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Development of the detection of benzophenone in recycled paper packaging materials by ELISA

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An enzyme-linked immunosorbent assay method was developed here for benzophenone (BZP) detection in package paper samples based on polyclonal antibody. 4-Aminobenzophenone (ABZP) was used as hapten and conjugated with bovine serum albumin for antibody production. Cross-reactivity results showed that the antibody can be recognised BZP, ABZP and Michler's ketone very well (IC_{50} value was 31.2 ± 7.8 ng/mL, 35.9 ± 8.3 ng/mL and 43.7 ± 8.2 ng/mL, respectively). After optimisation of the effects from pH, ionic strength and organic solvents on immunoassay, we established a rapid method and used for BZP detection in fortified package paper samples with good recovery data (98.5 and 99.7%) at 100 and 600 ng/g spiked level.)

Keywords: benzophenone; ELISA; polyclonal antibody; package paper samples

Introduction

Xenobiotics leaching from packaging into food have been characterised with high public and regulatory awareness in recent years. Benzophenone (BZP) can be used as one of the most commonly used photo-screen agents in the formulation of ink and also used as photoinitiator in the production of the polyethylene coating film. Previous work has shown that BZP can permeate through packaging to food (Johns, Jickells, Read, & Castle, 2000; Sagratini et al., 2008; Sanches-Silva et al., 2008; Triantafyllou, Akrida-Demertzi, & Demertzis, 2002). Several studies have verified the potential toxicity of BZP, such as carcinogenesis (Rhodes et al., 2007), sensitisation (Cook & Freeman, 2002) and endocrine disrupting (Hsieh et al., 2007; Muncke, 2009). The Food Standards Agency (UK) conducted a survey in 2000 and showed that no health effects would be expected in an individual's lifetime with daily intake value levels lower than 0.01 mg/kg body weight. Specific migration limit (SML) set by law (EU Commission Directive 2002/72/EC) is 0.6 mg/kg, which is the maximum quantity tolerated to migrate from plastic material used to food itself. Up to date, however, BZP and other photoinitiators used in ultraviolet (UV)-cured inks are not regulated with regards to potential food contamination activity. Almost all food packaging need ink printing procedure and a variety of xenobiotics may migrate into foodstuffs.

Considering the wide use and potential migrating of BZP, many studies have investigated the detection of BZP in packaging and food. Song, Park, and

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Komolprasert (2000) detected the suspected contaminants including BZP in recycled package paper/paperboard by GC with flame ionisation and electron capture detectors. Pastorelli, Sanches-Silva, Cruz, Simoneau, and Losada (2008) used HPLC-DAD and GC-MS to determine the content of BZP in different categories of cake packaging. Sagratini et al. (2008) developed GC-MS and LC-MS-MS techniques to detect five ink photoinitiators including BZP in packaged beverages. The most significant contamination was from BZP and was found in all samples with concentration ranging from 5 to 217 μg/L.

Until now, almost all analytical methods for BZP detection are based on the chromatographic measure especially by GC. As a fast and high throughput detection method, enzyme immunoassy provides a good prospective in screening the remainder or leaching of BZP in food. This text presents an enzyme-linked immunosorbent assay (ELISA) method to determine the leachate solutions of BZP from spiked virgin package paper.

Materials and methods

Chemicals and reagents

BZP (analytical reagent CAS no. 119-61-9), polyvinyl alcohol (polyvinylpyrrolidone [PVP], purity 87–89%) and polyethylene glycol (PEG, MW 4000) were procured from Aladdin chemistry Co., Ltd., Shanghai, China. 4-Aminobenzophenone (ABZP, CAS no. 1137-41-3 purity > 98%) was obtained from J&K Chemical Ltd., Shanghai, China. 4, 4′-Bis(dimethylamino) benzophenone (also named as Michler's ketone [MK], purity > 97%, CAS no. 90-93-7) was bought from Tokyo Chemical Industry Co., Ltd., Japan. 3, 3′, 5, 5′-Tetramethylbenzidine (TMB) were from Fluka, Buchs, Switzerland. PVP was obtained from Beijing Biodee Biotechnology Co., Ltd., China. Proteins including bovine serum albumin (BSA) and ovalbumin (OVA) were from BoAo Co., Shanghai, China. Gelation (Type A, gel strength 300 g, Bloom) and D-trehalose (purity 98%) were from Biodee Biotechnology Co., Ltd., Beijing, China. Goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase was from Hua Mei Co., Shanghai, China. All other chemicals and reagents were analytical grade or higher were obtained from East China Chemicals Co., Ltd., Wuxi, China.

