Research Article

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A one-step incubation ELISA kit for rapid determination of dibutyl phthalate in water, beverage and liquor

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Abstract: A one-step incubation ELISA kit based on monoclonal antibody against dibutyl phthalate (DBP) was developed. After optimizing concentrations of coating antigen, antibody and composition of the assay buffer, an inhibition curve was plotted. The IC_{50} is $29.6 \text{ ng}\cdot\text{mL}^{-1}$, and the detection limit for DBP is $3.6 \text{ ng}\cdot\text{mL}^{-1}$. Compared with other ELISA methods, this ELISA kit had a simpler sample preparation, costed less time for detection and could detect more types of sample. The recoveries of DBP in water, beverage and liquor samples were range from 78% to 110.4%, the range of coefficient of variations is 7.7-15.3%. The cross reactivity was very low (<1%) except that for butyl benzyl phthalate (3.9%) and the di-isobutyl phthalate (12.5%). The detection results in liquor showed good correlation with those from GC-MS. All data above indicated that this kit could be used as the fast and high-throughput screening of DBP in water, beverage and liquor.

Keywords: dibutyl phthalate; ELISA kit; one-step incubation; beverage; liquor.

1 Introduction

Phthalate acid esters (PAEs) are ubiquitous industrial chemicals that are used as plasticizers, lubricants, cosmetics ingredients, and other applications. The commonly used PAEs are diethylhexyl phthalate (DEHP), dimethyl phthalate (DMP), butyl benzyl phthalate (BBP), dibutyl phthalate (DBP) and di-n-octyl phthalate (DOP) [1].

Several phthalates, including DBP, have demonstrated weak oestrogenic activity in vitro. A study of 168 men performed by Duty et al suggested that some phthalate monoesters are associated with lower sperm concentration, lower motility and increased percentage of sperm with abnormal morphology in humans [2]. In a study published in 2005, Swan reported that human phthalate exposure during pregnancy results in decreased anogenital distance in baby boys [3]. The phthalate-related syndrome of incomplete virilization in prenatally exposed rodents indicates the relationship between abnormal development of male genital and phthalate exposure. In recent studies, in utero exposure to phthalates in general has been shown to have anti-androgenic effects on the foetus [4,5]. Additionally, phthalates exposure could have side effects on the sexual development of women. In 2004-2007, a multiethnic longitudinal study of 1,151 girls (6-8 years of age) from five cities was performed to investigate the associations of phthalate and pubertal development. A positive trend was observed for phthalates with breast and pubic hair development [6]. In a zebrafish embryo toxicity test, DBP showed the highest toxicity, with a lethal concentration required to kill 50% of the population (LC_{50}) of $0.63 \text{ mg}\cdot\text{kg}^{-1}$ in six PAEs. Additionally, BBP, DBP, DEHP and DINP demonstrated oestrogenic activity [7]. Due to their toxicity, DEHP, DBP and BBP have been identified as reprotoxic, category 2 compounds by EU in 2005 [8].

Due to exposure from dietary ingestion and many commonly used products, the PAEs have found their way into every corner of our lives. Based on the concentrations

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of phthalate metabolites in 183 urine samples, the estimated daily intakes of DBP in China were $12.2 \mu\text{g}\cdot\text{kg}^{-1}$ bw/day, exceeding the suggested tolerable daily intake of $10 \mu\text{g}\cdot\text{kg}^{-1}$ bw/day by European Food Safety Authority [9]. Digestive intake was dominated the human exposure to phthalates [10]. Phthalate esters in food are mainly due to contamination from plastic food packaging and processing. Phthalates are fat-soluble compounds and can very easily enter the food via fats and oils, which then become phthalate-enriched.

