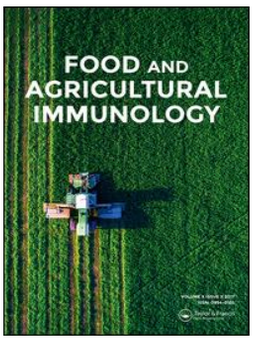

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Development of molecularly imprinted biomimetic immunoassay method based on quantum dot marker for detection of phthalates

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ABSTRACT

In this study, a direct competitive molecularly imprinted biomimetic immunoassay method was developed for determination of the plasticizer dibutyl phthalate. Water-soluble carboxyl quantum dots were used as the marker. A hydrophilic molecularly imprinted polymer synthesized directly on the surface of a 96-well plate was used as biomimetic antibody. Under the optimal conditions, the limit of detection and the sensitivity of the method were 0.011 and 0.136 mg/L respectively. The cross-reaction values of two structural analogs were 4.75% and 6.89%, this indicaties that the method has the good specificity. Edible oil samples spiked with dibutyl phthalate were assayed, and good recoveries ranging from 80.0% to 111.5% were obtained. The method was used to detect dibutyl phthalate residues in liquors, and there were no significant differences obtained using the developed method and those obtained using gas chromatography.

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Biomimetic immunoassay; molecular imprinting; quantum dots; dibutyl phthalate

Introduction

Phthalic acid esters, as a class of plasticizers, are often added to plastic food-packaging materials to increase the plasticity and ductility of the plastic (Benjamin, Pradeep, Josh, Kumar, & Masai, 2015; Sørensen, 2006; Sun, & Zhuang, 2015a). However, such plasticizers tend to migrate into foods as a result of environmental factors such as storage temperature and food polarity (Keresztes, Tatar, Záray, & Czegeny, 2013; Salazar-Beltrán, Hinojosa-Reyes, Ruiz-Ruiz, Hernández-Ramírez, & Guzmán-Mar, 2018; Shi et al., 2012). These plasticizers are hormonal persistent organic pollutants, they have nephrotoxicity and reproductive toxicity, and teratogenic mutagenic effects. They can enter the human body through the respiratory system and digestive tract. Long-term contact is a serious threat

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to human health (Seth, 1982; Sun, & Zhuang, 2015b). The specific migration limits of some phthalate plasticizers in food packaging materials are therefore limited by the European Union, the United States, and some other countries and regions (Salazar-Beltrán et al., 2018). Since a plasticizer incident in Taiwan in 2011, China has specified maximum residue limits for phthalate plasticizers in foods (0.2 mg/kg), and strictly monitors phthalate contents in foods (Zhang et al., 2017).

It is therefore necessary to establish sensitive and rapid methods for detecting plasticizer contamination in food and packaging materials. Phthalate plasticizers are generally detected by gas chromatography (GC), high-performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), and liquid chromatography-mass spectrometry (LC-MS) (Leng, Chen, & Wang, 2015; Sendón et al., 2012). Although GC and LC are widely used, they are susceptible to interference by complex substrates, and they have the disadvantages of low sensitivity and long operating times. GC-MS and LC-MS have strong anti-interference abilities and give accurate qualitative analysis (Grimalt & Dehouck, 2016), but the expensive instruments needed and complicated procedures make these methods inconvenient.

Immunoassays have the advantages of high specificities, sensitivities, and selectivities compared with conventional instrumental analyses (Haas-Lauterbach, Immer, Richter, & Oehler, 2012; Kuang, Xu, Cui, Ma, & Xu, 2010; Xu et al., 2016). However, traditional immunoassays have many shortcomings, such as the small-molecule compounds must synthesize haptens to have immunogenicity, the preparation process is tedious, and the physical and chemical properties of biological antibodies are unstable. Their application has been limited. The design and synthesis of low-cost biomimetic antibodies with high specificity, good stability, and performances comparable to those of biological antibodies are therefore important.

