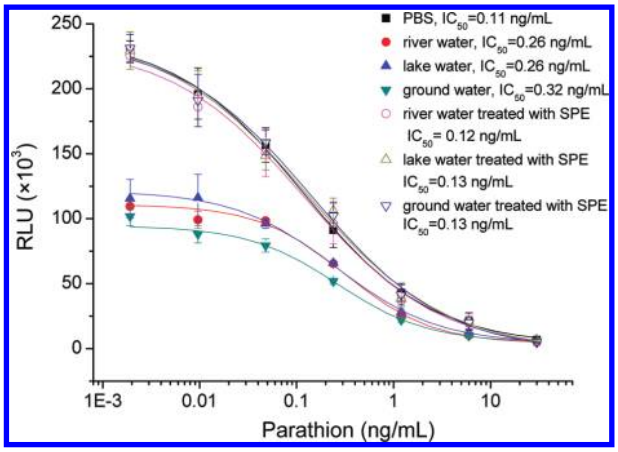

Figure 2. Matrix effects of three environmental water samples (before and after SPE pretreatment) on dcCL-EIA performance.

this further dilution may also result in a reduction of assay sensitivity. To enable the proposed dcCL-EIA to be used as a broad-specificity screening method for the seven OPs with enough sensitivity, SPE pretreatment of environmental water samples was performed.

SPE is used extensively as a cleanup procedure and also as a preconcentration technique that can increase the sensitivity of an analytical method for environmental samples.[26] Graphitized carbon black adsorbent exhibited some unique properties, such as homogeneous surface, nonspecific and nonporous, and was useful for the simultaneous extraction of pesticides. Therefore, in this study a graphitized carbon black column was used as a pretreatment of the water samples to remove interfering substances and to concentrate the analytes. First, the three environmental water samples were pretreated with SPE and then used to construct dose−response curves for parathion. As shown in Figure 2, the matrix effects of three water samples were completely eliminated after the pretreatment with SPE. Second, 100 mL of water sample was passed through the SPE column and the final residue was redissolved with 5 mL of assay buffer (PBS_2 containing 5% methanol). Since no matrix effects were observed for all three water samples, this demonstrates that a concentration of 20 times lower of the analytes can be measured after the pretreatment with SPE. Therefore, the combination of SPE pretreatment and dcCL-EIA (SPE−dcCL-EIA) showed that the LOD for the proposed seven OPs was below 0.05 ng/mL and the limit of quantitation (LOQ) was below 0.1 ng/mL, as shown in Table 1. The sensitivity of the

Table 1. The Sensitivity of SPE−dcCL-EIA to Seven OPs (n = 3)[a]

analyte	IC_{50} (ng/mL)	LOD[b] (ng/mL)	linear range[b] (ng/mL)
parathion	0.007	0.001	0.002−0.07
coumaphos	0.002	0.001	0.001−0.01
phoxim	0.06	0.01	0.01−0.33
quinalphos	0.09	0.01	0.03−0.88
triazophos	0.12	0.01	0.02−1.41
dichlofenthion	0.19	0.02	0.04−0.87
azinphos-ethyl	0.23	0.03	0.05−1.26

[a]The assay buffer is PBS_2 containing 5% methanol. [b]LOD is the limit of detection (IC_{15}), and linear range is the lower (IC_{25}) and the upper (IC_{80}) limit of quantitation.

Table 2. Recoveries of OPs from Spiked Environmental Water Samples by SPE−dcCL-EIA and SPE−HPLC−MS/MS

