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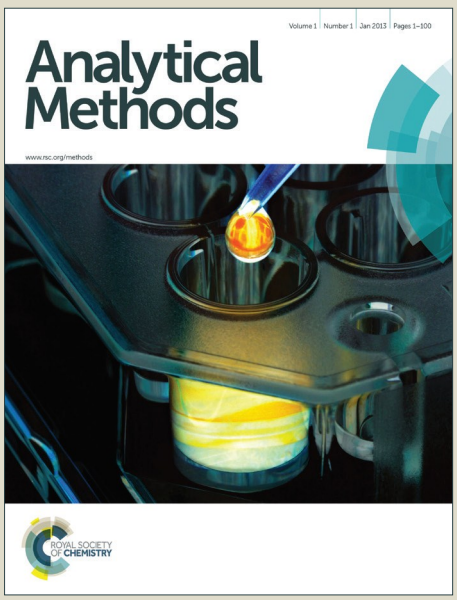

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A highly sensitive direct competitive enzyme-linked immunosorbent assay for the detection of di-(2-ethylhexyl) phthalate (DEHP) in infant supplies

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Abstract

A sensitive and specific direct competitive enzyme-linked immunosorbent assay (dc-ELISA) was studied for the detection of di-(2-ethylhexyl) phthalate (DEHP) based on antigen-coating format in this paper. The DEHP-specific polyclonal antibody was raised in rabbits and used to construct the dc-ELISA for measurement of DEHP. The conjugates of the antibody with horseradish peroxidase (HRP) were used as the detection probe. Under the optimal conditions, the assay had a detection limit (LOD) about 0.0042 ng/mL, with an apparent linear range of 10^{-3}-10^{3} ng/mL (R^2=0.998). The cross-reactivity with other five structurally related phthalate esters was below 1%. The recoveries of DEHP ranged from 80.8% to 119.2% indicated that the method was successfully applied to the determination of DEHP in infant supplies.

1. Introduction

Phthalate esters (PAEs) are widely used as additives in the manufacturing of polyvinyl chloride (PVC) plastics to make them flexible and workable.[1-3] Because of their properties as plasticizers, PAEs were used widely in industrial and household products such as children's toys, baby care products, personal care products, chemical stabilizers in cosmetics, lubricants, medical devices including blood bags and intravenous tubing.[4-9] PAEs have been produced in large quantities since the 1930s, and, in 2010 year, the global production of PAEs was 4.9 million tons, which accounts for 84% of total plasticizer production.[10,11] PAEs can leach from plastic products into environment over time. Release of PAEs into the environment during manufacture, use, and disposal has recently been reviewed.[3,12,13] In view of their high production volume, common use, and widespread environmental contamination, humans are exposed to these compounds through ingestion, inhalation, and dermal exposure on a daily basis.[14] Therefore, PAEs are a threat to the health of humans. The recent researches have shown that PAEs may reduce male's anogenital distance and sperm counts, causes testicular atrophy and disrupts fetal endocrine function.[15-18]

Di-(2-ethylhexyl) phthalate (DEHP) belongs to phthalate esters family, which are used primarily as plasticizers and produced in

very large amounts each year,[9,19] accounts for 50 % of total phthalate production.[2,10] It has been listed as priority pollutants by the U.S. Environmental Protection Agency (EPA), and the European Environment Agency.[20,21] The Agency for Toxic Substances and Disease Registry (ATSDR) estimates that the maximum daily exposure to DEHP for the general population is about 2 mg/day. However, occupational and medical exposures can reach much higher levels.[22] According to the U.S. EPA's current management plan, DEHP and the other seven phthalates are listed as the priority pollutants among phthalate esters.[20]

At present, the existing detecting methods for DEHP include gas chromatography (GC),[23] high-performance liquid chromatography (HPLC),[24] and GC/MS spectrometer.[25] However, these traditional analytical methods require many sample preparation steps, including sampling, handing and preconcentration. The immunoassays such as enzyme-linked immunosorbent assays (ELISA) draws researcher's attention because they are simple, rapid, selective, highly sensitive and cost-effective.[26,27,28] ELISA is widely used in the detection of small molecular substances, hormone, and protein.[29-31]

In this study, a direct competitive enzyme-linked immunosorbent assay (dc-ELISA) was presented for the determination of DEHP based on the polyclonal antibody highly

specific against DEHP. This method was successfully applied to the analysis of DEHP in infant supplies. The validation of these assays with spiked samples was discussed in details.