Molecularly imprinted polymers (MIPs) are synthetic material, which can be prepared according to different requirements (Venkatesh et al., 2017). Many results showed that the affinities and selectivities of MIPs are similar to those of natural biomolecular recognition systems (Altintas et al., 2015; Jiang, He, Gong, Gao, & Xu, 2019; Wang, Jiang, Ju, Qiao, & Xu, 2018). More importantly, MIPs have stable physical and chemical properties and can be used in a variety of complex environments. With structure-activity designability, specificity, and high stability, MIPs have been widely used in catalysis (Wulff & Liu, 2012), purification (Asliyuce et al., 2012), and enzyme-linked immunoassays (Chianella et al., 2013; Smolinska-Kempisty et al., 2016). MIPS are therefore ideal biomimetic antibodies for immunoassays.

For immunoassay technology, labelling techniques are needed. However, the use of enzymes as markers has disadvantages, e.g. enzyme molecules are large, and their size interferes with the specific recognition of spatial sites and reduces the sensitivity and specificity of biomimetic immunoassays (BIAs). The development of new markers for BIAs is therefore important. Quantum dots (QDs) are nanocrystals of semiconductor materials and have received increasing attention in recent years. QDs have many useful optical and chemical properties such as symmetric emission distributions, and high quantum yields, brightness, and light stability (Das, Bandyopadhyay, & Pramanik, 2018; Khan, Khan, & Zulfequar, 2017; Li, Qian, & Ren, 2005). QDs have been widely used in molecular biology (Tan et al., 2018), cell biology (Zhao et al., 2018), immunobiology (Li & Ding, 2016; Liao et al., 2019), and various detection applications (Gao, Zhao, Chen,

& Fan, 2018; Gui, Jin, Wang, & Tan, 2017; Jiang, Wu, Xu, Qiao, & Xu, 2017; Kumar, Joanni, Singh, Singh, & Moshkalev, 2018; Yi & Shen, 2015). QDs are smaller than traditional enzyme and are therefore more suitable markers than enzymes in BIAs.

Dibutyl phthalate (DBP) is one of the most widely used plasticizers. In this study, a sensitive direct competitive molecularly imprinted biomimetic immunoassay (MIBIA) method for DBP detection was established. Hydrophilic MIP membranes were used as artificial antibody and water-soluble carboxyl QDs as markers. The experimental conditions were optimized, and the applicability and accuracy of the proposed method were evaluated.

Materials and methods

Materials and reagents

Edible oil and liquor samples were purchased from rt-mart in March 2019 (Taian, China).

Diethyl phthalate (99.5%), diisobutyl phthalate (98%), and DBP (99%) were supplied by the Beijing Dikma Technology Co., Ltd. (Beijing, China). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 99%) was purchased from the Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). *N*-Hydroxysuccinimide (NHS, 98%) and methacrylic acid (MAA, analytical grade) were supplied by the Shanghai Macklin Biochemical Technology Co., Ltd. (Shanghai, China). Ethylene glycol dimethacrylate (EGDMA), 2-methylpropionitrile (AIBN), anhydrous ethanol, iron powder, ammonium chloride, ethyl acetate, anhydrous sodium sulfate, citric acid, sodium citrate, 2-(*N*-morpholino)ethanesulfonic acid (MES), methanol, acetone, and *n*-hexane were obtained from the Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). The acetone and *n*-hexane were chromatographic grade, and all other reagents were analytical grade. MAA and AIBN were purified before use. Water-soluble carboxyl QDs were obtained from the Wuhan Jiayuan Quantum Dots Co., Ltd. (Wuhan, China). MD34 dialysis bags were obtained from the Solarbio Life Sciences Technology Co., Ltd. (Beijing, China). A 0.22 μm filter film was purchased from the Jiangsu Green Union Science Instrument Co., Ltd. (Taizhou, China). Double-distilled water (DDW, 18.2 MΩ cm), which was used in all experiments, was prepared by using an Aike ultrapure water instrument (Chengdu, China).