		recovery ± SD[a] (%)					
		river water		lake water		wastewater	
analytes (ng/mL)	spiked level	dcCL-EIA	HPLC−MS/MS	dcCL-EIA	HPLC−MS/MS	dcCL-EIA	HPLC−MS/MS
parathion	0.1	86.5 ± 12.5	76.5 ± 6.1	101.2 ± 14.1	81.2 ± 8.1	95.4 ± 12.1	75.4 ± 7.5
	1.0	102.3 ± 14.6	89.8 ± 7.9	91.3 ± 9.5	84.1 ± 5.7	114.7 ± 18.9	91.5 ± 6.9
coumaphos	0.1	81.4 ± 9.8	71.2 ± 4.2	113.3 ± 15.3	69.5 ± 10.3	95.3 ± 11.3	102.3 ± 10.2
	1.0	73.8 ± 15.1	103.6 ± 10.2	81.1 ± 12.0	89.3 ± 4.8	102.4 ± 16.5	85.4 ± 10.2
phoxim	0.1	124.5 ± 14.5	82.5 ± 5.6	107.4 ± 10.7	102.5 ± 6.7	131.7 ± 17.8	75.3 ± 7.8
	1.0	95.3 ± 11.0	76.8 ± 4.0	121.5 ± 19.7	79.7 ± 7.1	103.5 ± 9.6	95.5 ± 6.4
quinalphos	0.1	126.6 ± 13.7	112.3 ± 8.1	98.7 ± 11.2	85.6 ± 8.0	86.1 ± 11.7	71.6 ± 5.9
	1.0	83.4 ± 10.2	74.9 ± 6.4	103.6 ± 18.9	91.2 ± 7.3	104.7 ± 20.1	85.6 ± 4.7
triazophos	0.1	95.1 ± 8.5	85.3 ± 8.3	102.1 ± 14.0	100.7 ± 5.6	94.0 ± 13.9	74.5 ± 6.5
	1.0	107.8 ± 9.1	91.2 ± 3.6	113.6 ± 15.3	75.6 ± 11.2	108.6 ± 16.7	101.9 ± 7.1
dichlofenthion	0.1	62.5 ± 17.9	73.8 ± 10.2	91.0 ± 6.8	81.2 ± 8.4	73.3 ± 21.2	81.6 ± 6.0
	1.0	79.1 ± 12.3	81.2 ± 6.0	108.1 ± 13.1	73.9 ± 6.9	125.6 ± 17.3	84.7 ± 8.9
azinphos-ethyl	0.1	131.5 ± 16.2	102.3 ± 4.9	114.7 ± 10.5	101.1 ± 2.1	89.9 ± 9.7	89.3 ± 9.1
	1.0	107.5 ± 9.3	78.6 ± 5.7	95.3 ± 12.5	85.7 ± 5.4	101.2 ± 12.5	87.2 ± 10.3

[a]SD = standard deviation, $n = 3$.

developed SPE−dcCL-EIA can satisfy the analytical demand for these OPs in drinking waters set by European Union Directives.

Determination of Recovery. To determine the sensitivity and reproducibility of the proposed SPE−dcCL-EIA, recovery tests from spiked environmental samples were performed. Three environmental water samples were spiked with each of the seven OPs at two concentrations (0.1 and 1.0 ng/mL) and determined by both SPE−dcCL-EIA and SPE−HPLC−MS/MS. The results are shown in Table 2. The recoveries from three spiked environmental water samples by SPE−dcCL-EIA ranged from 62.5% to 131.7%, and the mean recovery was 100.6%. The coefficients of variation (CVs) ranged from 6.8% to 20.1%, and the mean CV was 13.5%. For SPE−HPLC−MS/MS, the recoveries ranged from 69.5% to 112.3%, and the mean recovery was 85.8%. The CVs ranged from 2.1% to 11.2%, and the mean CV was 7.1%. Although recoveries for some OPs are quite low (60−80%) and show high discrepancies (CV% from 15−20%), the mean recoveries (100.6%) and CVs (13.5%) were acceptable. Recoveries for pesticides from spiked water matrix by immunoasssays were always reported to range from 60% to 150%, and CVs were usually between 10% and 20% for an optimized assay.[27] This reproducibility of immunoassay is acceptable for a screening purpose.

dcCL-EIA Screening and HPLC−MS/MS Analysis of Real Water Samples. The quantitative analysis of individual OPs is not possible using a broad-specificity immunoassay because the affinity of the antibody varies between different compounds and the relative amounts of the compounds are unknown in the real samples.[10] However, it is feasible to develop a broad-specificity immunoassay as a semiquantitative screening method.[23] Percentage inhibition was used to determine whether a sample contained OPs. When samples showed a percentage inhibition (PI) lower than 15% (LOD of the developed SPE−dcCL-EIA), they were regarded as negative, and they were considered positive when the PI was higher than 15%. The sample was positive with a higher value of PI. Twenty-three environmental water samples (including ten river water samples, eight lake water samples, and five groundwater samples) were collected and determined by SPE−dcCL-EIA and SPE−HPLC−MS/MS simultaneously. The results are displayed in Table 3. Of the thirty-three water