2. Materials and Methods

2.1 Reagents

DEHP and o-phenylenediamine (OPD) were obtained from Shanghai Chemical Reagent, Co. (Shanghai, China). Bovine serum albumin (BSA), ovalbumin (OVA), and horseradish peroxidase (HRP) were purchased from Sigma (St. Louis, MO, USA). 25% glutaraldehyde was supplied by Hefei BoMei Biotechnology Co. (Hefei, China). Freund's complete adjuvant (lanoline:mineral oil 1:2, with heat-killed mycobacterium tuberculosis) and Freund's incomplete adjuvant (lanoline: mineral oil 1:2) were prepared in our laboratory. New Zealand White rabbits were obtained from Shuanghe Pharmaceutical Co. (Wuhu, China). All reagents were of analytical grade unless specified otherwise.

2.2 Buffer and Solutions

Coating buffer (CB) was 0.05 mol/L pH 9.6 carbonate buffer. Assay buffer (PBS) was 0.01 mol/L pH 7.4 phosphate-buffered saline, containing 0.137 mol/L NaCl and 0.00134 mol/L KCl. Washing buffer (PBST) was PBS with 0.05% (v/v) Tween-20. Blocking buffer was 1%

OVA dissolved in PBS. The substrate solution was 4 mg OPD dissolved in 10 mL pH 5.0 citric acid-phosphate, and 15 μL 30% H_2O_2 was added before use. Stop solution was 2 mol/L sulfuric acid.

2.3 Instruments

Absorbance measurement was performed on Synergy HT multi-detection microplate reader (Bio-Tek Instruments, Inc., USA). Polystyrene microtiter plates (96-well) were purchased from Gene Company, Ltd. (Shanghai, China). UV spectra were recorded on a spectrophotometer (UV-3010, Hitachi, Japan). Immune reactions were carried out in an electric heated incubator (Shanghai, China), and the temperature was kept at a certain range (37±0.5°C) throughout the experiment. The pH values of all of the buffers and solutions were measured by a pHS-3C pH meter (Shanghai Yoke Instrument Co., LTD, China).

2.4 Synthesis of Hapten

As the immune system does not recognize small molecules, such as phthalate, the small molecules are generally not able to stimulate the immune response in vivo. Therefore, a synthesis analog of the target analyte DEHP is required.[33] Di-(2-ethylhexyl) 4-nitro phthalate (4-DEHNP) and di-(2-ethylhexyl) 4-amino phthalate (4-DEHAP) as hapten derivative were synthesized according to the method of

dimethyl phthalate.[32] Briefly, Under the protection of nitrogen, 4-nitrophthalic acid (2.5 g, 0.012 mol) was dissolved in thionyl chloride ($SOCl_2$) with stirring. The mixture then reacted for 3 h at 4°C, and the excess $SOCl_2$ was evaporated under reduced pressure. 4 mL 2-ethyl hexanol was added and reacted for 30 min under the condition of ice-bath. The solution then reacted for 12 h at 40°C. Next, the mixture was transferred to a separating funnel, the water layer was removed and the organic layer was washed with distilled water for three times. The solvent was evaporated under reduced pressure, and 3.2 g 4-DEHNP was obtained.

4-DEHNP (1.0 g, 0.0023 mol) was dissolved in 40 mL benzene and 5 mL concentrated hydrochloric acid. Then, 1.7 g zinc powder was added, After reacting for 30 min, an additional 1.7 g zinc dust was added and the mixture was stirred for 12 h at RT. 200 mL of distilled water was added and the pH of the mixture was adjusted to approximately 7.0 by adding 1 mol/L sodium hydroxide. Then the mixture was transferred to a separating funnel and the benzene layer was removed. The combined benzene extracts were distilled under reduced pressure to obtain the yellow crude products. The crude products were recrystallized from ethanol, 0.6 g 4-DEHAP was obtained. The products were characterized by IR (KBr) and ^{1}H NMR.