Solutions of phosphate-buffered saline (PBS, 50 mmol/L sodium phosphate, 154 mmol/L NaCl, pH 7.0), PBS with 0.05% Tween-20 (PBS/T), and borate-buffered saline (BBS, 180 mmol/L boric acid, 5 mmol/L borax, pH 7.4) were used. A DBP stock solution (in methanol) was diluted to 5, 1, 0.2, 0.04, 0.008 mg/L. These solutions were used to obtain a standard detection curve.

Apparatus

A ProElut PSA solid phase extraction (SPE) column was supplied by the Beijing Dikma Technology Co., Ltd. (Beijing, China). Microlon 96-well plates were purchased from Beijing Biolead Biology Sci & Tech Co., Ltd. (Beijing, China). The 96-well plates were washed in a ST-36wt microplate washer (Kehua Bio-engineering Co., Ltd., Shanghai, China). Fluorescence detection was performed with a Spectra Max M5 multifunctional

microarray (Molecular Devices Co., Ltd.); the excitation wavelength and emission wavelength were 425 and 525 nm, respectively.

A Shimadzu 2010 GC system equipped with a flame ionization detector (FID) was used to determine DBP in liquor samples. An Rtx-5 elastic quartz capillary column (30 m × 0.32 mm × 0.25 m) was used. The carrier gas was nitrogen at a constant flow rate of 1.5 mL/min, the injection volume was 1.00 μL, the FID temperature was 280°C. The temperature programme was as follows: The temperature was maintained at 60°C for 2 min, increased to 220°C at 20°C/min and maintained for 3 min, and then increased to 280°C at 5°C/min and maintained for 5 min.

Synthesis of DBP haptens

The haptens were synthesized from 4-nitrophthalic acid by esterification, reduction, and diazotization. First, dibutyl 4-nitrophthalate was synthesized as follows. 4-Nitrophthalic acid (10 g) was added to *n*-butanol (20 mL), and then 1 mL of concentrated H_2SO_4 was added as a catalyst. The mixture was refluxed at 120°C for 12 h. The liquid was cooled to 70°C and washed three times with 15% Na_2CO_3 solution (100 mL). The oil was separated from the liquid with a separating funnel, and water and unreacted *n*-butanol were removed by vacuum distillation with a reduced-pressure rotary evaporator. A yellow oily liquid (dibutyl 4-nitrophthalate) was obtained after cooling, and the yield was 89%.

Dibutyl 4-nitrophthalate (7.0 g) was added to methanol (150 mL), and iron powder (11.2 g) and ammonium chloride (10.7 g) were added to the solution. The solution was condensed and refluxed under stirring at 80°C for 12 h. When the reaction was finished, the residue was removed by filtration. The mixed solution was neutralized with NaOH (80 mL, 1 mol/L). After 150 mL of ethyl acetate was added, the ethyl acetate layer was removed and the solution was dried with anhydrous sodium sulfate. 4-Aminodibutyl phthalate was obtained by resteaming with anhydrous ethanol, and the yield was 90%. The reaction solution was placed in a dialysis bag and dialyzed with DDW for 5 d; the DDW was changed twice a day. The DBP haptens were freeze-dried and stored.

Preparation of QD–hapten conjugate

Fluorescent carboxyl QDs were conjugated with DBP haptens via the following procedure. The water-soluble fluorescent QDs (25 mg) were dispersed in MES buffer (1 mL, 0.1 mol/L, pH 4.7) and then EDC (4.8 mg) and NHS (5.8 mg) were added. The activated fluorescent carboxyl QDs were obtained by reaction at 37°C for 30 min. After centrifugation, the DBP haptens (0.2 mg) and activated fluorescent carboxyl QDs were mixed with BBS (50 mmol/L, pH 8.5). The QD–hapten conjugates were obtained by reaction for 3.5 h at 37°C. After ultrafiltration, the conjugates were resuspended in PBS buffer (0.02 mol/L, pH 7.4). The QD–hapten conjugates were stored at 4°C.

