J Fluoresc (2010) 20:1167–1173
DOI 10.1007/s10895-010-0663-8

ORIGINAL PAPER

An Indirect Competitive Fluorescence Immunoassay for Determination of Dicyclohexyl Phthalate in Water Samples

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Received: 16 January 2010 / Accepted: 30 March 2010 / Published online: 14 April 2010

Abstract A sensitive and specific indirect competitive fluorescence immunoassays (FIA) has been developed for the quantitative determination of dicyclohexyl phthalate (DCHP) using an antigen-coated plate format. The polyclonal antibodies raised against dicyclohexyl 4-amino phthalate conjugated to bovine serum albumin (BSA) by the amino diazotization linkage method. Antiserum with a sufficiently high titer was generated in rabbits and fluorescein isothiocyanate (FITC) was used as sensitive labels to construct the fluorescence immunoassay (FIA) for measurement of targeted compounds. Under optimized FIA condition, the quantitative working range was from 0.1 to 200 μg L^{-1} with a limit of detection of 0.05 μg L^{-1}. Other similar phthalate compounds do not interfere significantly in the analysis using this immunoassay technique, and the cross-reactivity rates were less than 10%. Four kinds of water samples (tap water, lake water, river water and leachate) had been detected in this assay, the recovery was 91.3–107.8%. The proposed fluorescence immunoassay turned out to be a powerful tool for monitoring of dicyclohexyl phthalate in water samples at trace level.

Keywords Dicyclohexyl phthalate · Fluorescence immunoassay · Polyclonal antibodies · Water sample

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Introduction

Phthalate esters are high production volume chemicals used to impart flexibility to polyvinyl chloride plastics as well as many other applications. Phthalate esters are found in many commonly used products, including children's toys, health and beauty supplies (e.g. cosmetics and perfumes), medical equipment (e.g. dialysis tubing and intravenous bags), and the enteric coating of some pharmaceuticals [1].

As a result of the large quantities produced, phthalate esters have become environmental pollutants [2]. Phthalate esters readily migrate from such products, and their metabolites have been detected in several human bodily fluids, including maternal urine during pregnancy, breast milk and amniotic fluid [3–6]. Some phthalate esters are suspected carcinogens that may affect the male reproductive system [7, 8]. There are some reports on phthalate ester pollution on aquatic environments adjacent to industrial sites. Some studies have also reported that phthalate esters exercise acute or chronic toxicity toward aquatic organisms [9, 10].

Dicyclohexyl phthalate (DCHP) is used as a plasticizer or blocking inhibitor in a wide variety of products [11]. Some endocrine-mediated effects such as reduced prostate weight, testicular dysfunction, reduced anogenital distance, areola/nipple retention and thyroid dysfunction have been already detected in the two-generation reproductive study [12]. Otake et al. have detected phthalate esters in samples of indoor air from 27 houses in the Tokyo Metropolitan area, the median concentration of dicyclohexyl phthalate was o.11 μg/m^3 [13].

The typical analytical methods used to determine phthalate esters in environmental samples are based mainly on

chromatographic techniques including gas chromatography, high-performance liquid chromatography, and mass spectroscopy [14–16]. The instrumental methods provide sensitive and specific techniques, but they are very laborious, expensive, and need skilled personnel; moreover, the extraction and cleanup processes involve numerous steps that are time-consuming and unsuitable for monitoring a large number of samples [17]. Presently, there is a need for acceptable, rapid, reliable, sensitive, and cost-effective assays for determining the presence of phthalates. Indeed, suitably designed immunoassay-based techniques can meet these requirements [18]. Due to their low cost, simplicity, versatility and relatively high specificity, immunoassays are widely used in life science research, drug discovery, clinical diagnostics and environmental monitoring [19, 20]. Additionally, immunological methods provide an opportunity to screen large sample volumes quickly [21, 22]. Yanaihara et al. had measured several phthalates in plastic disposable gloves. The sensitivity achieved is good, with a limit of detection (LOD) of about 50 ng L^{-1} for diethyl phthalate and a working range of between 0.64 and 10,000 ng/ml for di-n-butyl phthalate [23].