2.5 Conjugation of Protein and Hapten

4-DEHAP was covalently attached to BSA and OVA by diazotization method. Briefly, 4-DEHAP (0.04 g, 0.0001 mol) was mixed with 200 μL HCl (12 mol/L) and 1.0 mL redistilled water was added. Then 1.2 mL 0.1 mol/L sodium nitrite were added dropwise and reacted for 45 min at 4°C. Approximately 0.007 g urea was added to remove the excess sodium nitrite, and BSA or OVA solution (120 mg dissolved in 25 mL sodium borate) was added slowly. The pH of the mixture was adjusted to approximately 9.2, and stirred in an ice bath for 2-6 h. Finally, the conjugates (DEHAP-BSA or DEHAP-OVA) were dialyzed in redistilled water (pH 7.0) that was changed with fresh water twice a day for 5-7 d at 4°C. The structures of all conjugates were detected by the UV-vis spectrophotometer, and then the hapten density (the number of hapten molecules per molecule of protein) of conjugates was estimated directly according to the following formula:[11]

$$\text{Hapten density} = (\varepsilon_{\text{conjugation}} - \varepsilon_{\text{protein}}) / \varepsilon_{\text{hapten}}$$

Where, ε is molar extinction coefficient, the ε values at the wavelengths of maximal absorbance (λ_{max}) of conjugate, protein and hapten are taken into account in our calculations.[32]

2.6 Preparation of the Polyclonal Anti-DEHP Antibodies

The polyclonal antibodies were achieved from three male New Zealand white rabbits by intradermal injection of DEHAP-BSA conjugates. All

animal experiments were performed in compliance with the relevant laws and institutional guidelines. The approach was carried out based on previous methods.[32] The specific antibody was stored at -20°C until use.

2.7 Synthesis of HRP-antibody Conjugate

HRP-antibody conjugates were prepared by a modified glutaraldehyde method. Firstly, 0.1 mL 25% glutaraldehyde was added into 0.4 mL HRP solution (10 mg HRP dissolved in 0.05 mol/L pH 9.6 carbonate buffer). After the solution was incubated for 2 h at 37°C, 2 mL cold anhydrous ethanol was added. The mixture was centrifuged for 10 min at 415(×g). After being washed twice by using ethanol (80%, v/v), the precipitate was dissolved in 1.0 mL 0.05 mol/L pH 9.6 carbonate buffer. Then 1.0 mL anti-DEHP antibody was added and reacted for 12 h at 4°C. Because there were many impurities in the conjugates, it must be purified. In this study, we purified the conjugates by using a saturated ammonium sulfate precipitation method and dialyzed against PBS (pH 7.2) overnight at 4°C. The conjugate was stored in a refrigerator for use. The purified antibody-HRP conjugates were tested by UV spectral absorption and the absorbance was read at 280 nm and 403 nm. The concentration of labelled HRP and IgG, the mole ratio of HRP with antibody were calculated according to the following equations:

$$C_{\text{Labelled HRP}}\,(\text{mg/mL}) = A_{403}\times 0.42$$

$$C_{\text{IgG}}\,(\text{mg/mL}) = (A_{280}-A_{403}\times 0.42)\times 0.94\times 0.62$$

$$\text{Mole ratio of HRP with antibody} = 4C_{\text{Labelled HRP}} / C_{\text{IgG}}$$

Where $C_{\text{Labelled HRP}}$, C_{IgG} are the concentration of labeled HRP and anti-DEHP antibodies, respectively; A_{280}, A_{403} are the absorption values of conjugates at 280 nm and 403 nm, respectively.[32]

2.8 Direct Competitive ELISA

A dc-ELISA was developed by using the DEHAP-OVA conjugate as coating antigen. The 96-well microplate was coated with the coating antigen (DEHAP-OVA diluted in coating buffer, 100 μL/well) and incubated for 2.5 h at 37°C. The plate was washed three times for 3 min with PBST, and the paper towel was used to remove the excess of liquid by inverting the microplate and tapping it on the bench. Then the non-specific binding sites were blocked with 1% OVA (v/v) solution (0.1 g OVA diluted in 10 mL PBS, 200 μL/well), and incubated for 1.0 h at 37°C. After the plate was washed three times, 50 μL 20 μg/mL HRP-antibodies and 50 μL DEHP standard or sample solution were added into each well and the plate was incubated at 37°C for 2.0 h. After three washes, 100 μL of substrate solution was added per well. After incubated for 30 min at 37°C, the enzymatic reaction was stopped by adding 50 μL of 2 mol/L sulphuric acid. The

absorbance was read at 490 nm by a microplate reader.