Preparation of molecularly imprinted film on surface of 96-well plate

DBP (0.288 g) and MAA (0.258 g) were dissolved in a mixture of DDW (8 mL) and acetonitrile (12 mL); the solution was stirred for 30 min. AIBN (35 mg) and EGDMA (1.508 mL) were added, and the mixture was stirred continuously for 1 h. The mixture

(200 μL) was placed in each well of a 96-well plate, and the plate was left in the dark at 38° C for 18 h under nitrogen. The plate was washed with DDW and methanol to remove the unreacted solution. After ultrasonic extraction with methanol/acetic acid (320 mL, 7:1, v/v) for 8 h and methanol (300 mL) for 4 h, the plate was dried at 37°C for 4 h.

For comparison, a non-imprinted polymer (NIP) was synthesized via the same method, but without addition of DBP.

Direct competitive MIBIA method

The plate was washed three times with a PBS/T solution. The QD–hapten conjugates were diluted with BBS (pH 7.6) at a ratio of 1:30 (v/v). The methanol solution was added to the blank wells (100 μL) and control wells (100 μL). BBS buffer was added to the blank wells (100 μL) and a standard solution (100 μL) was added to allocated wells. The QD–hapten conjugates (100 μL) were immediately added to the control wells and allocated wells. The 96-well plates were mechanically stirred (400 r/min) in the dark for 1 h at room temperature and then washed five times with PBS/T. The fluorescence values were recorded. The inhibition rates of the direct competitive reaction of DBP at different concentrations were calculated according to the following equation:

$$\mathrm{IC\%} = \{1 - [(F_{\mathrm{sample}} - F_{\mathrm{blank}})/(F_{\mathrm{control}} - F_{\mathrm{blank}})]\} \times 100.$$

IC%: the inhibition rate of standard solutions to competitive reaction; F_{sample}: the average fluorescence value of standard solutions; F_{blank}: the average fluorescence value of the blank wells; F_{control}: the average fluorescence value of the control wells. Finally, the plate was ultrasonically extracted with methanol/acetic acid (320 mL, 7:1, v/v) for 8 h and then with methanol (300 mL) for 4 h for the next MIBIA procedure.

Sample preparation

The accuracy and applicability of the MIBIA method were evaluated by assaying edible oil samples which spiked with DBP. Standard solutions of DBP (1 mL; 0.05, 0.1, and 0.2 mg/L) were added to 50 mL samples. The samples were allowed to stand for 3 h and then completely transferred to a test tube. Acetonitrile-saturated *n*-hexane solution (40 mL; the purification solution) and *n*-hexane-saturated acetonitrile solution (80 mL; the extraction solution) were added to the test tube, and ultrasonication was performed for 20 min. The solution was centrifuged at 8000 r/min for 10 min. The extraction procedure was repeated. The lower solution layers were collected and dried by rotary evaporation at 38°C. After accurate redissolution in methanol (1.0 mL), the solution was analyzed by the MIBIA method and the DBP level was calculated.

Liquor was selected as the sample for comparative analysis. The pretreatment method was similar to that used for edible oil, except that no purification solution was added during pretreatment. Then acetone (5 mL) and *n*-hexane (5 mL) were sequentially added to the SPE column to activate the column bed, and the effluent was discarded. The extraction solution was flowed through the SPE column at the rate of 1 mL/min. The effluent was collected, *n*-hexane (5 mL) and 4% acetone/*n*-hexane (5 mL) were sequentially added to the column, and the effluent was collected. The solution was dried by rotary evaporation at 38°C and then redissolved in 1.0 mL of *n*-hexane for GC

analysis (1.0 mL of methanol for MIBIA analysis). Before GC analysis, the solution was filtered through a 0.22 μm filter film.

