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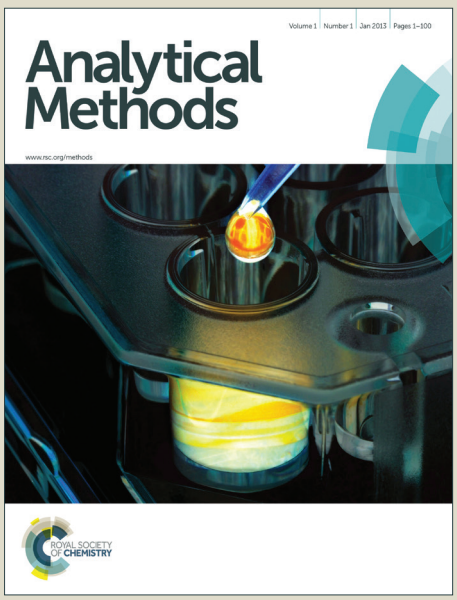

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A sensitive heterogeneous biotin-streptavidin enzyme-linked immunosorbent assay for the determination of di-(2-ethyl hexyl) phthalate (DEHP) in beverages using a specific polyclonal antibody

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ABSTRACT: Di-(2-ethyl hexyl) phthalate (DEHP) is one of the long-chain or high-molecular-weight phthalic acid diesters (PAEs) family, which is the most commonly used as plasticizer and additive. However, DEHP may cause birth defects, sexual dysfunction, even cancers and possibly heart disease, etc. In order to detect DEHP with high sensitivity and specificity, an indirect competitive biotin-streptavidin enzyme-linked immunosorbent assay (BA-ELISA) has been established in this study. A specific polyclonal antibody (pAb-DEHP) targeting DEHP was obtained firstly, and the procedures of BA-ELISA were optimized for the determination of DEHP in beverages. Under optimal conditions, good linearity was achieved within a range of 0.021 to 12.948 μg Ł$^{-1}$. The limit of detection (IC_{10}) was 0.0074 μg Ł$^{-1}$ and the median inhibitory concentration (IC_{50}) was 0.526 μg Ł$^{-1}$. The BA-ELISA was highly selective, with low cross-reactivity values with DEHP analogues (lower than 7%). Finally, the assay was successfully used to detect DEHP in beverages; the concentrations of DEHP in the samples ranged from 1.18 μg Ł$^{-1}$ to 40.68 μg Ł$^{-1}$. Satisfactory recoveries (89.07-109.33%) and coefficient of variation (CV) values (5.97 to 10.68%) were obtained, which further confirmed that this proposed BA-ELISA immunoassay is sensitive, rapid and accurate for monitoring DEHP in the environment.

Key words: DEHP; Biotin-streptavidin; Enzyme-linked immunosorbent assay (ELISA); Polyclonal antibody; Beverages

1. Introduction

Phthalate esters are used as plasticizers in a wide variety of commercial and personal care products, including building materials, clothing, detergents, electronics, medical devices, packaging, skin care products, toys, insect repellent, and medication coating, to improve flexibility and durability[1-3]. In addition, PAEs are used as an additive to various foods for improving the taste and quality[4]. Based on the above, the global production of PAEs is estimated to be about 5 million tons in 2010[5]. Due to their potential adverse effects on the reproductive system, some tissues and organs of the body[6-8], PAEs which are seen as ubiquitous endocrine disrupting chemicals (EDCs), have been regulated by the Council of the European Union, the United States Environmental Protection Agency (EPA) and many other countries' government departments[9-11].

As one of the long-chain or high-molecular-weight PAEs family, DEHP has been the most commonly used as plasticizer and additive[12]. However, DEHP is a known reproductive and developmental toxicant at high doses in living species[13]. In recent years, numerous biomonitoring study (>85%) show that significant abundant of DEHP is migrated from foodstuff to the human bodies in daily life, and then makes harmful affection on human's health and safety[14-16]. Actually, beverages occupies a large proportion in the diet to maintain human life. Nevertheless, little data is available on the pollution levels and the residual concentrations of DEHP in beverages for our study region: Shanghai, China. Hence, it is an enormous important for monitoring DEHP in beverages in Shanghai, China.

The determination of DEHP in various environmental matrixes including beverages has been largely based on chromatographic analysis, such as GC-MS[17], gas chromatography-flame ionization detection (GC-FID)[18], gas chromatography-low resolution-mass spectrometry with electron impact ionization (GC-EI-MS)[19] , gas chromatography-electron capture detector (GC-ECD)[20], high performance liquid chromatography (HPLC)[21] , liquid chromatography-mass spectrometry (LC-MS)[22], liquid chromatography-photodiode array detector (LC-DAD)[23], etc. Although all the above instrument analysis methods are certainly suitable and accurate for DEHP analysis in all sorts of environmental samples, these instruments has much more disadvantages, *i.e.* generally time-consuming, complex and labor-intensive sample pretreatment procedures, expensive instrumental detection analysis system, and more skill to operation, which restrict their widespread application for rapid determination of environmental contaminants.