Immunogen synthesis

ABZP (20 mg, equivalently 1×10^{-4} M) was dissolved in 6 mL dimethylformamide (DMF), mixed with 1 mL of 2 M HCl and then diluted the mixture to 20 mL with water. Sodium nitrite solution (1%, m/m) was added dropwise and stirred for 15 min at 4°C. The resulting solution was added dropwise with stirring to protein solution which was prepared in advance by dissolving 110 mg BSA (or 80 mg OVA) in 20 mL carbonate–bicarbonate solution (CBS, pH 9.6, Na_2CO_3 1.59 g, $NaHCO_3$ 2.93 g, diluted with 1 L H_2O). The pH of reaction mixture was maintained at 9.5 during the procedure and continued to be stirred for 2 h in an ice bath. The reaction mixture was then dialysed in PBS (0.02 M, pH 7.2, NaCl 8.0 g, KCl 0.2 g, KH_2PO_4 0.2 g, Na_2HPO_4 $12H_2O$ 2.9 g in 1 L water) for at least 72 h and the dialysis solution was changed three times per day. The purified conjugates were stored in aliquots at −20°C.

Product analysis was done by UV spectrogram (2128 PCS spectrophotometer, Unic, Shanghai, China).

Immunisation

Two New Zealand white rabbits (1.5–2.0 kg, female) were injected intradermally at multiple sites on the back with immunogen in Freund's complete adjuvant (1 mg/mL). Three weeks later, booster injections were given with immunogen emulsified with Freund's incomplete adjuvant. Blood was taken from ear vein 10 days later after injection since the third boostering. Serum was isolated by centrifugation and tested for polyclonal antibody production.

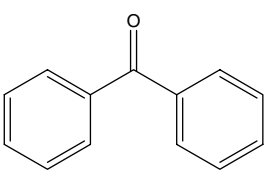
Enzyme-linked immunosorbent assay (ELISA) development

The titres of the antisera were determined by checkerboard method. One was selected for next optimisation cycle as it showed the greater binding to coated antigen and inhibition at the lower concentrations of BZP based on indirect ELISA. The ability of the antibody to recognise several structurally related compounds including ABZP, BZP and MK (Figure 1). The cross-reactivity (CR) values were calculated based on the formula below:

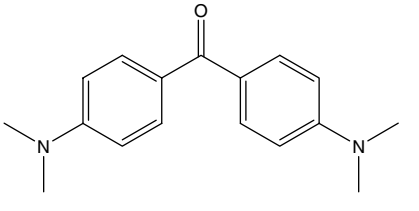
$$\%\mathrm{CR} = (\mathrm{IC}_{50}\ \mathrm{BZP}/\mathrm{IC}_{20}\ \text{structural analogue}) \times 100.$$

Effects of organic solvents, pH, ionic strength and contents in different buffers on assay performance of ELISA are evaluated.

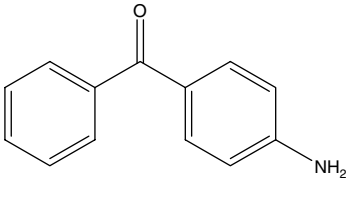
The procedure was as follows: 100 μL/well of 1.2 μg/mL of coating antigen (ABZP–OVA conjugate) in CBS (containing 0.5% PVP) was coated on microtiter plate and incubated at 4°C overnight. After washing with the wash solution (300 μL/well, phosphate buffered saline with Tween 20 [PBST], containing 0.05% Tween 20 and 1% PEG in PBS) four times, blocking solution (150 μL/well) was added and held at 37°C



Benzophenone, BZP CAS.No. 119-61-9



Michler's ketone, MK, CAS.No.90-93-7



4-aminobenzophenone, ABZP, CAS.No. 1137-41-3

Figure 1. Chemical structure for BZP, MK and ABZP.

for 2 h. The solution was then removed and 50 μL/well of serially diluted standards (BZP or BZP analogues) in PBST (containing 2% PVP) were added followed by 50 μL/well of antiserum solution (The dilutian ratio was 1/16,000 with PBST (containing 0.5% BSA, v/v) for the antiserum) in each well. Following 30 min at 37°C, the wells were washed three times again and 100 μL/well of goat anti-rabbit IgG labelled with peroxidase (diluted 1/5000 in 0.5% BSA in PBST) was added and the plates were incubated at 37°C for 30 min. After another washing cycle, the plates were added with the substrate solution (1 mg/mL TMB and 0.1% H_2O_2 in 4 mM citric acid, 100 μL/well). The reaction was allowed in darkness at 37°C for 15 min and stopped with 2 M H_2SO_4.The absorbance at 450 nm was measured.

Analysis of spiked sample

Virgin package paper which had been validated with no BZP residues was used for recovery tests. Package paper was cut into 1 × 1 cm pieces and weighed 1 g for each use. A solution of 100 μL (1 μg/mL or 6 μg/mL BZP in methanol) was absorbed using package paper pieces (1 g) which was put into tubes for standing overnight at 4°C.