Data analysis

The analysis of variance method (SAS Institute, Cary, NC, USA) was used to evaluate the differences between the results obtained by the MIBIA and GC methods.

Result and discussion

Optimization of conditions for hydrophilic MIP preparation

The template molecule, functional monomer, and crosslinker ratios used for preparing the MIP membrane were optimized to ensure that the prepared hydrophilic membrane had a high adsorption capacity. The various ratios and adsorption capacities are shown in Figure S1. Ratios of 1:2:2 gave the lowest adsorbed amount (4.536 μg/well) and ratios of 1:3:8 gave the largest adsorbed amount (9.595 μg/well). Ratios of 1:3:8 were therefore used in subsequent experiments.

Characterization of hydrophilic MIPs and DBP haptens by FT-IR spectroscopy

The IR spectra of the MIP before and after ultrasonic extraction and that of the NIP are shown in Figure 1(A). The stretching vibration peak for –OH appeared at 2920 cm^{-1} before MIP extraction and at 2933 cm^{-1} after MIP extraction. This red shift indicates that hydrogen bonds were formed between the template molecule, i.e. DBP, and the functional monomer, i.e. MAA. For the MIP before extraction, the stretching vibration peak of the benzene skeleton was observed at 1575 cm^{-1} but this peak was not present in the IR spectrum after extraction. The peak at 2361 correspond to C–H symmetric stretching of methyl groups, and those at 1491 and 1448 cm^{-1} correspond to C–H asymmetric stretching of methyl and methylene groups, and the MIP after ultrasonic extraction were similar to those for the NIP. All these observations can be explained by DBP removal from the MIP by extraction.

The FT-IR spectrum of the DBP haptens is shown in Figure 1(B). The primary amine N–H asymmetric and symmetric stretching vibrations appeared near 3349 and 3390 cm^{-1}, respectively. This shows that the nitro group of the DBP hapten was successfully reduced to an amino group.

Thermogravimetric analysis

The thermogravimetric analysis curve for the MIP membrane is shown in Figure S2. The result shows that the weight of the hydrophilic MIP membrane did not obviously change below 400°C. The prepared hydrophilic MIP membrane therefore had good thermal stability.

Adsorption capacity and adsorption kinetics tests

The specific adsorption capacity of the hydrophilic MIP membrane for DBP was determined by investigating the adsorption capacity and adsorption kinetics. The adsorption