Considered the health effects of phthalates, the Chinese Ministry of Health (MOH) released an administrative notice on the maximum residue limit (MRL) of phthalates in food and food additives on 13 June 2011 (MOH Notice 551): DEHP ($1.5 \text{ mg}\cdot\text{kg}^{-1}$), DINP ($9.0 \text{ mg}\cdot\text{kg}^{-1}$) and DBP ($0.3 \text{ mg}\cdot\text{kg}^{-1}$). The US Food and Drug Administration (FDA) published a guidance document on the use of phthalates in products in 2012. The draft guidance recommends that the pharmaceutical industry should avoid the use of DBP and DEHP as excipients in drugs and biological products.

Chromatographic analyses including HPLC-MS, and GC-MS are the most used methods for PAEs determination [11-14]. Although these methods are sensitive and accurate, they are also labourious, expensive, time-consuming and unsuitable for effectively monitoring the PAEs in environment and food. By contrast, the enzyme immunoassay method has excellent selectivity due to the high specificity of the antibodies, and therefore, the sample pre-treatment requirements are relatively simple and inexpensive. Multiple samples can be measured simultaneously, making this method suitable for the rapid detection and screening of a large number of samples.

The first study of the antibodies against PAEs was reported in a patent by Yanaihar et al [15]. Zhuang et al. have developed several ELISA methods and prepared antibodies against PAEs [16-19]. However, the antibodies used in these works were polyclonal antibodies. A monoclonal antibody specific to DBP was produced by Wei et al., who developed an indirect competitive enzyme-linked immunosorbent assay (icELISA) method employing direct coating of hapten on polystyrene microtiter plates [20]. The limit of detection (LOD) of their method was $0.426 \text{ ng}\cdot\text{mL}^{-1}$ for DBP. In their study, polystyrene was oxidized and directly linked to hapten. However, besides the additional time of the pre-coating process, the entire analysis required almost 3 h and contained two incubation steps.

In this work, based on the DBP monoclonal antibody developed in our lab, a one-step incubation rapid ELISA kit for DBP was developed. After the optimization of icELISA, the kit could detect the DBP in water, beverages

and liquor. The detection time was decreased to 30 min, which was faster than the methods mentioned above. The results of DBP detection by ELISA kit were consistent with the results of the detection by GC-MS.

2 Experimental

2.1 Reagents and chemicals

Dibutyl phthalate (DBP), diethylhexyl phthalate (DEHP), dimethyl phthalate (DMP), butylbenzyl phthalate (BBP), 4-nitrophthalic anhydride, and 1-butanol were purchased from J&K Scientific Ltd. (Beijing, China). Methyl 4-hydroxybenzoate, ethyl 4-hydroxybenzoate, isopropyl 4-hydroxybenzoate, butyl 4-hydroxybenzoate, isobutyl 4-hydroxybenzoate, pentyl 4-hydroxybenzoate, *n*-heptyl 4-hydroxybenzoate, and butyl formate were of analytical grade and were obtained from Sinopharm Chemical Reagent Beijing Co., Ltd. (Beijing, China). Bovine serum albumin (BSA), complete and incomplete Freund's adjuvant, and goat anti-mouse IgG-HRP (IgG-HRP) were obtained from Sigma-Aldrich (St. Louis, MO, USA). HAT, HT, Cell-culture media (DMEM), foetal bovine serum and supplements were obtained from Gibco Life Technologies Corporation (Carlsbad, CA, USA). 3,3',5,5'-Tetramethylbenzidine (TMB) substrates, blocking buffer and immobilized biomolecule stabilizer were purchased from Biopanda Diagnostics (UK). Clonotyping ELISA kit was obtained from SouthernBiotech, and 96-well microplates were purchased from Corning-Costar (Tewksbury, MA, USA). Chromatography-grade reagents such as hexane, methanol, ethanol, *N,N*-dimethylformamide (DMF) and dimethyl sulphoxide (DMSO) were purchased from Thermo Fisher Scientific (MA, USA). All of the other reagents were of analytical grade or higher.

2.2 Apparatus

The cells were incubated in a SANYO MCO-15AC incubator (Moriguchi-shi Osaka, Japan). For ELISAs, a plate washer (ELx50/8) purchased from BioTek (Bad Friedrichshall, Germany) was used. Immunoassay absorbance was recorded using a Thermo Scientific Multiskan MK3 Microplate spectrophotometer (Thermo Fisher Scientific, Inc. MA, USA). The UV-2802 UV/Vis spectrophotometer used in this work was purchased from UNICO Instruments Co., Ltd. (Shanghai, China).