However, ELISA which is based on the principle of molecular biology, is a rapid, easy-to-operation, cost-effective and reliable screening methods for determination of environmental contaminants in environmental samples, and has become increasingly prevalent and far-reaching[24-26]. In the meantime, some ELISA methods had been used for detecting PAEs[27-29]. To improve the sensitivity of ELISA, chemiluminescence immunoassay (CL-ELISA), fluorescence-enzyme immunoassay (FL-ELISA) and BA-ELISA have been developed based on traditional ELISA assays for detecting environmental contaminants[30-32]. Among these assay, BA-ELISA can reduce the nonspecific reaction with reagents because of higher affinity and specificity between

streptavidin and biotin[33]. Besides, BA-ELISA has 8-fold higher sensitivity than the traditional competitive ELISA using the same antibody and coating antigen[34].

Therefore, in this study, a highly specific, rapid and sensitive indirect competitive BA-ELISA has been established for the detection of DEHP in beverages. Firstly, a specific polyclonal antibody targeting DEHP was obtained based on optimal immunization primarily. Subsequently, to reduce background interference, several physiochemical factors that influence assay performance, such as optimal coupling concentration of DEHP coating antigen and biotinylated pAb-DEHP (Bio-pAb-DEHP), incubation time, blocking solution, the concentration of streptavidin-horseradish peroxidase (SA-HRP), pH of the buffer, ionic strength and organic solvent were studied and optimized. Under the optimized conditions, the proposed BA-ELISA immunoassay was implemented to determine DEHP in beverages sampled from Auchan (China) investment Co. Ltd in Shanghai, China. Finally, the BA-ELISA results about DEHP in beverages were further compared with those by GC-MS analysis.

2. Materials and methods

2.1. Reagents and Solutions

The standard of DMP, DEP, DPrP, DBP, DIBP, DEHP, DINP (100.00%) were purchased from Accustandard, Inc (New Haven, Connecticut, USA). The organic materials for DEHP hapten synthesis were purchased from J&K Chemical (Beijing, China). Biotinylated N-hydroxysuccinimide ester (BNHS), freund's complete adjuvant (CFA) and freund's incomplete adjuvant (IFA) were purchased from Sigma-

Aldrich Co. LLC (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), dimethyl formamide (DMF), 25% glutaraldehyde solution, ammonium sulfate, coomassie brilliant blue G250, Tween 20, $NaHCO_3$, Na_2CO_3, KCl, NaCl, Na_2HPO_4, KH_2PO_4 ·$12H_2O$, 3, 3', 5, 5'-tetramethylbenzidine (TMB), hydrogen peroxide (H_2O_2) and polyethylene glycol 20,000 (PEG 20,000) were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Bovine serum albumin (BSA), Ovalbumin (OVA), and SA-HRP was purchased from Sangon Co. Ltd. (Shanghai, China). All reagents were of analytical grade unless specified otherwise. The details of the buffers and solutions were described in the electronic supplementary information (ESI). All animal studies were performed in compliance with the relevant laws and the institutional guidelines, and the institutional committee that has approved the experiments.

2.2. Materials and instruments

Hapten was purified through column chromatography using silica gel (40μm average particle size) from Shanghai Sanpont Co. Ltd. (Shanghai, China). Fourier transform infrared spectrometry was performed on a Nicolet 6700 instrument (Thermo Fisher Scientific, Inc., USA). The ^1H Nuclear Magnetic Resonance (NMR) Spectrometer was an Avance III 400MHz instrument (Bruker, Inc., Switzerland) with $CDCl_3$ solution. Ultraviolet-visible (UV-vis) spectra were obtained on a DU-800 spectrophotometer (Beckman Coulter, Inc., Brea, CA). Ultra-pure water used was prepared using a Milli-Q System (18.2 kΩ) (Millipore, Bedford, MA, USA). Polystyrene 96-well microtiter plates were purchased from Sango Biotech Co. Ltd

(Shanghai, China). Immunoassay absorbance was measured with a Multiskan photometer in dual wavelength mode (450-630 nm) purchased from Thermo LabSystems (Vantaa, Finland). GC-MS analysis was performed on GCMS-QP2010 Gas Chromatography and Mass Spectrometer (Shimadzu Scientific Instruments, Inc., Japan).

2.3. Synthesis of DEHP hapten

DEHP molecules do not contain functional groups that can connect with proteins directly. Therefore, DEHP hapten must be synthesized through esterification and reduction firstly as Fig.1 showed. The results of the hapten synthesis and characterization are given below.