After the samples returning to room temperature, 10 mL methanol was added into the tube then ultrasonic extraction of 2 min was carried out. The extracts were collected and the residues were re-extracted with tetrahydrofuran (THF, 10 mL). The solutions were merged together and dried with nitrogen stream. The residues were dissolved with 2 mL methanol and vortexed for 1 min, and then diluted into final volume of 20 mL with PBST (containing 2% PVP) for ELISA analysis.

Results and discussion

Antigen characterisation

Considering to the amino group on hapten (ABZP), diazotisation method was used to get antigen. The maximum UV absorption wavelength of ABZP is 340 nm and the typical protein absorption peak is at 280 nm. The UV spectrum of ABZP–BSA showed both adsorption nature of BSA and ABZP and was quite different from the spectrogram of ABZP or BSA alone (Figure 2). Similar analysis was carried out for ABZP–OVA conjugates.

Optimisation of enzyme-linked immunosorbent assay (ELISA) conditions

Because BZP is lipophilic, a water miscible organic cosolvent is needed to ensure the solubility. Methanol and THF were tested in this assay. The concentrations of cosolvents significantly influenced the assay sensitivity (IC_{50}) and absorbance. Generally, the optical density (OD) values would increase with higher concentration of methanol (0–10%, Figure 3a) in assay buffer while the OD values went down with more THF contents (0–10%, Figure 3b). Results in Figure 3 showed that the lowest IC_{50} (67.3 ng/mL) was found at 0% THF content and smal changes of IC_{50} value for BZP were found when methanol concentration ranged from 0 to 10%. Thus, methanol was used as cosolvent finally for subsequent experiments.

To evaluate potential interferences that may be encountered in real sample analysis, the effects of pH and ionic strength on the ELISA system were tested in this study.

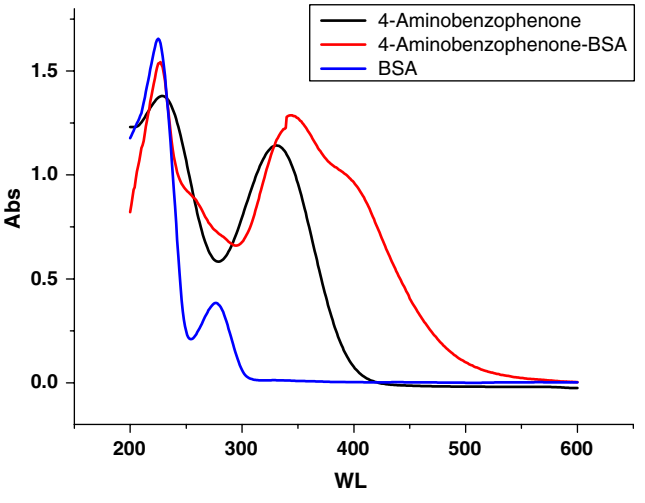


Figure 2. The ultraviolet-visible spectrum of hapten ABZP, carrier protein BSA and the complete antigen of ABZP-BSA. In the optimised ELISA, buffer PBST at pH6 (containing 10% methanol, 3.6% NaCl and 2% PVP) was used to prepare the standard solution. The optimised concentration for coating antigen was 1.2 μg/mL, while the dilution ratio of antiserum was 1/16,000. Final standard curve for B2P was established under the conditions above (Figure 4).