Figure 1: The synthetic route of DBP-BSA.

An Agilent GC-MS System (7890A+5975C) with an Agilent DB-5MS column (30 m x 0.25 mm x 0.25 μm) (California, USA) was used.

2.3 Hapten synthesis

Concentrated sulphuric acid (1 mL) was added slowly to a solution of 4-nitrophthalic anhydride (2.5 g) in 50 mL 1-butanol. The mixture was stirred and heated at reflux (120°C) for 7 h. The residue was washed with 10 mL of 10% (w/v) aqueous Na_2CO_3 three times and was evaporated to remove water and 1-butanol at 80°C . The oily residue (0.15 g) was dissolved in benzene (28 mL), and zinc dust (0.28 g) was added to the solution. Then, hydrochloric acid (8.2 mL) was added in three portions. After 18 min of stirring at room temperature (RT), more zinc dust (0.28 g) was added, and the mixture was stirred at RT for 12 h. The benzene layer was washed with water (34 mL) and dried over anhydrous sodium sulphate (5 g) three times. The benzene layer was removed by evaporation, and a yellow crude solid of dibutyl 4-aminophthalate (DBAP) was obtained. The product was analysed by TLC and nuclear magnetic resonance spectroscopy (NMR).

DBAP (0.0466 g) was added to a stirred mixture of concentrated hydrochloric acid (0.1 mL), water (3 mL) and DMF (2 mL) at 5°C . NaNO_2 (0.2 M) was added dropwise until the solution turned the potassium iodide starch test paper blue. The mixture was stirred at 5°C for 30 min. BSA (160 mg) in sodium borate (40 mL, pH 9.0) was added dropwise. The colour of the mixture changed from colourless to a reddish orange. After 2 h of stirring, the mixture was dialyzed against phosphate buffered saline (PBS, 10 mM, pH 7.4) that was changed twice a day for three days at 4°C . The mixture dialyzed was the DBAP-BSA conjugate (Figure 1).

2.4 Preparation of antibodies

DBAP-BSA (100 μg) in 0.1 mL sterilized PBS was emulsified with an equal volume of Freund's complete adjuvant. This solution was injected intraperitoneally into each of several 7-week-old female BALB/c mice. After 3 weeks, the mice were boosted with another 100 μg DBAP-BSA that was emulsified with Freund's incomplete adjuvant (1:1). Ten days after injection, serum was collected from the caudal vein of each mouse, and titres of antisera were determined by indirect ELISA. A positive mouse was sacrificed for cell fusion after four immunizations. The spleen was removed aseptically, and splenocytes were fused with the cells of the Sp2/0 murine myeloma cell line in the presence of polyethylene glycol, molecular weight 4000. After fusion, the cells were suspended in DMEM HAT medium containing 16% foetal bovine serum (FBS) and added to 96-well microplates. After 7 days, culture supernatants from each well were assayed using indirect competitive ELISA. Hybridoma cells in the ELISA-positive wells were subcloned by the limiting dilution method. Female BALB/c mice were treated with intraperitoneal injection of pristine (0.2 mL), and then intraperitoneally injected with cloned hybridoma cells (1.0×10^7 in PBS). Antibodies were purified from the ascitic fluids by protein-A-agarose affinity chromatography [21].

2.5 Optimization of icELISA

Checkerboard titration was used to select the appropriate concentrations of coated antigen, antibody and IgG-HRP. The checkerboard assays were performed using indirect competitive ELISA (ic-ELISA).

Microplates were coated overnight at 4°C with 100 μL per well of DBAP-BSA in 0.05 M sodium carbonate buffer (pH 9.6). After the incubation at 4°C , the microtiter plates

were washed three times with PBST (0.01 M, pH=7.4, 0.05% Tween 20) after each incubation step. The wells were blocked with 200 μ L blocking buffer. After another washing step, the plates were vacuum dried, vacuum sealed and stored in 4°C.