Fig. 1

Production of Di-(2-ethyl hexyl) 4-nitrophthalate (4-DEHNP): 4-Nitrophthalic acid (10.0 g, 47.5 mmol) was dissolved in 44.6 mL of 2-ethyl hexanol and then 1.65 mL of concentrated sulfuric acid was added. The mixture was refluxed for 6 hours at 120 ℃, and the solvent was evaporated under reduced pressure. The oily residue was washed with ice-water mixtures and then a yellow oily liquid was obtained. Subsequently, the yellow oily liquid was washed with 10% Na_2CO_3 solution until this washing solutions turned colorless. After the crude product was recrystallized from cold ethanol, the resulting 19.2 g 4-DEHNP was obtained, $C_{24}H_{37}NO_6$, m.w.: 435.55, yield: 92.8%. IR (KBr) ν (cm^{-1}): 3048.89 (C-H, Ar stretching vibration), 2928.61, 2860.47, 2732.86 (C-H stretching vibration), 1731.20 (C=O absorption band), 1611.98,1463.37, 1350.78 (C=C skeletal vibration), 1532.84, 1350.78 (-NO_2

stretching vibration), 1412.61 (d-O-$CH_2CH(CH_2CH_3)CH_2CH_2CH_2$- absorption band), 1278.95, 1128.22 (C-O-C absorption band), 854.03 (C-H, Ar plane bending vibration).

Production of Di-(2-ethyl hexyl) 4-aminophthalate (4-DEHAP): 4-DEHNP (2.0 g, 4.6 mmol) was dissolved in 230 mL of benzene, and 2.8 g of purified zinc dust was added. Then 8.2 mL of concentrated hydrochloric acid was added in portions. After stirring for 15 minutes at room temperature, another 2.8 g of zinc dust was added and the mixture was stirred at room temperature for 12 hours. Then, 280 mL of cold water was added to the reaction mixture and the mixture was neutralized with 1 mol L^{-1} NaOH solution. The mixture was transferred to a separatory funnel and the benzene layer was removed later. The aqueous layer was extracted with benzene. The combined benzene extracts were washed with water and dried over anhydrous sodium sulfate. After evaporation under vacuum, the pale yellow crude solid was obtained and purified by silica gel column chromatography (n-hexane : acetic acid = 15 : 1) to give 1.23 g 4-DEHAP, $C_{24}H_{39}NO_4$, m.w.: 405.57, yield: 66.1%, and m.p.: 34-36 ℃. IR (KBr) ν (cm^{-1}): 3473.78, 3376.37 (-NH_2 stretching vibration), 2958.83, 2930.61, 2873.27, 2860.21 (C-H stretching vibration), 1714.16 (C=O absorption band), 1603.78, 1569.83, 1382.04 (C=C skeletal vibration), 1463.19 (d-OCH_2-$CH(CH_2CH_3)$ -CH_2-CH_2-CH_2- absorption band), 1280.38, 1127.72 (C-O-C absorption band), 1628.00, 835.80 (C-H, Ar plane bending vibration). ^{1}H-NMR ($CDCl_3$): δ 7.68 (1H, d, ArH), 7.24 (1H, d, ArH), 6.76 (1H, dd, 1H), 4.19 (2H, q, -NH_2), 4.14 (2H, t, -O-CH_2), 4.12 (2H, t, -O-CH_2), 1.64 (2H, m, O-CH_2-CH-), 1.62-1.04 (16H, m, O-CH_2-

CH(CH_2CH_3)-CH_2-CH_2-CH_2-), 0.95-0.87 (12H, t, O-CH_2-CH(CH_2CH_3)-CH_2-CH_2-CH_2-) ppm.

2.4. Preparation of immunogen and coating antigen

As a contact portion between hapten and carrier protein, linking arm could become an antigenic determinant to determine the specificity of conjugating with antibody[35]. As shown in Fig.1, the diazotization method and the glutaraldehyde method were used for preparing immunogen (BSA-DEHP) and coating antigen (OVA-DEHP), respectively (see ESI). UV-vis spectrophotometer was used to identify all conjugates, and then the coupling ratios were estimated based on mole absorbance ε and calculated using the following equation[36,37]:

$$\text{Coupling ratio} = \frac{\epsilon_{\text{conjugate}} - \epsilon_{\text{protein}}}{\epsilon_{\text{hapten}}} = \frac{(\text{OD}_{\text{Conjugate}} - \text{OD}_{\text{protein}}) \times \text{C}_{\text{hapten}} \times \text{M}_{\text{protein}}}{\text{OD}_{\text{hapten}} \times \text{M}_{\text{hapten}} \times \text{C}_{\text{protein}}} \quad (1)$$

2.5. Preparation of Bio-pAb-DEHP

Rabbit polyclonal anti-DEHP antibodies (pAb-DEHP) were prepared as follows: two male New Zealand white rabbits were immunized with BSA-DEHP through subcutaneous and intramuscular injections with the immunogen. The initial immunisation was performed by injecting 1.0 mg of BSA-DEHP dissolved in 0.5 mL normal saline and emulsified with 0.5 mL of CFA. Twenty days after the injections, the rabbits were boosted six times at two week intervals by injecting a solution of 1.0 mg of the immunogen dissolved in 0.5 mL normal saline and emulsified with 0.5 mL of IFA. The serum titer was determined by ELISA. After antiserum titer outreached 60,000 with immunization at 3 months, pAb-DEHP were separated and purified from rabbit serum through ammonium sulfate precipitation method, and subsequently was

dialyzed against PBS for 5 d, freeze-dried and stored at -20 ℃.