2.9 Cross-reactivity

The specificity of the immunoassay was characterized by cross-reactivity (CR). Seven structurally related substances were selected for the CR test. Standard solution of each compound was dissolved in methanol and diluted in PBS with the concentration range of approximately 10^{-3} to 10^{3} ng/mL. The CR values were calculated according to the following equation:

CR= (IC_{50} of DEHP/ IC_{50} of other structurally related substances) ×100%

2.10 Sample Preparation

Five kinds of infant supplies: inflatable toy, pacifier, teether, plastic duck and baby diapers, were chosen to evaluate the performance of dc-ELISA. According to the previous methods, the samples were cut into pieces of less than 3 mm^2. Approximately 1 g of each sample was transferred into a flask and shaken with 10 mL of distilled water, then incubated for 12 h at 50°C. The pH of filtered aqueous samples was adjusted to approximately 7.4 with 1 mol/L NaOH or 1 mol/L HCl. Then 10 mL water samples were extracted with hexane using a separatory funnel two times. The hexane extract was dried, and the volume was made up to 1 mL using methanol (100%, v/v) for analysis.[33] Inflatable toy, teether,

plastic duck and baby diapers concentrated solution were diluted to 100 times before use, except that the concentrated solution was used directly for pacifier.

3. Results and Discussion

3.1 Optimization of the Coating antigen and HRP-antibody Concentration

The DEHAP-OVA was detected by UV-vis spectrophotometer. The conjugation molar ratio of hapten to OVA was 12:1. The protein content of DEHAP-OVA was 7 mg/mL, determined by UV absorption method. According to the UV spectral absorption value at 280 nm and 403 nm, the concentration of labelled HRP and IgG was 0.76 and 1.67 mg/mL, the mole ratio of HRP with antibody was 1.82, indicated that the conjugation of antibody with HRP was successful.

Checkerboard titration method was used to select the suitable concentrations of coating antigen and HRP-antibody dilution. Coating antigens were dispensed in rows with different concentrations (2-10 μg/mL) and the HRP-antibody was diluted at a concentration range of 10-30 μg/mL. Under the same concentration of coating antigen, the concentration of HRP-antibody (20 μg/mL) was considered to be suitable for the following test[11, 32]. Under the same concentration of HRP-antibody, the absorbance was increased with the change of concentration of coating

antigen, so the optimal concentration of coating antigen was 7 μg/mL (as shown in Fig. 1).

(Fig. 1 near here)

3.2 Optimization of assay conditions

In this study, to monitor the amounts of DEHP in infant supplies, a highly sensitive detection scheme is required, so we need to optimize some important reaction conditions, such as the coating conditions, concentration of blocking solution, incubation time, pH and ionic strength.

The different times and temperatures have affected the immobility of coating antigen in the plates. Four different coating conditions were studied: 4°C 12 h, 37°C 2 h, 37°C 2.5 h, 37°C 3 h. The coating antigen that incubated for 2.5 h at 37°C had the maximum absorbance.

The blocking step is important in the ELISA method to avoid nonspecific absorption. Three kinds of blocking solution were selected for the detection: 1% (v/v) OVA, 1% (v/v) OVA dissolved with 0.05% (v/v) Tween-20 and 1% (v/v) nonfat dry milk. The result showed that 1% (v/v) OVA was the suitable blocking solution due to the lowest background.

The concentration of blocking solution might affect the competitive reaction. The concentration of blocking solution from 1% to 5% (v/v) was

studied. The absorbance was decreased obviously. In order to save chemicals, we chose 1% (v/v) as the optimal blocking concentration.

Incubation time of the competition reactions have been studied, specifically from 1 h to 6 h at 37°C. According to the ELISA, the optimal incubation time was 1-3 h. The reaction had maximum absorbance and a higher sensitivity at 2 h.

The immunoassays reported in this paper are stable under a neutral or slightly alkaline condition. To evaluate the effect of the pH on the immunoassay, different phosphate buffers within the pH range of 6 to 9 were tested. The absorbance intensity was high between pH 7.0 and 7.5. Therefore, the pH value around 7.4 was considered as the optimum for the study. The same behavior has been observed for other phthalate esters.[34]

The ionic strength is the important factor in immunoassays. Under the same pH value, the influence of different ionic strengths of assay buffer (PBS) was studied. An increasing concentration of PBS (from 0.005 to 0.05 mol/L) was prepared. The absorbance intensity was significantly affected by ionic strength, with a maximum value at the PBS concentration of 0.01 mol/L. So 0.01 mol/L PBS was selected of this study.