2.6 Development of ELISA methods

After the temperature of the microplates reached the room temperature, 50 μ L/well standard or sample solution, 50 μ L/well antibody and 50 μ L/well HRP-conjugated IgG were added and incubated at 37°C for 20 min. Following a washing step, 100 μ L/well TMB substrates were added. The colour development was stopped after 10 min by adding 50 μ L/well stop solution (2 M H₂SO₄). The absorbance was detected at 492 nm with 630 nm as reference wavelength.

2.7 Optimization of the assay buffer

Methanol, ethanol, DMF and DMSO were mixed with PBST in several ratios, and then, DBP was dissolved in the buffer for the preparation of the standard solution.

2.8 Development of the standard curve of ELISA kits

DBP was diluted with assay buffer to the concentrations of 0, 5, 15, 45, 135, and 405 ng·mL⁻¹. After the ELISA procedure, the results were expressed as the binding percentage:

$$\text{Binding (\%)} = (B/B_0) \times 100,$$

where B is the mean absorbance in the presence of the DBP standard sample or analysed sample and B₀ is the mean absorbance in the absence of DBP. A standard curve was plotted based on the binding (%) and DBP concentrations.

2.9 Sample preparation for ELISA

For the bottled water and carbonated beverages, the sample (5 g) was transferred to a clean, dried glass tube, and chromatography-grade hexane (2 mL) was added. For liquor and some beverages such as orange juice or tea drinks, the sample (1 g) was transferred to the tube, and water (4 mL) and chromatography-grade hexane (2 mL) was added. All tubes were capped and vibrated. After vibrating for 3 min, 1 mL of the supernatant was transferred

to another clean, dried glass vessel and evaporated naturally. The residues were dissolved in assay buffer (1 mL) for a further ELISA test. The samples for which the DBP content exceeded the highest concentration of the standard solution were diluted with assay buffer and re-analysed.

2.10 Accuracy, specificity and precision of the ELISA kit

The LOD was determined as the DBP concentration that gives a 10% inhibition (IC₁₀). Three concentration levels of DBP in fortified samples were investigated: 0, 0.3 and 0.6 μ g·mL⁻¹. Three sets of five samples each purchased from a supermarket were spiked with the DBP standard. The IC₅₀ value is obtained by calculating DBP concentration corresponding to 50% of B₀. Cross-reactivity (CR) for each DBP analogue was calculated according to [20,22]:

$$\text{CR \%} = (\text{IC}_{50} \text{ value of DBP}) / (\text{IC}_{50} \text{ value of DBP analogue}) \times 100$$

2.11 Storage stability study

Excess ELISA kits were stored at 4°C and were used to perform the standard curve experiments every month. Another set of kits was stored at 37°C and was used to carry out the ELISA method measurements every day. The B₀ and IC₅₀ values of each kit were investigated.

2.12 Validation with GC-MS

Overall, 24 liquor samples collected from the markets were evaluated with an ELISA kit and the national standard method (GB/T 21911-2008, Determination of phthalate esters in foods). The sample (5 mL) was added to hexane (2 mL) and vortexed vigorously for 1 min. After standing to allow the formation of distinct layers, 1 mL of the upper layer was transferred for GC-MS analysis.

DBP was detected by gas chromatographic with a DB-5MS column and with helium as the carrier gas. 1 microliter of sample was injected in splitless mode at 250°C with the oven program as follows: 60°C, for 1 min; increase at a rate of 20° C·min⁻¹ up to 220 °C, hold for 1 min; and increase at a rate of 5° C·min⁻¹ up to 280°C, hold for 4 min. The MS analysis was performed in the selected ion monitoring (SIM) mode, and the detector was operated in the electron impact (EI) mode at 70 eV. The transfer line

and source temperatures were both maintained at 280°C. DBP SIM ions (m/z): 149, 223, 205, and 104.