Fluorescent detection methods have led to major improvements in bioanalytical applications because of their extraordinary sensitivity and selectivity. Fluorescence immunoassays (FIAs) employ a fluorescent signal for analyte detection [24]. FIAs based on selective antigen–antibody binding and a fluorescence label have gained increasing importance in recent years and have become a common clinical chemistry procedure for the analysis of a wide range of analytes, such as drugs, hormones, and proteins. They have also found application in environmental analysis for the determination of pesticide contaminants in water [25–31]. Competitive immunoassays are quite easy and fast to perform since they do not require any separation steps. Consequently, they can be easily automated and miniaturised. Furthermore, only small quantities of substances are necessary, making them cost-effective [32]. Zhuang et al. have detected DCHP pollution in several water samples by using an antibody-coated plate format fluorescence immunoassay with a detection limit of about 1.0×10^{-4} mg L^{-1}, and a dynamic range of 3.0×10^{-4}~0.1 mg L^{-1} [33]. In this work, an antigen-coated plate format was researched, and a sensitive and selective competitive fluorescence immunoassay is assessed for the determination of dicyclohexyl phthalate in water and food packaging samples.

Materials and methods

Apparatus

Fluorescence measurement was performed on a Synergy HT Multi-Detection Microplate Reader (Bio-Tek instruments, Inc. USA). Polystyrene microtiter plates were purchased from Gene Company L td. (Shanghai, China). The pH of all buffers and solutions were measured with pHs-2C pH meter (Shanghai, China). Immune reactions were carried out on an electric heated incubator and temperatures were kept in a certain range (T±0.1 °C) throughout the experiment.

Reagents

Goat anti-rabbit IgG were obtained from Beijing Dingguo changsheng Biotechnology Co., Ltd. (Beijing, China). Fluorescein isothiocyanate (FITC) were obtained from sigma. Bovine serum albumin (BSA) and Ovalbumin (OVA) were purchased from Sigma. Freud's incomplete adjuvants (lanoline/mineral oil 1:2, concussed by ultrasonic cleaner for 3 h) and incomplete adjuvants (Freud's incomplete adjuvants with Bacille Calmette-Guerin vaccine) were prepared in our laboratory.

The stock solution of anti-DCHP antibody (100 μg mL^{-1}) was stored at 4 °C and protected from light. Dicyclohexyl phthalate (DCHP) was obtained from Shanghai Chemical Reagent Co. (Shanghai, China), and purified. Standard DCHP solution (1.0 mg mL^{-1}) was dissolved in ethanol and stored at 4 °C. Buffers for the immunoassay procedure were prepared by routine methods. Immunochemicals were obtained from Sino-American Biotechnology Co. (Shanghai, China).

In this work, all reagents used were of analytical-reagent grade unless specified and tridistilled water was used for the preparation of solutions in the whole procedure.

Buffer solutions: phosphate-buffered saline (PBS; 0.01 mol L^{-1} sodium phosphate, 0.137 mol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, pH 7.4), PBS-T (PBS containing 0.05% Tween 20), and coating buffer (0.05 mol L^{-1} sodium bicarbonate buffer, pH 9.6).

Hapten conjugation

Because the target DCHP is of a small molecular weight (MW 330.4), it requires conjugation to carrier proteins in order to be immunogenic. Dicyclohexyl 4-nitrophthalate (4-DCHNP) and dicyclohexyl 4-amino phthalate (4-DCHAP) as hapten derivative were synthesized in our previous work [34]. Then DCHAP was conjugated to BSA via amino diazotization linkage. The concentrations of DCHAP remaining in the reaction mixture were determined by measuring the corresponding fluorescence at λex=307 nm, λem=468 nm on the assumption that the fluorescence of DCHAP remained unchanged before and after labeling and the fluorescence of DCHAP in DCHAP–BSA conjugate had changed after labeling. The concentrations of DCHAP in DCHAP–BSA conjugate were calculated as the concentrations of added

DCHAP minus the concentrations of DCHAP remaining. The concentration of BSA was calculated as that added. The approximate molar ratio of DCHAP to BSA was 19.