3.3 Standard Curve

Under the optimal conditions, the calibration curve of DEHP by the method of dc-ELISA was obtained. The standard samples were dissolved in methanol and diluted in PBS with the concentration range of approximately 10^{-4} to 10^{4} ng/mL. Fig. 2 suggest that the regression equation was A=0.6470-0.034lg*c* (R^2=0.998), with a linear range of approximately 10^{-3} to 10^{3} ng/mL, which is determined as the concentrations causing 20-80% inhibition of color development.[35] The LOD was defined to be three times standard deviations of A_0, A_0 is the absorbance without the analyte.[36] LOD of this method was 0.0042 ng/mL, which was lower compared with the result taken by the HPLC (0.18-0.86 ng/mL).[5] The inhibition of the ELISA method was calculated as: Inhibition=$(1-A/A_0)\times100\%$, A is the absorbance at the related dose of the analyte, and A_0 is the absorbance at 0 dose of the analyte.

(Fig. 2 near here)

3.4 Immunoassay specificity

The specificity of ELISA was evaluated by CR of the antibodies with seven structurally related phthalate esters: DMP, DEP, DPrP, DBP, DAP, DCHP, 4-DEHAP. The results were summarized in Table 1. The CR of the antibodies with each of the first six listed related phthalate esters was below 1%. This might be due to the different functional groups and sterile hindrance between the DEHP and the first six listed structurally related

phthalate esters. However, the high CR of 4-DEHAP that was used as the hapten in this study might be due to the same aromatic structure, but 4-DEHAP is not present in the real samples. Therefore, the developed method can be applied for the detection of DEHP.

(Table 1 near here)

3.5 Analysis of the infant supplies and standard addition recovery experiments

In this study, we prepared five real samples for the experiment. According to the standard curve, the DEHP concentrations in real samples were calculated. Results were listed in Table 2. Compared with the national standard,[37] the DEHP levels of inflatable toy, teether and plastic duck were greater than national standard, the DEHP levels of pacifier and baby diapers within certain limits. The recoveries of DEHP were ranged from 80.8% to 119.2%, and indicated the recovery of dc-ELISA were satisfactory.

(Table 2 near here)

4. Conclusions

In this paper, the direct competitive ELISA was performed successfully for the detection of DEHP in infant supplies. This method has the specific advantages of sensitivity, simplicity, and reliability.

Acknowledgements

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Figure captions:

Fig. 1. Optimization of coating antigen and HRP-antibody dilutions carried out by a non-competitive checkerboard titration method. Each point represents the mean ± SD (standard deviation, n=3).

Fig. 2. Calibration curve of DEHP by dc-ELISA under the optimized conditions: the coating antigen that incubated for 2.5 h at 37°C; 1% (v/v) OVA as the blocking solution; incubation time of the competition reactions was 2h; 0.01mol/L PBS (pH 7.4) as assay buffer. Each point represents the mean ± SD (n=3).

Table 1 Cross-reactivity of DEHP structurally related phthalate esters.

Analogues	Cross-reactivity (%)
Diethylhexyl phthalate(DEHP)	100
Dimethyl phthalate(DMP)	<0.01
Diethyl phthalate (DEP)	0.06
Dipropyl phthalate (DPrP)	<0.01
Dibutyl o-phthalate (DBP)	<0.01
Diamyl phthalate(DAP)	<0.01
Dicyclohexyl phthalate (DCHP)	0.17
Diethylhexyl 4-aminophthalate(4-DEHAP)	57

Table 2 Determination of DEHP in real samples and the recovery experiments.