Ethical approval: The conducted research is not related to either human or animal use.

3 Results and discussion

3.1 Preparation and characterization of monoclonal antibody

Five monoclonal antibodies showed good affinity to DBP (data not shown). The antibodies titers were assessed by indirect competitive ELISA (icELISA). The antibody of 1D2 had good titers and the lowest IC_{50} value for DBP. The isotype of antibody 1D2 was IgG_1 and the light chain was kappa, as determined by the SouthernBiotech Clonotyping ELISA kit. The affinity constant of antibody reached $6.3 \times 10^7 \text{ L} \cdot \text{mol}^{-1}$, which was evaluated by indirect non-competitive ELISA as described by Beatty et al²³.

3.2 Optimization of ELISA

To optimize the conditions of the coating process, the type, pH and concentration of the coating buffer were investigated carefully. Citrate buffer, phosphate buffered saline and carbonic acid buffer were compared in this work. Among these solutions, the carbonic acid buffer was found to be the most efficient buffer (data not shown). The B_0 values under different pH and different concentrations of carbonic acid buffer are shown in Figure 2. The highest B_0 value was obtained when the buffer concentration was 50 mM at pH 9.6, indicating that maximum immobilization of DBP-BSA conjugates in the plate well occurred. Therefore, the carbonic acid buffer (50 mM, pH=9.6) was chosen as the coating buffer in the subsequent experiments.

In this experiment, organic solvents were utilized to increase the solubility of DBP²⁰ and modify the interaction between the DBP and its monoclonal antibody in the assay buffer. Four water-miscible organic solvents such as methanol, ethanol, DMF and DMSO were used to prepare the standard solution and the sample solutions. As shown in Figure 3, compared to the DMF and DMSO solutions, the B_0 values were higher in the methanol or ethanol solutions, indicating greater DBP antibody binding onto the immobilized DBP-BSA conjugates. The experimental group with a higher B_0 and lower IC_{50} value means that the solution was more suitable for the specific binding between the DBP antibody and the antigenic determinant

Figure 2: Effect of pH and concentrations of carbonic acid buffer on B_0 value ($n=3$).

Figure 3: The influence of organic solvent on the B_0 and IC_{50} .

of DBP. When 20% methanol was used as the standard solution, the highest B_0 value and lowest IC_{50} value were obtained (Figure 3a); therefore, this solution was selected for further optimization assay.

Table 1: Optimization of concentrations of coating antigen, antibody and IgG-HRP.

Concentration of antigen/($\mu\text{g}\cdot\text{mL}^{-1}$)	Concentration of antibody/($\mu\text{g}\cdot\text{mL}^{-1}$)	Dilution ratio of HRP-IgG	B_0	RSD%	$IC_{50}/(\text{ng}\cdot\text{mL}^{-1})$
0.8	100	1/4000	2.539	7.8%	74
	50	1/2000	2.167	6.9%	63
	50	1/4000	1.813	7.3%	48
	25	1/2000	1.291	11.6%	29
0.4	100	1/2000	2.017	7.6%	44
	100	1/4000	1.726	8.5%	35
	50	1/2000	1.442	13.2%	22
	50	1/4000	1.108	15.1%	16
0.2	200	1/2000	1.390	16.7%	45
	200	1/4000	1.238	16.3%	37
	100	1/2000	1.085	17.9%	16
	100	1/4000	0.940	--	--

The results of the checkerboard determination (Table 1) indicated that the concentrations of DBP-BSA, antibody and HRP-IgG all affected the IC_{50} value. The IC_{50} value changed slightly with the changes in the HRP-IgG concentration, while the changes in the concentrations of DBP-BSA and antibody had a marked effect on the IC_{50} value. While lower IC_{50} values were obtained at lower antigen and antibody concentrations, the B_0 value was lower and the relative standard deviation (RSD) was higher at the same time. For a commercial ELISA kit, the B_0 value was critical for long-term storage, and the RSD was critical for the accuracy of the final results. Taking these results together, we chose the concentration of the DBP-BSA used for coating to be $0.4 \mu\text{g}\cdot\text{mL}^{-1}$; the concentration of antibody was $100 \text{ ng}\cdot\text{mL}^{-1}$, and that of IgG-HRP was 1:4000, for which the B_0 value was higher than 1.5, the RSD was less than 10% and the IC_{50} value was low.