Bio-pAb-DEHP were prepared as follows: 5.0 mg of pAb-DEHP was dissolved in CBS at the concentration of 1.0 mg·mL^{-1}. The antibody solution was mixed with 1.0 mg·mL^{-1} BNHS in DMSO in the mass ratio of 1:10. The mixture was stirred for 4 h at room temperature and then dialyzed against PBS for 3 d, and was stored at -20 ℃.

2.6. Heterologous indirect competitive BA-ELISA

Heterologous indirect competitive ELISA, based on the immobilisation of coating antigens, was performed in 96-well microplates as follows: the microplates were coated with the coating antigen in 100 μL of CBS overnight at 4 ℃. After three times washing (200 μL/tube of PBST), the unbound active sites were blocked with 200 μL/tube of blocking reagent, and were incubated at 37 ℃ for 60 min. After a second washing step, the DEHP standard or sample (50 μL/tube) and Bio-pAb-DEHP (50 μL/tube) were added and the mixture was incubated for 60 min at 37 ℃. After another washing step to remove unbound Bio-pAb-DEHP, SA-HRP (dilution 1:1000, 100 μL/tube) was added and the mixture was incubated for 60 min at 37 ℃. After an additional five times washing, 100 μL of TMB substrate solution was added. The enzymatic reaction was stopped by adding 50 μL of 2 mol·L^{-1} sulphuric acid after 15 min sufficient colour development. The absorbance of each well was immediately recorded in dual-wavelength mode (450 nm as test and 630 nm as the reference).

The standard curve of BA-ELISA is determined by plotting inhibition (%) against the logarithm of the standard concentration of DEHP and negative control, and the linear range was used for quantification of DEHP concentration in the

samples. The IC_{50}, the concentration at which a compound inhibited a particular phenomenon by 50%, was used to evaluate the sensitivity of the methods. Analogously, the limit of detection (LOD) is evaluated in terms of IC_{10}.

$$\text{Inhibition (\%)} = \frac{(A_{max}-A_{min})-(A_s-A_{min})}{A_{max}-A_{min}} \times 100\% \quad (2)$$

where A_{max} was the absorbance in the absence of DEHP, A_{min} was the absorbance of the blank sample, and A_s was the absorbance of DEHP at the standard concentration.

2.7. Cross-reactivity

The specificity of the optimized BA-ELISA assay was evaluated by measuring cross-reactivity (CR) of the Bio-pAb-DEHP using a group of DEHP structural analogues. The CR values were calculated as follows:

$$\text{CR (\%)} = \frac{IC_{50} \text{ of DEHP}}{IC_{50} \text{ of analogues}} \times 100 \quad (3)$$

2.8. Sample preparation

All the beverages were purchased from Auchan (China) investment Co. Ltd. in Shanghai, China. Further details of these samples were provided in the Table 3. Besides, these samples collected in this study were all popular brands that leaded by a wide margin in Shanghai market.

To avoid PAEs contamination, all glassware used in the study was immersed with acetone for at least 30 min, and then were washed with hexane three times. All the blanks, standards, spiked samples and real samples were undergone similar extraction method (see ESI). The treated sample was divided into two fractions: one for the BA-ELISA detection and the other for GC-MS analysis.

3. Results and discussion

3.1. Characterization of immunogen, coating antigen, and antibody

From the UV spectrum (Fig.2), several characteristic absorption peaks of DEHP hapten, protein, and conjugates appeared at 286 and 309 nm (for hapten), 227 and 278 nm (for BSA), and 234, 241, and 268 nm (for OVA). However, the characteristic peaks of BSA-DEHP and OVA-DEHP were shown at 329 nm and 343 nm, respectively. The results revealed that the DEHP hapten was conjugated into the protein successfully. Moreover, the coupling ratio was calculated using the Equation.1 above. The coupling ratio was 20 for BSA-DEHP and was 36 for OVA-DEHP.

Fig.2

The immunogen BSA-DEHP was injected into New Zealand White rabbits, which improved immunity for 15 weeks, with the highest antibody titer at 1:150,000. The concentration of immunogen, coating antigen and Bio-pAb-DEHP were determined by coomassie blue staining, *i.e.* 4.14 mg mL^{-1}, 1.09 mg mL^{-1} and 13.17 mg mL^{-1}, respectively.