Anti-DCHP antibody preparation

The DCHAP–BSA conjugate was used as an immunogen to immunize two female New Zealand white rabbits (rabbit 080401, rabbit080402). Immunogen (500 μg) in 1.0 mL of 0.9% normal saline was emulsified with an equal volume of Freund's complete adjuvant, and the emulsion was then injected subcutaneously. After 3 weeks, the animals were boosted with an additional 500 μg immunogen that was emulsified with Freund's incomplete adjuvant (1:1, *v*/*v*). The boosts were given every 2 weeks, and blood samples were drawn 7 days after each boost to check the titer of antibodies by using the indirect fluorescence immunoassay test. 4 months later, the final serum had titer against DCHP with dilutions of 1:64000. The blood was collected in the glass tube. The antiserum was obtained by centrifugation and the IgG fraction of the antiserum was isolated by precipitation with saturated ammonium sulfate solution. After dialysis against PBS, the purified IgG fractions were lyophilized and aliquoted into vials, and stored at −20 °C until use.

Conjugation of fluorescein isothiocyanate and goat anti-rabbit IgG

Fluorescein isothiocyanate (FITC) and goat anti-rabbit IgG (goat anti-rabbit IgG/FITC 1:10) were conjugated according to the Marshall method [35]. Common procedure of conjugation: The appropriate amount of goat anti-rabbit IgG was brought to an equal volume of phosphate-buffered saline (0.01 mol L^{-1}, pH 7.1). FITC was dissolved in the sodium bicarbonate buffer (0.5 mol L^{-1}, pH 9.5) and then mixed into the protein solution. The reaction proceeded with stirring for 4 h at 20 °C. Thereafter the conjugates were dialysed against phosphate-buffered saline pH 7.1 for 4 h and treated with Sephadex G-50 for filtration of the remaining free dye [36]. Then the solution of goat anti-rabbit IgG-FITC and a known amount of standard FITC solution were determined by measuring the corresponding ultraviolet absorption at λ=495 nm, 280 nm and 260 nm. From the standard curves for FITC, the concentrations of FITC and IgG were calculated to be 1.96 μg mL^{-1} and 0.099 mg mL^{-1}. The approximate molar ratio of FITC and IgG (n_{FITC}/n_{IgG}) was 8.1.

Fluorescence immunoassay procedure

The fluorescence immunoassay was performed in 96-well Microtiter immunoassay plates. Unless otherwise specified, the incubation step was performed at 37 °C, PBS (0.01 mol L^{-1}, pH 7.4) was used as diluent, and PBST was used as washing solution throughout the experiment. DCHAP-BSA solution (100 μL, 25 μg mL^{-1} in 0.05 mol L^{-1} sodium carbonate buffer, pH 9.6) was coated on microtiter plates and incubated at 4 °C overnight. The plates were washed three times, and nonspecific binding sites were blocked with 150 μL OVA (1%) in PBS at 37 °C for 0.5 h. After another wash step, mixture (100 μL $well^{-1}$) of sample solutions or various standard antigen solution (diluted in PBS) and anti-DCHP IgG (30 μg mL^{-1} in 0.01 mol L^{-1}PBS, pH 7.4) were added, and the reaction was incubated at 37 °C for 2.0 h. The well was then again washed as before and 100 μL of goat anti-rabbit IgG-FITC (a 1:20 dilution of the labeled antibody solution) was added and after incubation at 37 °C for at 2.5 h. The well was then again washed three times. The plate was read using an automatic detection microplate reader at λex=485 nm, λem=528 nm. The fluorescence intensity was measured and standard curves were obtained by plotting the fluorescence intensity difference ($F–F_0$) values against the DCHP concentration, where F_0 is the fluorescence intensity in the absence of labeled antibody.

Results and discussion

Titers and antisera screening

Specific polyclonal antiserum titer was based on noncompetitive indirect fluorescence immunoassay. The avidity of the different antiserum versus the coating antigens was determined by measuring the binding of serial dilutions (1/1000 to 1/128000, 100 μL $well^{-1}$) of each antiserum to the antigen-coated microplates (2 μg mL^{-1}, 100 μL $well^{-1}$) [37]. The plates were processed as described above without analyte and read using an automatic detection microplate reader at λex=485 nm, λem=528 nm.