3.3 Standard curves of icELISA for DBP

The standard curve was obtained under the optimized conditions described above (Figure 4). The concentrations of DBP were 0, 5, 15, 45, 135, and $405 \text{ ng}\cdot\text{mL}^{-1}$. The IC_{50} value was calculated as $29.6 \text{ ng}\cdot\text{mL}^{-1}$, and LOD (IC_{10}) was calculated as $3.6 \text{ ng}\cdot\text{mL}^{-1}$. Thus, this kit is less sensitive than Wei's immunoassay for DBP ($IC_{50}=14.6 \text{ ng}\cdot\text{mL}^{-1}$) [20] but is similar to Kuang's assay ($IC_{50}=33.6 \text{ ng}\cdot\text{mL}^{-1}$) [24]. However, both of their methods require two-step incubations and more than 1 h, while the incubation time of this kit was less than theirs and the IC_{50} was sufficiently low for the MRL in China ($0.3 \mu\text{g}\cdot\text{mL}^{-1}$ in food).

Figure 4: The Standard curve for DBP detection by ELISA.

3.4 Cross-reactivity

Eleven phthalate esters were tested for cross reactivity (CR) using the ELISA kit. As shown in Table 2, for all of the tested compounds, the obtained CR values were very low except those for BBP (3.9%) and DIBP (12.5%).

3.5 Recoveries in some consumer products

The detection of DBP in bottled water and liquor has been reported by Zhang et al. and Xu et al., respectively [16,25]. These two types of samples were reasonably clean compared to the beverages, and therefore, these two samples did not require dilution prior to extraction. For the detection of DBP in carbonated beverages and tea-flavoured beverages, Sun et al. developed a method using

Table 2: Cross reactivity for ELISA kit analysis of DBP analogue.

Compound	IC ₅₀ /(ng·mL ⁻¹)	Cross-reactivity %
dibutyl phthalate(DBP)	29.6±1.2	100
butyl benzyl phthalate(BBP)	762.8±23.2	3.9
di(2-ethylhexyl)phthalate(DEHP)	>9100	<1
di-isobutyl phthalate(DIBP)	236.1±16.0	12.5
methyl 4-hydroxybenzoate	>9100	<1
ethyl 4-hydroxybenzoate	>9100	<1
isopropyl 4-hydroxybenzoate	>9100	<1
butyl 4-Hydroxybenzoate	>9100	<1
isobutyl 4-Hydroxybenzoate	>9100	<1
pentyl 4-Hydroxybenzoate	>9100	<1
heptyl 4-Hydroxybenzoate	>9100	<1
butyl formate	>9100	<1

Table 3: Recoveries of DBP spiked in samples (n=5).

Samples	Spiked level/($\mu\text{g}\cdot\text{mL}^{-1}$)	Average Recovery (%)	RSD (%)
Bottled water	0.3	110.4	7.7
	0.6	102.5	8.0
Carbonated beverages	0.3	87.9	10.0
	0.6	97.9	15.3
Juices and tea drinks	0.3	80.3	9.5
	0.6	98.0	13.0
Liquor	0.3	81.7	10.2
	0.6	78.0	7.8

ultrasonic processing for 30 min three times [26]. This sample processing requires much time and is not suitable for a rapid ELISA kit, and hence, we developed a new preparation for the beverages in this work.

Five samples from a retail store were spiked with 0.3 and 0.6 $\mu\text{g}\cdot\text{mL}^{-1}$ of DBP. The samples were mixed, stored at room temperature overnight, and then analysed using the ELISA kit. The recoveries of DBP in various samples ranged from 78% to 110.4%, and the range of the coefficient of variations was 7.7-15.3% (Table 3). The high recoveries indicated that the sample preparation could extract the DBP with satisfactory results.