3.2 Optimisation of BA-ELISA

To develop a sensitive immunoassay method, several parameters were optimized. Firstly, the concentrations of coating antigen and Bio-pAb-DEHP were determined using a checkerboard assay. In this immunoassay, different blocking solutions, such as gelatin, OVA, skimmed milk powder (SMP), PEG 20,000, and PVA, were dissolved in PBS and their background values were compared. Otherwise, the effects of different ionic strengths, pH in PBS buffer, concentrations of SA-HRP, incubation time and solvent matrix effects, were determined from the DEHP standards and the relative

antibody titers in PBS. All determinations were performed repeat eight times and the mean absorbance values were calculated. The IC_{50} and the maximum absorbance (A_{max}) were used to assess the optimum conditions for the assays[38].

As the primary influencing factor, the optimum concentrations for Bio-pAb-DEHP and the coating antigen were used to improve the sensitivity of the immunoassay (details seen in Table 1). According to checkerboard titration, the optimum reagent concentrations were those that resulted in the maximum absorbance (A_0) of approximately 1.0 and the lowest antibody and coating antigen concentrations. The optimal concentrations of OVA-DEHP was 2.03 μg mL^{-1} and Bio-pAb-DEHP was at 1:500 dilution (1.95 μg mL^{-1}).

Table 1

Given that blocking is advantageous to eliminate unoccupied sites on the tubes, so different blocking solutions, such as gelatin (0.1%, 0.5% and 1%), 1% OVA, 1% SMP, 1% PEG20,000, 1% PVA in PBS were compared (as showed in Fig.3a). An optimum blocking reagent should achieve the minimal background interference, *i.e.* the lowest absorbance value. 0.5% gelatin blocking solution achieved the minimal background interference (0.075). The background values of the other blocking solutions as follows: 0.1% gelatin (0.085), 1% gelatin (0.082), 1% OVA (0.109), 1% SMP (0.094), 1% PEG20,000 (0.135), 1% PVA (0.097). Therefore, 0.5% gelatin was selected as the blocking solution in the following experiments.

Fig. 3

Immunoassay performance was determined under different ionic strengths (NaCl

concentrations ranging from 0.05 mol Ł$^{-1}$ to 2.00 mol Ł$^{-1}$; showed in Fig.3b). A_{0max} decreased from 1.264 to 0.462 with increasing salt concentration. Salt concentrations lightly affected the sensitivity, with the IC_{50} ranging from 1.913 μg Ł$^{-1}$ to 6.986 μg Ł$^{-1}$. The lowest IC_{50} (1.913 μg Ł$^{-1}$) was obtained at an ionic strength of 0.10 mol Ł$^{-1}$. Hence, a salt concentration of 0.10 mol Ł$^{-1}$ was selected for the buffer in the subsequent assay.

The antibody-antigen binding reaction is under a dynamic balance, so this reaction is characterized by weak intermolecular bonds, and is easily affected by pH. So, the pH of optimum assay buffer was adjusted to 5.00-9.00. It was found that the pH had an insignificant effect on the sensitivity of the assay (showed in Fig.3c). The A_{0max} values decreased with increasing pH, the IC_{50} and A_{0max} varied in the ranges of 1.253-37.799 μg Ł$^{-1}$ and 0.684-1.362 A.U., respectively. The best combination of IC_{50} and A_{0max} (IC_{50} =1.253 μg Ł$^{-1}$, A_{0max}=0.987), was obtained at pH 7.40. Thus, pH 7.40 was used in the further immunoassay.

In addition, the dilutions of SA-HRP (500, 1000, 1500, 2000 and 3000) were investigated, and then the dilution of 1000 was determined (IC_{50}=0.813 μg Ł$^{-1}$, A_{0max}=1.095, showed in Fig.3d). Furthermore, immunoassay performance was also determined by different incubation time (incubation times ranging from 15 to 90 min). From the Fig.3d, we can see that although A_{0max} value increased with incubation time increasing, the lowest IC_{50} (0.991 μg Ł$^{-1}$) was obtained at 60 min. Hence, an incubation time of 60 min was selected for the competitive reaction between antigen and antibody.

Considering that different organic solvents' concentrations make a difference in

the matrix effect about resulting interference, so three water miscible organic solvents were added into immunoassay system (showed in Fig.3f). These results indicated that lower amounts of organic solvent (< 5%) negatively affected the performance of the assay, and the PBS solution containing 5% DMSO (v/v) which obtained the lowest IC_{50} value 0.809, was used to improve the analyte solubility in the future assay.