Table 3. Screening Results of Environmental Water Samples by SPE−dcCL-EIA and SPE−HPLC−MS/MS

samples[a]	dcCL-EIA (PI,[b] %)	estimation[c]	HPLC−MS/MS[d] (ng/mL)
1	4.7	−	ND[e]
2	12.5	−	ND
3	−3.4	−	ND
4	2.8	−	ND
5[f]	41.2	++	triazophos (0.10)
6	1.9	−	ND
7	0.7	−	ND
8[f]	79.3	+++	azinphos-ethyl (0.12), triazophos (0.11)
9	1.9	−	ND
10	10.5	−	ND
11	−4.1	−	ND
12	0.6	−	ND
13	−2.5	−	ND
14	8.5	−	ND
15	16.7	+	ND
16	−3.2	−	ND
17	2.1	−	ND
18	3.8	−	ND
19	−3.9	−	ND
20	−2.6	−	ND
21	9.1	−	ND
22	6.7	−	ND
23	8.3	−	ND

[a]Samples 1 and 2 are river water collected from the Zhujiang River. Samples 3 to 10 are river water collected from small rivers in Guangzhou City. Samples 11 to 18 are lake water colleted in Guangzhou City. Samples 19 to 23 are wastewater collected from the campus of South China Agricultural University and nearby living areas. [b]Percent inhibition (PI) was calculated using the equation $[(A_0 - A_x)/A_0] \times 100$, where A_0 is the absorbance of the control at 450 nm and A_x is the absorbance of the samples. [c]+++ strong positive; ++ medium positive; + weak positive; − negative. [d]HPLC−MS/MS was developed to determine parathion, coumaphos, phoxim, quinalphos, triazophos, dichlofenthion, and azinphos-ethyl simultaneously. [e]ND, not detected. [f]Samples 5 and 8 are river water collected from small rivers near the farmlands.

samples, three positive samples (samples 5, 8, and 15) were picked out by the SPE−dcCL-EIA, in which the PI was higher than 15%. The SPE−HPLC−MS/MS analysis indicated that sample 5 contained a trace amount of triazophos (0.10 ng/mL) and sample 8 contained small amounts of triazophos (0.11 ng/mL) and azinphos-ethyl (0.12 ng/mL). Triazophos is reported to be stable and is often observed in the environment,[28] while azinphos-ethyl is one of the OPs that are most often detected in poisoning cases.[29] Both samples 5 and 8 are river waters collected from small rivers near farmlands, suggesting the potential use of these OPs in the farming practices. However, sample 15 (PI of 16.7%) was proved to be a false positive by SPE−HPLC−MS/MS, which may be due to the other potential OPs that can cross-react with the antibody.

Both false positive and false negative results can be found during the application of immunoassays in environmental samples. This can be attributed to susceptibility of antigen−antibody interaction by interference. In this study, no false negative results were found among forty-four positive (including spiked and naturally occurring) water samples. The reasons may be that the SPE pretreatment procedure was well established, which resulted in sufficient extraction of OP from original environmental water samples, and the dcCL-EIA had adequate sensitivity to provide a response for trace OP residues in water samples. Of twenty-one OP-free water samples, one false positive result was found. The false positive rate was about 4.8%, which is lower than the tolerable false positive rate for a screening method (10% to 25%) set by the U.S. Environmental Protection Agency's Office of Solid Waste.[30]

The results indicated that the proposed SPE−dcCL-EIA is ideally suited as a monitoring method for OP residues prior to chromatographic analysis. It is able to pick out positive samples from a large number of water samples. In practice, the positive samples screened out by SPE−dcCL-EIA can then be further analyzed by HPLC−MS/MS to determine which OP residues are present and the concentration of these residues. This approach can lead to a reduction in cost and time of analysis in confirming positive samples. Consider, for example, the analysis of the twenty-three water samples in this study: when using the proposed SPE−dcCL-EIA screening, followed by analysis with HPLC−MS/MS, the cost is reduced by 3-fold compared to when using HPLC−MS/MS without preliminary screening. To date, a large number of immunoassay-based kits for pesticide analysis have been commercialized and applied in practice.[31] The proposed SPE−dcCL-EIA is feasible to be transposed to a commercial kit since the SPE column and CL substrate are commercial, and the coated plates and mAb-HRP have been stabilized and standardized. The lifetime for CL substrate, the coated plates (evacuated), and mAb-HRP is twelve months at 4 °C. Further research will focus on the application of the commercial SPE−dcCL-EIA kits on a large-scale screening survey of environmental water samples.

■ ASSOCIATED CONTENT

Supporting Information

Table S1 presenting the cross-reactivity of OPs with the developed dcCL-EIA and Table S2 presenting the optimization of physicochemical parameters of dcCL-EIA were available. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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