Fluorescence immunoassay optimization

To monitor the low amounts of DCHP, a highly sensitive detection scheme is required, such as coating antigen and antibody concentration, length and temperature of the antigen coated, pH, incubation time and temperature of antibody and ionic strength.

Concentration of coating antigen and antibody

With goat anti-rabbit IgG-FITC dilution 1:20, the optimal working concentrations of coating antigen and antibody were determined by checkerboard titrations (Fig. 1). Coated antigens were dispensed in the columns used at six

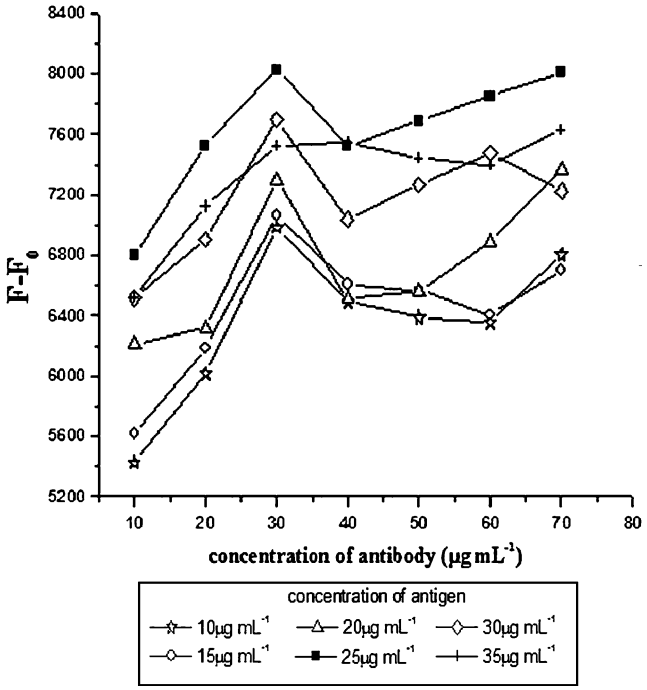

Fig. 1 Influence of coating antigen and antibody concentration on the competition step of DCHP FIA

concentrations (10–35 μg mL^{-1}), and antibodies were dispensed in the rows at seven concentrations (10–70 μg mL^{-1}). The results indicate that the fluorescence intensity difference increased while the concentration of coating antigen increased and while the concentration of antibody increased. In order to get a high sensitivity and reduce the cost, the coating antigen concentration of 25.0 μg mL^{-1} and antibody of 30.0 μg mL^{-1} were recommended in this assay.

Concentration of goat anti-rabbit IgG-FITC

The optimal working dilution of goat anti-rabbit IgG-FITC (1: 5, 1: 10, 1: 20, 1: 30, 1: 40, and 1: 50, in PBS buffer) was tested on our protocol (Fig. 2). The result shows that immunoassays for detection of DCHP are more sensitive with the goat anti-rabbit IgG diluted 10 to 20 times than other tested diluents.

Length and temperature of the antigen coated

Length and temperature are important in the reaction of antigen and antibody, low temperature could raise the binding fraction of antigen and antibody, and high temperature could accelerate the reaction. Different coated times and temperatures for the antigen-coated were studied. The results showed that antibody coated at 4 °C overnight were better than at 37 °C for 2 or 3 h. The coated plate

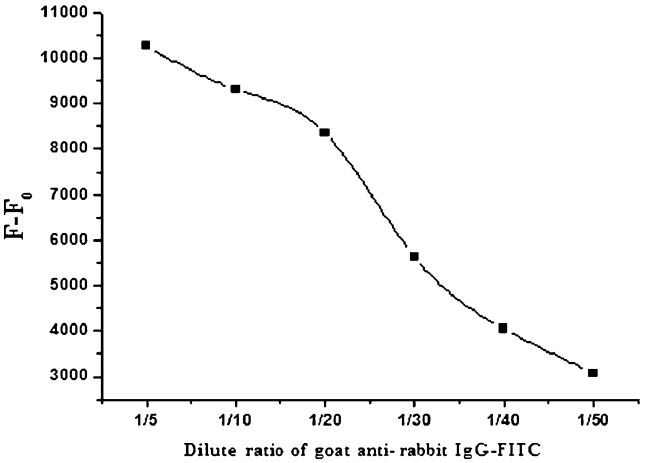

Fig. 2 Influence of goat anti-rabbit IgG-FITC concentration on the competition step of DCHP FIA with antibody concentration 30.0 μg mL^{-1} and antigen concentration 25.0 μg mL^{-1}

located at 4 °C overnight was therefore recommended in this assay as usual.