3.2. Sensitivity and stability of BA-ELISA

Under optimal conditions, a series of diluted concentrations of DEHP standard sample (0 μg Ł$^{-1}$, 0.001 μg Ł$^{-1}$, 0.01 μg Ł$^{-1}$, 0.05 μg Ł$^{-1}$, 0.1 μg Ł$^{-1}$, 0.25 μg Ł$^{-1}$, 1 μg Ł$^{-1}$, 2.5 μg Ł$^{-1}$, 5 μg Ł$^{-1}$, 10 μg Ł$^{-1}$, 25 μg Ł$^{-1}$, 50 μg Ł$^{-1}$) were reacted using the indirect competition BA-ELISA to construct standard curve (showed in Fig.4). Correlation coefficient of DEHP standard curve was 0.9850; besides, the slope and intercept were 21.57 and 56.01 respectively, *i.e.* $Y = 21.57\text{Log}C_{DEHP} + 56.01$. The linear working range, which is determined as the concentration range that causes 20 - 80% color inhibition[34], was 0.021-12.948 μg Ł$^{-1}$. The LOD of the DEHP assay, represented as IC_{10}, was 0.0074 μg Ł$^{-1}$; and the IC_{50}, which is a key criterion for evaluating the sensitivity of BA-ELISA, was 0.526 μg Ł$^{-1}$.

Fig. 4

3.3. Specificity of BA-ELISA

The specificity of immunoassay can be generally evaluated in the ability of the antibodies to combine with only the target molecule, *i.e.* cross-reactivity (CR) indirectly. The CR values were evaluated using some similar structure analogues about DEHP, such as dimethyl phthalate (DMP), diethyl phthalate (DEP), dipropyl

phthalate (DPrP), dibutyl phthalate (DBP), diisobutyl phthalate (DIBP), disononyl phthalate (DINP), 4-DEHNP, 4-DEHAP, and were calculated using Equation 3. The chemical structures of these analogues and the CR results were shown in Table 2. 4-DEHNP and 4-DEHAP showed higher CR values (*i.e.* 15.68% and 19.45%, respectively), because 4-DEHNP and 4-DEHAP were DEHP derivative. But 4-DEHNP and 4-DEHAP are not present in beverage samples. In all cases, there was a low CRs (below 7%) between DEHP and other structurally similar compounds, indicated that the pAb-DEHP exhibited high affinity and was suitable for the specific detection of DEHP at low levels.

Table 2

3.4. Determination of DEHP in beverages and recovery tests

The proposed BA-ELISA was used to detect DEHP residues in beverages collected from Auchan (China) investment Co. Ltd. in Shanghai, China. DEHP was found in all the samples, and the concentrations ranged from 1.18 ± 0.052 μg Ł$^{-1}$ to 40.68 ± 0.126 μg Ł$^{-1}$ (Table 3). The concentrations of DEHP in beer and white liquor were much higher than other samples. This is because wine can enhance mellow and soft taste after the added plasticizers. These samples were also tested on GC-MS to evaluate the precision of BA-ELISA. In a general, the BA-ELISA results were slightly higher than the GC-MS results. This difference may be caused by the non-specific absorbance of reagents used in the method, including Bio-pAb-DEHP and SA-HRP. In addition, polyclonal antibody had cross-reactivity for other PAEs present in the samples, which were not measured by GC-MS and contributed to the BA-

ELISA-derived concentrations.

Table 3

The recovery of the spiked samples and the CV were calculated to evaluate the accuracy and precision of BA-ELISA. Four samples (samples B1, M1, W1 and W2) were spiked with DEHP standard concentrations ranging from 0.05-100 μg L^{-1} before extraction. Moreover, the spiked samples were treated as described before, and then were tested six times using BA-ELISA and GC-MS for comparison. Table 4 showed that the average recoveries of BA-ELISA were ranged from 89.07% to 109.33%, the CV was 5.97% to 10.68% (below 15%). Meanwhile, GC-MS showed recovery rates of 89.25% to 108.89% and CVs of 2.71% to 4.74%.

Table 4

4. Conclusions

This study firstly developed a highly sensitive and effective indirect competitive BA-ELISA for the rapid detection of DEHP in beverages on the basis of specific pAb-DEHP. Several physicochemical factors that influenced the performance of proposed BA-ELISA were studied and optimized. Under optimised conditions, the IC_{50} value and the LOD of the assay were 0.526 μg L^{-1} and 0.0074 μg L^{-1}, respectively. This established BA-ELISA could selectively determinate DEHP against a number of structural analogues, with negligible cross-reactivity below 7%. The BA-ELISA was used to detect the presence of DEHP in beverages, and satisfactory recoveries and variation coefficient were achieved for DEHP from the spiked samples. These results confirmed that this method would be a useful option for the sensitive and selective

detection of DEHP in real environmental samples.

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Compliance with Ethics Requirements

In order to comply with the ethical requirements, all the authors of this article declare that they have no conflict of interest. We declare that the laboratory animals cared or used in the experiment were following the institutional and national guidelines. And this article does not contain any studies with humans.

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Table 1 Optimal concentrations of Bio-pAb-DEHP and OVA-DEHP.