Sample	DEHP levels (ng/mL)	Added levels (ng/mL)	Total found levels (ng/mL)	Recovery [a] (%)	RSD (%) (n=6)
inflatable toy	11.37	1	12.38	101.3	3.6
		5	15.41	80.8	1.5
		10	22.18	108.1	2.1
pacifier	1.168	1	2.196	102.8	1.1
		5	5.666	80.8	3.3
		10	11.06	98.9	3.8
teether	12.16	1	13.21	104.6	2.4
		5	17.75	111.8	2.4
		10	22.03	98.7	3.5
plastic duck	39.45	1	40.55	110.0	1.2
		5	44.04	91.8	4.3
		10	50.91	114.6	3.5
baby diapers	1.536	1	2.626	109.0	1.3
		5	7.494	119.2	1.9
		10	13.44	119.0	2.3

[a]Recovery (%) = (Total found levels - DEHP levels)/ added levels

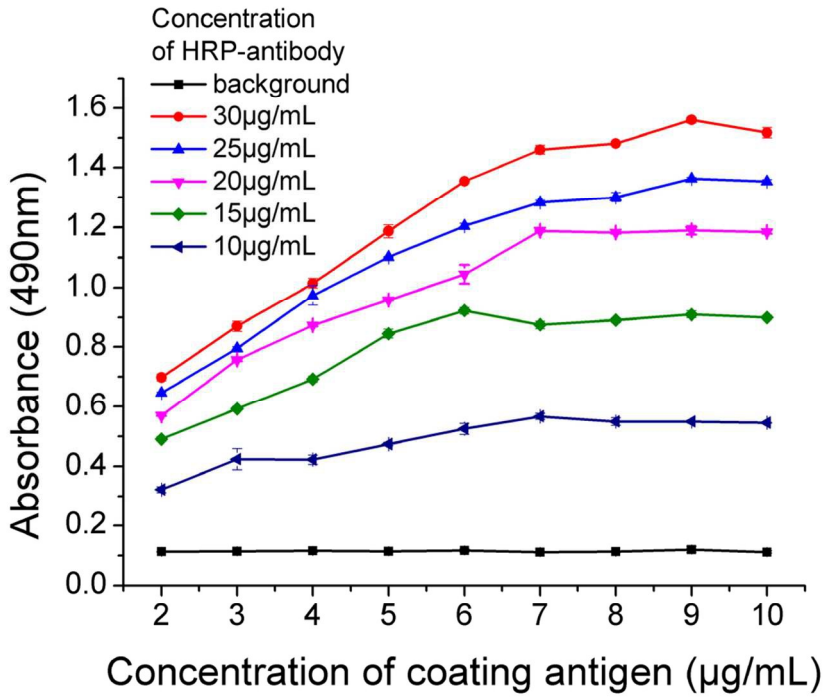

Fig 1
106x74mm (300 x 300 DPI)

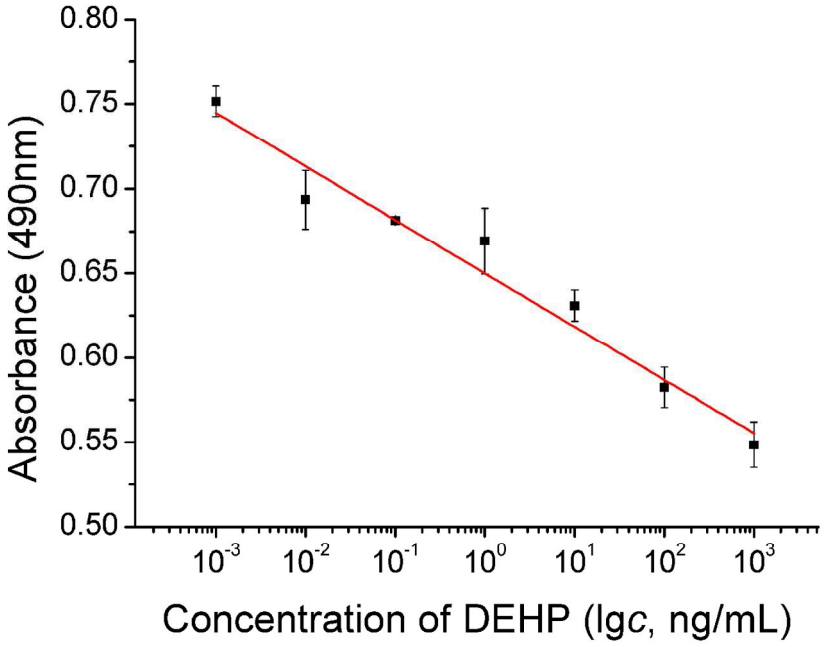

Fig 2
106x74mm (600 x 600 DPI)