Incubation time and temperature of antibody

Incubation time for antigen-antibody and antibody-goat anti-rabbit IgG-FITC reaction had been studied (Figs. 3 and 4), specifically from 1 h to 8 h at 37 °C. The antigen-antibody reacted fast in 2 h at 37 °C, and then slowed down, so 2 h was sufficient. The reaction of antibody and goat anti-rabbit IgG-FITC was fast in 2.5 h, and the fluorescence intensity was strong, but then there was no significant change with lengthening the time, so 2.5 h was chosen in this assay.

Influence of pH

Phosphate-buffered saline has most commonly been used in immunoassay. Different pHs were tested from 4.0 to 10.0.

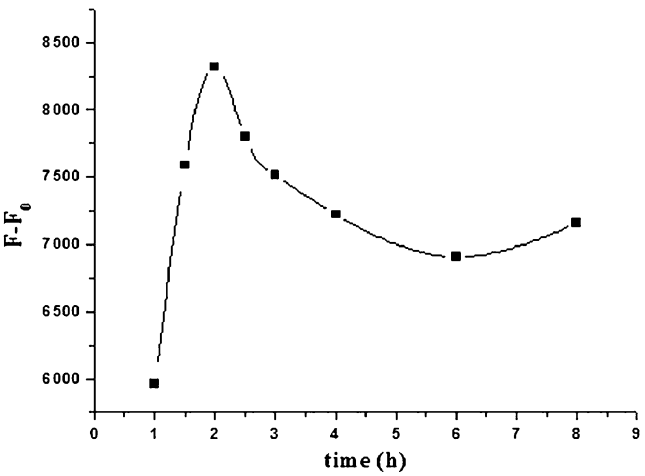

Fig. 3 Incubation time for antigen-antibody reaction

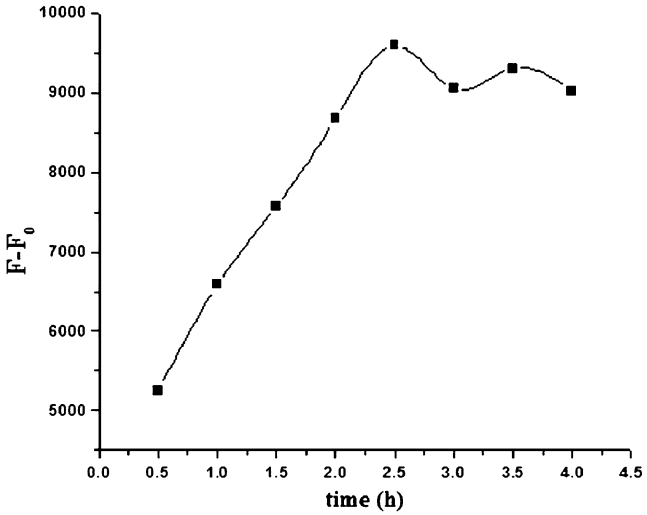

Fig. 4 Incubation time for antibody and goat anti-rabbit IgG-FITC reaction

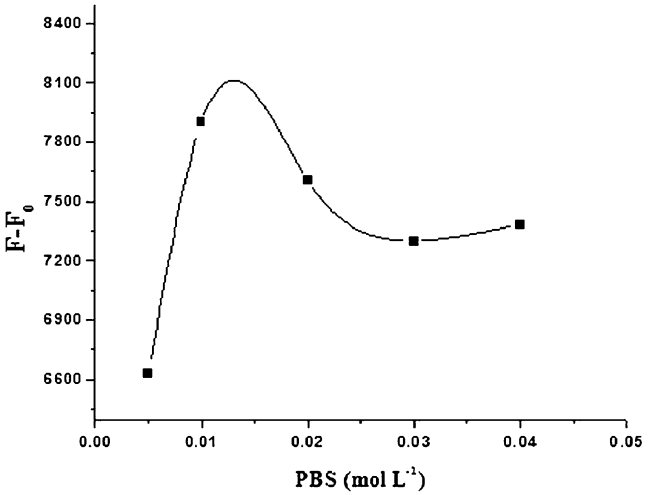

Fig. 6 Influence of buffer concentration (PBS) on the competition step of DCHP FIA