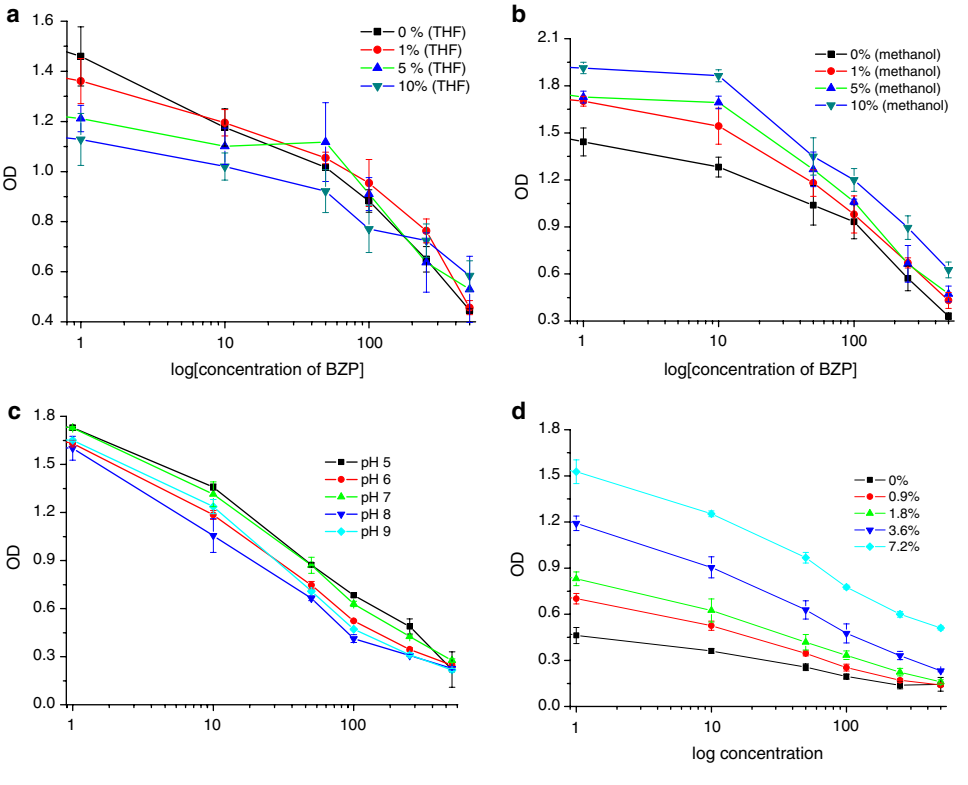


Figure 3. Effects of the organic solvents concentration (a,b), pH (c) and ionic strength (d) in assay buffer on the IC_{50} value of BZP.

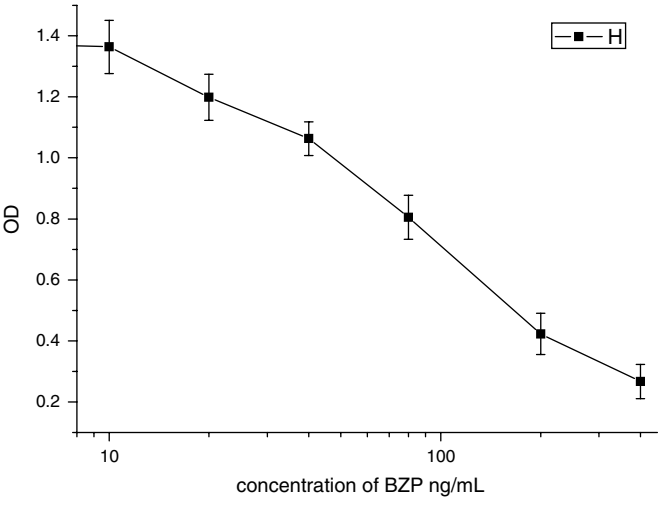


Figure 4. Standard curve of BZP by ELISA. The IC_{50} value was 31.2 ± 7.8 ng/mL. Each point of the curve represents the mean of 15 determinations and error bar indicates standard deviations (S.D).

Cross-reactivity (CR)

The prepared antibody can recognise two BZP analogues including ABZP and MK very well (IC_{50} was 35.9 ± 8.3 ng/mL for ABZP and 43.7 ± 8.2 ng/mL for MK) resulting the CRs value 86.9% and 71.4%, respectively (Table 1). These results provided information regarding the nature of the epitopes recognised by the antibody. The core chemical structure of epitope BZP was well exposed in the animal after inoculation of the antigen (ABZP–BSA conjugates).

Recovery studies

Two concentration levels of BZP (100 and 600ng/g) were used to spike the package paper samples. The recovery data shown in Table 2 was calculated based on five repeats on consecutive 3-day tests ($n = 15$). For 100 ng/g spiked level, the BZP recovery was 98.5% with inter-day coefficient of variation (CV, $n = 5$) 7.5% and intra-day CV 13.2%. Also, good recovery data of 99.7% for 600 ng/g level was obtained (CV values for inter-day or intra-day tests were 6.9% and 9.3%, respectively). The limit of detection (LOD), which was determinated by calculating the dose corresponding to 80% bound, was 45.6 ± 4.8 ng/mL in this test.

Table 1. Average 50% inhibition concentration (IC_{50}) in ng/mL and cross-reactivity (CR) listed for BZP analogues (ABZP and MK).

Compound	IC_{50} (ng/mL, $n = 5$)	CR (%)
BZP	31.2 ± 7.8	100
ABZP	35.9 ± 8.3	86.9
MK	43.7 ± 8.2	71.4

Table 2. Recoveries and coefficient variation of BZP in spiked package paper samples by ELISA.

		CV (%)	
Spiked level (ng/g)	Recoveries (%)	Inter-day ($n=5$)	Intra-day ($n=3$)
100	98.5 ± 3.6	7.5	13.2
600	99.7 ± 4.7	6.9	8.3

Conclusion

This is the first development of ELISA to determine BZP in package paper to our knowledge. The LOD of this simple method for determination of BZP in package paper samples was 45.6 ± 4.8 ng/mL, so that the method could be used for specific diffusion studies of BZP into foodstuff. Furthermore, both MK and ABZP are belonging to potential carcinogenic substances and this ELISA method can be used in these compounds control later.

Acknowledgements

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