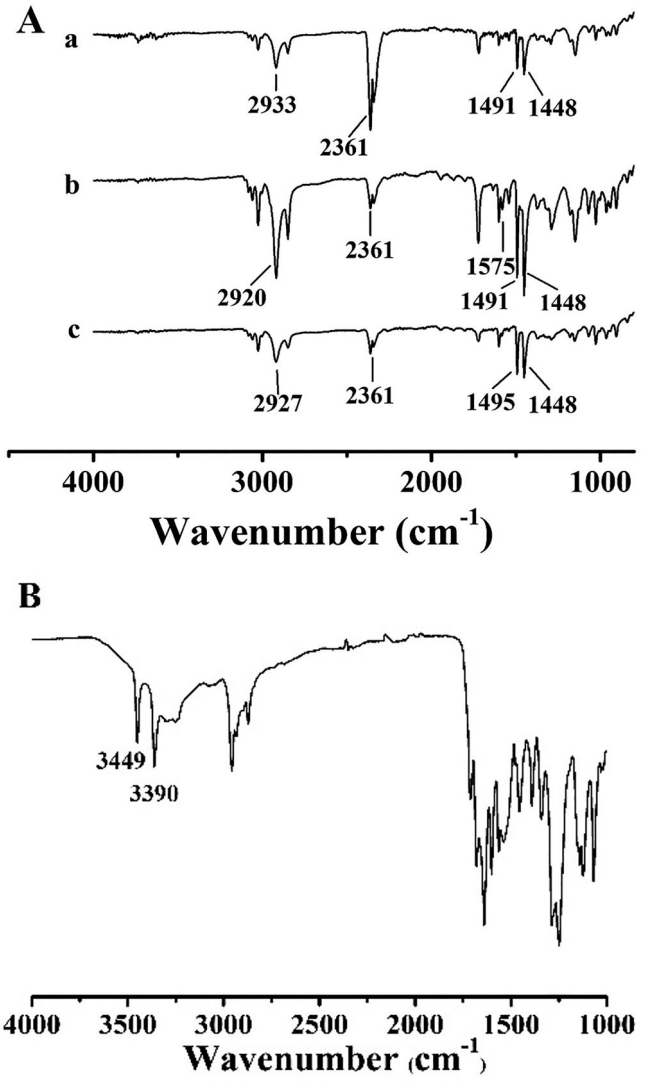

Figure 1. FT-IR spectra of (a) MIP after extraction, (b) MIP before extraction, and (c) non-imprinted film (A); FT-IR spectrum of QD–haptens (B).

capacity curve is shown in Figure 2. The adsorption capacities of the imprinted membrane and non-imprinted membrane both increased with DBP standard solution concentration increasing. At a DBP standard solution concentration of 300 mg/L, the quantity adsorbed by the MIP membrane was 13.83 μg/well, whereas the amount adsorbed by the NIP membrane was only 7.31 μg/well. The adsorption capacity of the MIP was much larger than that of the NIP (around twice as large), which indicates that the specific adsorption performance was good.

The adsorption kinetics for the MIP are tested. The results (Figure 3) show that adsorption reached almost equilibrium in 90 min, which indicates that the imprinted membrane exhibited with fast adsorption.

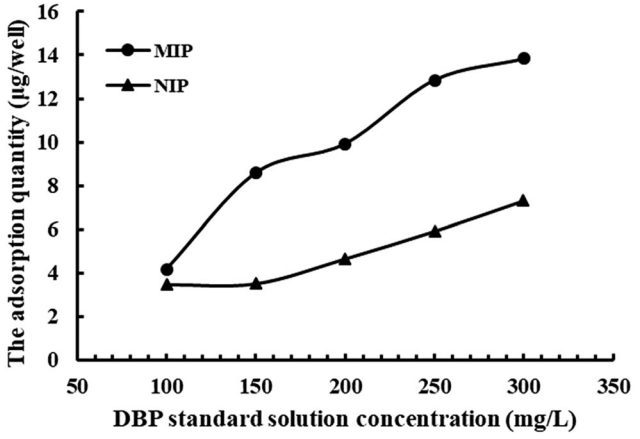

Figure 2. Adsorption capacity curves of the MIP and NIP toward DBP at concentrations of 100–300 mg/L.

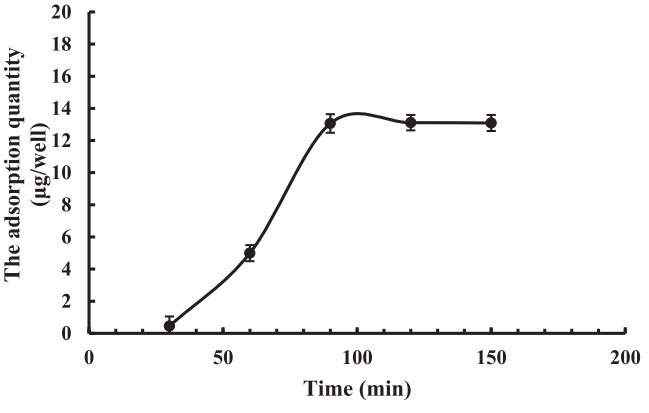

Figure 3. Dynamic curve of the MIP for DBP adsorption in 0–150 min.