Table 2 Cross-reactivity of Bio-pAb-DEHP with DEHP structural analogues.

Table 3 Concentrations of DEHP in milk and milk products by BA-ELISA and GC-MS.

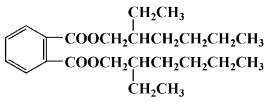
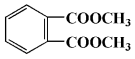
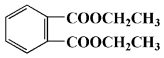
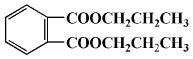
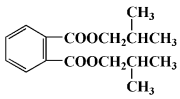
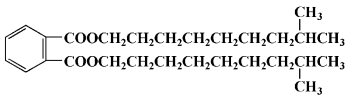
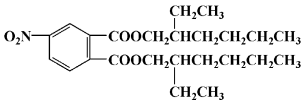
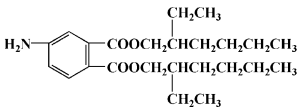
Table 4 Recovery of DEHP detected by BA-ELISA and GC-MS in spiked milk and milk products.

Table 1 Optimal concentrations of Bio-pAb-DEHP and OVA-DEHP.

Dilutions of biotinylated pAb-DEHP [a]	The concentration of OVA-DEHP (μg mL^{-1})					
	40.57	20.28	8.11	4.06	2.03	1.02
100	1.485	1.311	1.294	1.198	1.249	1.288
200	1.258	1.212	1.048	1.111	1.092	1.166
500	0.887	0.895	0.981	0.996	**1.035**	0.897
1000	0.634	0.738	0.000	0.674	0.572	0.466
2000	0.534	0.556	0.507	0.478	0.437	0.406
3000	0.482	0.380	0.397	0.298	0.330	0.375
Blank	0.093	0.089	0.092	0.097	0.101	0.099

Note: [a] The concentration of biotinylated pAb-DEHP was 0.979 mg mL^{-1}.

434 Table 2 Cross-reactivity of Bio-pAb-DEHP with DEHP structural analogues.

Analogues	Structure	IC_{50}（μg L^{-1}）	Cross-reactivity（%）
DEHP		0.526	100
DMP		7.547	2.03
DEP		7.708	3.73
DPrP		7.999	5.07
DBP		5.360	4.63
DIBP		11.274	5.17
DINP		37.974	6.32
4-DEHNP		3.024	15.68
4-DEHAP		1.979	19.45

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437 Table 3 Concentrations of DEHP in beverages by BA-ELISA and GC-MS.

Samples			Concentration (mean±SD) (μg·L^{-1}) (n=6)	
			BA-ELISA	GC-MS
Beverages	Beer	B1	10.67±0.088	8.34±0.023
		B2	12.39±0.107	9.65±0.029
		B3	10.05±0.096	8.72±0.020
	Mineral water	M1	1.92±0.059	1.21±0.026
		M2	1.79±0.067	1.73±0.022
		M3	1.18±0.052	＜LOD
	Tea flavored beverage	T1	1.22±0.039	0.98±0.011
		T2	1.38±0.051	1.06±0.015
		T3	1.46±0.042	1.23±0.012
	White liquor	W1	40.68±0.126	38.77±0.037
		W2	22.82±0.114	20.05±0.026
		W3	17.95±0.099	15.86±0.033

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Table 4 Recovery of DEHP detected by BA-ELISA and GC-MS in spiked beverages.

Samples			Sample concentration ($\mu g \cdot L^{-1}$)		Spiked Level ($\mu g \cdot L^{-1}$)	Average Recovery % and CV % (n=6)	
			BA-ELISA	GC-MS		BA-ELISA	GC-MS
Beverages	Beer	B1	10.67	8.34	5	109.33±6.53	108.89±2.96
					10	101.15±9.65	99.45±2.86
					20	95.28±7.42	88.73±2.71
	Mineral water	M1	1.92	1.21	0.5	108.54±6.78	102.39±3.91
					1	99.83±8.85	95.36±4.74
					5	92.34±7.96	89.25±2.86
	White liquor	W1	40.68	38.77	20	106.31±5.97	104.62±3.31
					50	96.04±6.66	98.69±3.85
					100	89.07±10.68	93.96±4.06
		W2	22.82	20.05	10	109.31±7.56	106.195±2.98
					20	100.86±5.35	98.36±4.33
					50	93.15±6.59	93.88±3.02

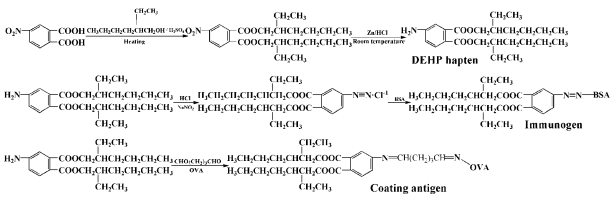
Figure Captions

Fig.1.The synthesis of DEHP hapten, immunogen, coating antigen.