Since the ELISA kit exhibited approximate and acceptable recoveries and the MRL requirements of DBP in

Figure 5: Validation of the assay with GC-MS. a, A typical GC-MS chromatogram for DBP in liquor samples. b, correlation of GC-MS and ELISA analysis of certain liquor.

food is 0.3 $\mu\text{g}\cdot\text{mL}^{-1}$, the ELISA kit could be applied to screen DBP residues in water, beverage, and liquor.

3.6 Storage stability study

Table 4 shows the summarized results of the accelerated stability study of the ELISA kit. According to the reference, 7 days at 37°C is equivalent to 1 year at 4°C [27]. The kits stored at 37°C showed performance that was similar to that of the kits stored at 4°C. The results collectively indicate that the DBP ELISA kit has a shelf life of at least 1 year.

3.7 Validation of the assay with GC-MS

Overall, 24 liquor samples were evaluated with the ELISA kit and the GC-MS. The typical GC-MS chromatogram for DBP is shown in Figure 5a. DBP was not detected in 6 samples using both methods, and the DBP values were in the range of 0.05-1.76 $\text{mg}\cdot\text{kg}^{-1}$ (GC-MS) in the other 18 samples. Good correlation was obtained between the results of the ELISA and GC-MS methods, as characterized

Table 4: Results for accelerated stability of ELISA kits. ($n=3$).

Stored at 4 °C			Stored at 37 °C		
storage period (months)	B_0	IC_{50} /(ng·mL ⁻¹)	storage period (days)	B_0	IC_{50} /(ng·mL ⁻¹)
0	2.066	29.6	0	2.064	29.6
1	1.985	31.7	2	1.965	32.7
2	1.954	30.6	4	1.827	28.6
4	1.885	26.5	6	1.821	30.5
6	1.893	32.2	9	1.773	34.2
8	1.935	30.1	12	1.659	35.1
10	1.788	28.0			
12	1.769	25.8			
14	1.723	24.7			

Table 5: Comparison of Several ELISA methods.

	This method	ELISA 1	ELISA 2	Biotin-Streptavidin ELISA
Antibody	monoclonal antibody	monoclonal antibody	polyclonal antibody	polyclonal antibody
Sample type	water, beverage and liquor	Liquor	White wine	Beverages and drinking Water
Estimated assay time	60 min	N/A*	65 min	More than 200 min
IC_{50} (ng·mL ⁻¹)	29.6	33.6	292.1	0.36
LOD (ng·mL ⁻¹)	3.6	21.5	64.5	0.005
Specificity	High	High	Moderate	High
Accuracy	Good	Good	Good	Good
Reference	This article	24	25	26

*N/A: not available

by the linear equation $y = 0.983x$, $R^2 = 0.9625$. This result further confirmed the reliability of the ELISA kit for the detection of DBP (Figure 5b).

3.8 Comparison with other ELISA methods

Three published ELISA methods for DBP detection were compared with the present method as shown in Table 5. Although the ELISA method reported in reference 26 is the most sensitive, it is also the most time-consuming. However, taking all factors together, the present method has the best combination of high sensitivity and analysis speed. Furthermore, the method has the more extensive applications for food than the other methods do.

4 Conclusion

A simple, fast and accurate ELISA kit for DBP was developed in this work. The IC_{50} was 29.6 ng·mL⁻¹, and the LOD of DBP was 3.6 ng·mL⁻¹. A simple sample preparation could alleviate or eliminate the matrix effect for water, beverages and liquor. The recoveries of DBP in the samples ranged from 78% to 110.4%, and the range of the coefficient of variations was 7.7-15.3%. The detection results in liquor showed good correlation to those obtained by GC-MS. Compared to other ELISA methods, this kit requires less time and can detect more types of samples than can the other ELISA methods for DBP; additionally, this kit has a shelf life of at least 1 year. The kit can be applied to the fast and high-throughput screening for DBP in water, beverages and liquor.

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