The assay performed better in neutral or basic media. The sensitivity was lower both in acidic and basic condition, especially in acidic condition. Therefore, a pH value of 7.4 was chosen as usual in this study (Fig. 5).

Ionic strength

Different concentrations of PBS, ranging from 0.5 to 4.0-fold of the original PBS buffer concentration and always 0.05% (*v*/*v*) Tween 20, were tested (Fig. 6). The results indicate that fluorescence intensity difference increased gradually as the buffer salt concentration increased. In this study, the optimum concentration of the buffer, which provided the highest fluorescence intensity, was 0.01 mol L^{-1} phosphate buffer, 0.15 mol L^{-1} NaCl, pH 7.4, containing 0.05% Tween 20 for assay.

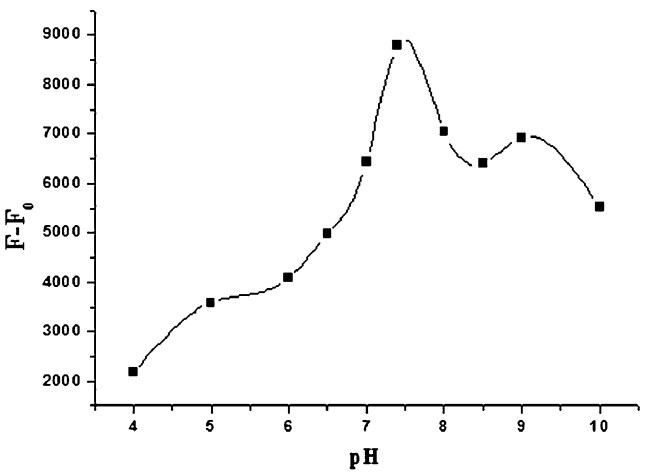

Fig. 5 Influence of pH on the competition step of DCHP FIA

Calibration

The calibration graph for DCHP antigen was constructed using the optimal conditions (Fig. 7). The calibration curve was obtained for DCHP determination by plotting ($F–F_0$) vs. DCHP concentration, and this gave a linear response in the range 0.1–200 μg L^{-1} (the regression equation was $F - F_0 = 8930 - 2290 \lg C$ (C: the concentration of DCHP, μg L^{-1}), correlation coefficient of 0.9964. The limit of detection ($3S_b/k$) was about 0.05 μg L^{-1} and IC_{50} was 34.0 μg L^{-1}.

Immunoassay specificity

The specificity of the FIA was determined using the diethyl phthalate (DEP), dipropyl phthalate (DPrP), dibutyl di(2-