Fig.2. The UV spectra of DEHP hapten, protein and conjugates; absorbance value at Characteristic peak, 329 nm: $OD_{BSA\text{-}DEHP}$=0.895, $OD_{DEHP\ hapten}$=0.783, OD_{BSA}=0.005; 343 nm: $OD_{OVA\text{-}DEHP}$=0.816, $OD_{DEHP\ hapten}$=0.258, OD_{OVA}=0.149; C_{BSA}: 0.25 g Ł$^{-1}$, C_{OVA}: 0.28 g Ł$^{-1}$, C_{hapten}: 0.05 g Ł$^{-1}$; protein and conjugate were dissolved in PBS buffer; hapten was dissolved in DMF.

Fig.3. Suitable operating conditions of the immunoassay method: (a) the blocking reagent, (b) ionic strength in PBS buffer, (c) pH of buffer, (d) concentrations of SA-HRP, (e) incubation time, and (f) the influence of different volume percentages of solvent on PBS buffer.

Fig.4. Standard curve for DEHP analyzed by BA-ELISA. The concentrations of DEHP were 0 μg Ł$^{-1}$, 0.001 μg Ł$^{-1}$, 0.01 μg Ł$^{-1}$, 0.05 μg Ł$^{-1}$, 0.1 μg Ł$^{-1}$, 0.25 μg Ł$^{-1}$, 1 μg Ł$^{-1}$, 2.5 μg Ł$^{-1}$, 5 μg Ł$^{-1}$, 10 μg Ł$^{-1}$, 25 μg Ł$^{-1}$, 50 μg Ł$^{-1}$. The linear working range was from 0.021 μg Ł$^{-1}$ to 12.948 μg Ł$^{-1}$. The linear equation was $Y = 21.57\text{Log}C_{DEHP} + 56.01$ (R^2=0.9850, n=16).



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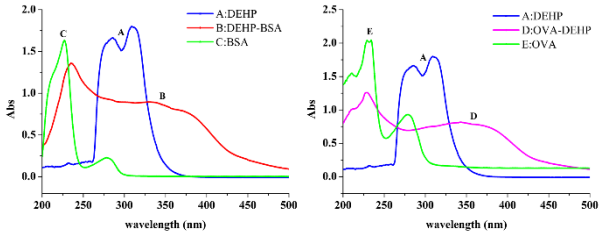

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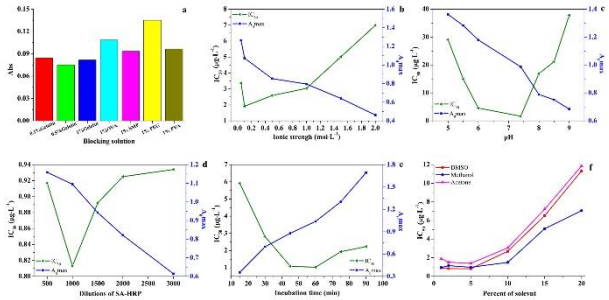

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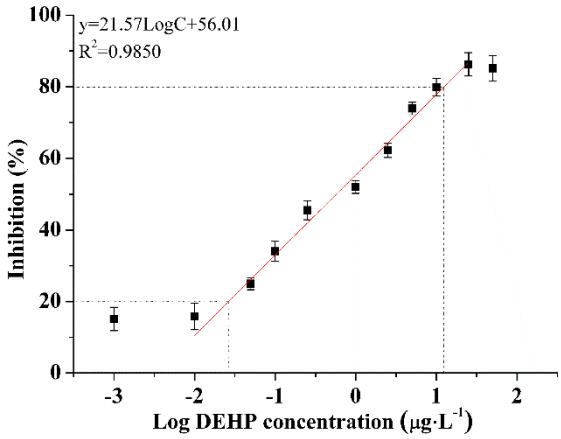

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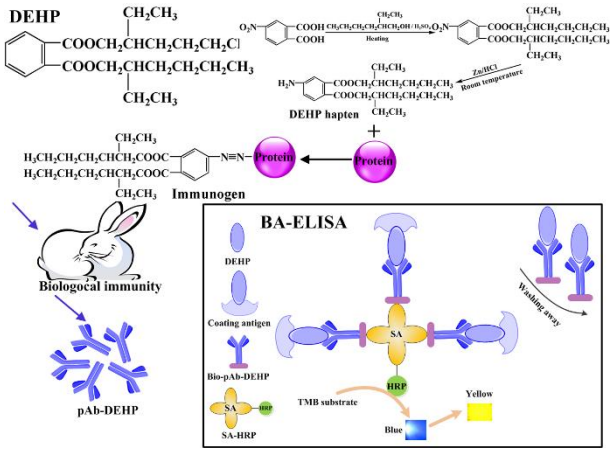

Graphical and textual abstract