Optimization of MIBIA conditions

The QD–hapten conjugate concentration was important in the competitive recognition process. The relationship between the fluorescence value and the dilution ratio is shown in Figure 4(A). A low dilution ratio with a high fluorescence value would lead to poor sensitivity and use of large quantities of materials. Too large dilution ratio with a low fluorescence value would lead to a large error. In this study, a dilution ratio of 1:30 was selected for subsequent experiments.

The buffer system used greatly affected the sensitivity of the method. As shown in Figure 4(B), the inhibition rates for all three buffer systems increased with increasing DBP standard solution concentration. The inhibition rate was higher in BBS than in DDW or citrate buffer solution (CPBS). BBS was therefore selected for the follow-up experiments.

The BBS pH was optimized. If the BBS pH is too high, the QD–hapten conjugate solubility is affected. In this study, the BBS of pH 7.4, 7.6, 7.8, and 8.0 was investigated. The results show that BBS of pH 7.6 gave the highest inhibition rate (Figure 4(C)).

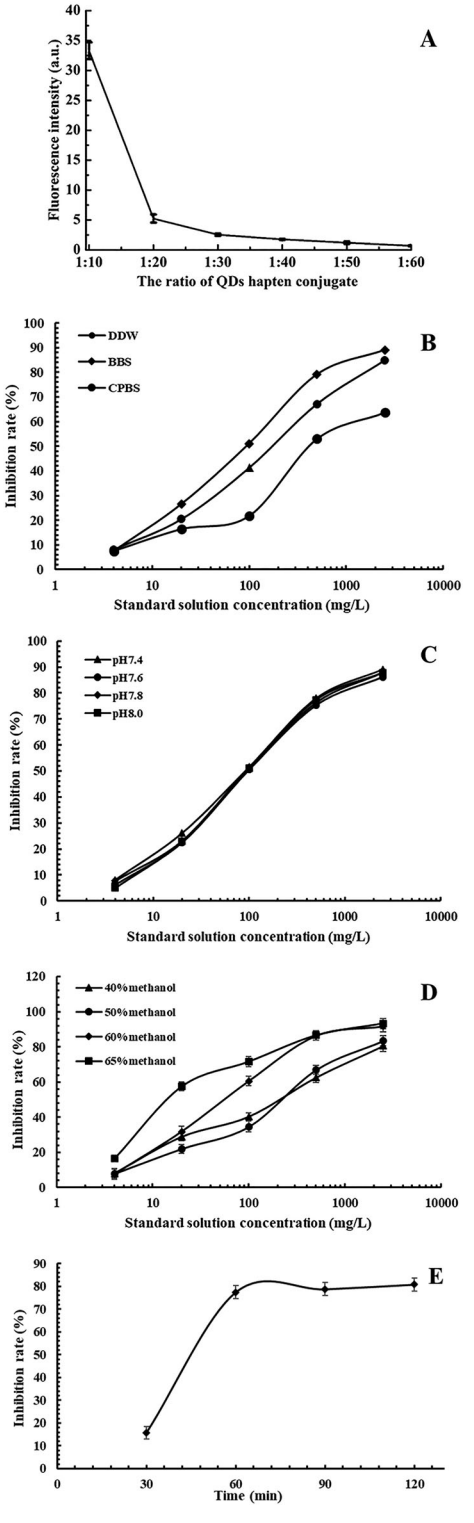

Figure 4. Effects of different buffer systems on inhibition rate (A); effects of BBS pH on inhibition rate (B); effects of methanol content in system on inhibition rate (C); effects of competitive reaction time on inhibition rate (D); QDs with different dilution ratios and their corresponding fluorescence values (E).

The effect of the methanol content of the buffer system on the sensitivity of the method was also studied (Figure 4(D)). Too little methanol led to low sensitivity, and too much methanol caused insolubility. When the methanol content was higher than 65%, precipitation was initiated. The results show that the system containing 65% methanol gave the highest inhibition rate.