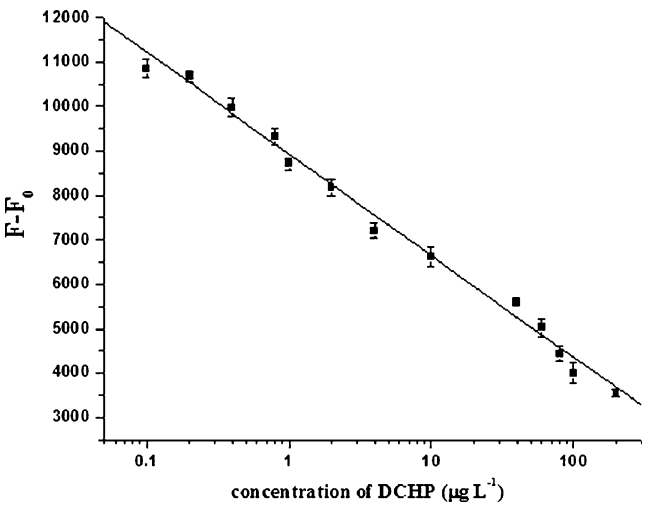

Fig. 7 The standard curve for detection of DCHP by fluorescence immunoassay

Table 1 Cross-reactivity of DCHP structurally related compounds

Coexisting substance	Cross-reactivity (%)
Dicyclohexyl phthalate (DCHP)	100
Diethyl phthalate (DEP)	5.8
Dipropyl phthalate (DPrP)	7.3
Di(2-ethylhexyl)phthalate (DEHP)	3.6
Diamyl phthalate (DAP)	8.7
Dicyclohexyl 4-amino phthalate (DCHAP)	14.6
Dicyclohexyl 4-nitrophthalate (DCHNP)	21.9

ethylhexyl) phthalate (DEHP), diamyl phthalate (DAP), dicyclohexyl 4-amino phthalate (DCHAP) and dicyclohexyl 4-nitrophthalate (4-DCHNP) which were dissolved in ethanol and prepared; the cross-reactivity rate is ($IC_{50,DCHP}$ /$IC_{50,\ coexisting\ substance}$)×100%.

Under the optimum conditions, the potential interference of a group of four structurally related phthalate esters (which are also environmental hormone-disrupting chemicals) and two hapten derivatives were tested. As displayed in Table 1. The cross-reactivities (CRs) of four structurally related phthalate esters were below 10%. Although the antibody showed relatively higher CRs to DCHAP and DCHNP, these DCHAP and DCHNP are not present in environmental samples such as serum, water and soil.

Sample analysis

Tap water, Jinghu Lake (Wuhu, China), Yangtze River water (Wuhu, China) and leachate from plastic drinking water bottles were used for analytical purposes. Tap water, lake water and the Yangtze River water samples were collected in bottles, filtered and adjusted to pH 7.0 with 0.1 mol L^{-1} HCl or 0.1 mol L^{-1} NaOH, then stored at 4 °C until required. Plastic drinking water bottles were cut into small pieces (ca. 0.5 cm^2). An accurately weighed sample was then transferred into a 100 mL flask filled with pure water. The flask was placed in the water bath kept at 50 °C to extract DCHP for 12 h. The aqueous sample, approximately 100 mL, was extracted with hexane using a separatory funnel. The hexane extract was dried and redissolved with ethanol during concentration to a volume of 1 mL or less. Standards and sample were run as nine replicates on different plates.

To perform this study, four kinds of water samples were analyzed with the optimized immunoassay. The recovery rates were approximately 91.3–107.8% (Table 2). The results show that the recovery and reproducibility of the proposed method are satisfactory.

Conclusions

This paper describes the development and evaluation of fluorescence immunoassay for the quantification of dicyclohexyl phthalate in the water. To achieve the best assay performance, several external factors affecting the specific immunochemical interactions (time, temperature, pH and ionic strength) have been demonstrated. The linear range was approximately 0.1–200 μg L^{-1} with a limit of detection (LOD) of about 0.05 μg L^{-1} and the cross-reactivity rates were less than 10%. The study demonstrated that the developed antiserum and fluorescence immunoassay procedure can be used to detect dicyclohexyl phthalate in environmental samples such as tap water, river water, lake water, and leachate from plastic drinking water bottle.

Table 2 Determination and standard addition results of DCHP in water samples (n=9)

Water sample (condensation)	DCHP levels (μg L^{-1})	Added (μg L^{-1})	Found (μg L^{-1})	Recovery (%)
Tap water	0.52	10	10.10	101.0
		20	18.93	94.7
		50	46.95	93.9
Lake water	0.87	10	9.13	91.3
		20	20.54	102.7
		50	47.96	95.9
River water	2.24	10	10.78	107.8
		20	21.20	106.0
		50	52.43	104.9
Leachate	5.38	10	9.32	93.2
		20	18.45	92.2
		50	51.89	103.8

Acknowledgements This work is supported by the National Natural Science Foundation of China (No. 20875004), the Natural Science Foundation of Anhui Province, China (Grant No. 2006KJ149B), the Anhui Postdoctoral Sustentation Fund, China and Program for Innovative Research Team in Anhui Normal University. We here express our deep thanks.

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