Figure 4(E) shows the relationship between the inhibition rate and the competitive reaction time. The figure shows that the inhibition rate increased gradually with increasing reaction time, and the reaction almost reached equilibrium in about 60 min, therefore the time needed for the MIBIA is 60 min.

Evaluation of direct competitive MIBIA method

In this study, a direct MIBIA method based on a molecular imprinting technique was used to detect DBP. A standard curve for the direct competitive MIBIA method was obtained under the optimal conditions (Figure 5). The method gave good sensitivity and selectivity, and limit of detection and sensitivity were 0.011 and 0.136 mg/L, respectively. These values are lower than the maximum residue limits in China. The limit of detection of the developed method is lower, and the operating procedure is simpler, than those of the traditional ELISA and chromatographic methods (Table S1). This method therefore has potential applications in trace analysis of DBP in food samples.

The specificity of the DBP detection method established in this study was evaluated by performing cross-reaction tests with two DBP structural analogs, i.e. DEP and DIOP. The cross reactivity was calculated as the ratio of the sensitivity (IC_{50}) of DBP to those of the structural analogs. The cross-reaction rates for DEP and DIOP were 4.75% and 6.89%, respectively. Furthermore, the inhibition rate with the imprinted membrane was higher than that with the non-imprinted membrane for the same DBP concentration. These results indicate that the method has good specificity.

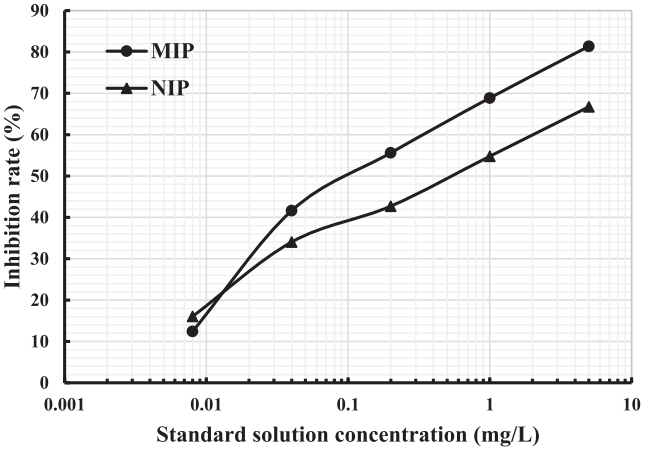

Figure 5. Direct competitive MIBIA standard curves for DBP with MIP and NIP at concentrations of 0.008–5 mg/L in 65% methanol/BBS solution.

Table 1. Analytical results and recoveries obtained using MIBIA method for determination of DBP in edible oil samples ($n = 3$).

Sample	Sample content (mg/L)	Spiked level (mg/L)	Found level (mg/L)	Recovery (±RSD, %)
Edible oil	0.268	0.050	0.308	80.0 ± 1.96
		0.100	0.352	84.0 ± 1.89
		0.200	0.491	111.5 ± 2.35

Accuracy and applicability of MIBIA method

The accuracy and applicability of the established MIBIA method were investigated by assaying edible oil samples spiked with DBP. Measurements were performed in triplicate for each concentration. The recoveries ranged from 80.0% to 111.5%, with a relative standard deviation of less than 3.0% (Table 1). These results show that this method has good accuracy.

Comparative analysis of liquor samples by MIBIA and GC methods was performed. The gas chromatogram of DBP in the liquor samples is shown in Figure S3. The DBP contents in the two liquor samples were determined by GC to be 1.39 ± 0.01 and 1.13 ± 0.01 mg/L, and the values obtained by using the MIBIA method were 1.31 ± 0.08 and 0.99 ± 0.11 mg/L. There were no significant differences between the results obtained by the two methods ($P > 0.05$). This indicates that the established MIBIA method could be used to detect trace DBP in food samples.

Conclusion

In this study, a direct competitive MIBIA was developed. This method has high sensitivity and accuracy, and can be used for actual sample detection.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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