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ORIGINAL PAPER

Development of sensitive direct and indirect enzyme-linked immunosorbent assays (ELISAs) for monitoring bisphenol-A in canned foods and beverages

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Abstract Enzyme-linked immunosorbent assays (ELISAs) are investigated in this work for the detection of bisphenol-A (BPA), a plastic monomer and a critical contaminant in food and environment. A series of polyclonal antibodies generated in vivo using BPA-butyrate-protein conjugate and BPA-valerate-protein conjugate were evaluated on direct and indirect competitive assay formats with five competing haptens (BPA-butyrate, BPA-valerate, BPA-crotonate, BPA-acetate, and BPA-2-valerate). Two indirect ELISAs and one direct ELISA exhibiting high sensitivity and specificity for BPA were developed. The 50 % inhibition of antibody binding (IC_{50}) values were 0.78±0.01–1.20±0.26 μg L^{-1}, and the limits of detection as measured by the IC_{20} values were 0.10±0.03–0.20±0.04 μg L^{-1}. The assays were highly specific to BPA, only displaying low cross-reactivity (3–8 % for the indirect assays and 26 % for the direct assay) for 4-cumylphenol (4-CP), at pH 7.2. The degree of cross-reaction of 4-CP was influenced by the antibody/hapten conjugate combination, assay conditions, and the assay format. The assays were optimized for the analysis of BPA in canned vegetables, bottled water and carbonated drinks. The limits of quantification for these three evaluated sample types, based on the spike and recovery data, were 0.5, 2.5, and 100 μg L^{-1}, respectively.

Keywords Bisphenol-A · Enzyme-linked immunosorbent assays · Canned corn · Bottled water · Carbonated drinks

Introduction

Bisphenol-A (BPA) is an indispensable monomer in the production of polyesters, polysulfones, polyether ketones, polycarbonate, and epoxy [1], with over 2.72 billion kg/year [2]. In particular, polycarbonate plastic has been used to make a variety of common products such as food and beverage containers, can linings, and dental composite fillings.

From the available toxicity data, BPA has potential to disrupt normal cell function by acting as an estrogen agonist as well as an androgen antagonist [3,4] and to influence the development of male and female reproductive tracts in utero [5,6]. More critically, BPA is associated with an increasing risk of cancer in the hematopoietic system since it can alter microtubule function and induce aneuploidy in some cells and tissues [7]. Additionally, pre-natal exposure to diverse and environmentally relevant doses of BPA may also increase endpoints which are considered markers of breast cancer risk in human [6].

As aforementioned, BPA is widely used in packaging materials and as a result of leaching it has been found widely in food containers, food products, processed beverages and in drinking water. In particular, BPA has been detected in fish (0.2–59 μg L^{-1}) [8], milk (<1.7–15.2 μg L^{-1}) [9], wine (<0.2–2.1 μg L^{-1}) [10], beverage (0.1–3.4 μg L^{-1}), vegetable (8.5–35 μg L^{-1}), and fruit (5.0–24 μg L^{-1}) [11]. BPA also has been found in environmental water (0.002–0.07 μg L^{-1}) [12], tap water (0.6 μg L^{-1}), and wastewater (10–2,500 μg L^{-1}) [13].

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Although the contents of BPA in the food and environment are lower than the BPA tolerable daily intake (0.05 mg kg^{-1} body weight) [14], the potential risk of explosure, especially in the prenatal, neonatal, and lactational stages is still unclear and under extensive investigation [6]. Subsequently, countries such as Canada, the European Union (EU), United Arab Emirates, and People's Republic of China have prohibited use of BPA in the production of baby food container. In Australia, the Food Standard Australia and New Zealand implemented a voluntary phase out of BPA use in baby bottle manufacturing, after conducted an independent risk assessment. Therefore, regular monitoring of BPA residues in environmental resources and in food is still necessary to ensure the safe levels are maintained until BPA is completely faded out from the manufacturing. The most frequently used methods for monitoring BPA are gas chromatography–mass spectrometry, liquid chromatography with tandem mass spectrometry, and high-performance liquid chromatography [15–19]. These techniques are highly sensitive and specific; however, most of them are cumbersome, expensive, and require extensive sample preparation and clean-up procedures prior to analysis [20]. Contrarily, enzyme-linked immunosorbent assays (ELISAs) offer many advantages that can complement instrumental analysis. They are of low operating cost, allow easy pretreatment of the samples, use small sample volume and are more suitable for rapid and in-field analysis.

Several BPA specific ELISAs with high sensitivity have been reported [21–25]. Some of the assays exhibit relatively significant cross-reaction with 4-cumylphenol (4-CP), and this could limit their application in food samples containing this major degradation product [25]. Additionally, none of these reported assays has been fully optimized in terms of the influence of assay format (i.e., direct assay vs. indirect assay) effects of competing haptenic structures on assay sensitivity and specificity (e.g., homologous vs. heterogeneous assays) and evaluation of matrix effects of foods and beverages.

In this study, a range of synthetic approaches for BPA haptens and a series of polyclonal antibodies raised against these haptens were extensively evaluated. Two indirect and one direct ELISA which were highly sensitive and specific to BPA were developed and characterized. Their analytical performance with food matrices was established by performing spike and recovery studies with canned corn, bottled water, and carbonated drinks.

Materials and methods

Materials

Ethyl 4-bromobutyrate, ethyl bromoacetate, ethyl 5-bromovalerate, ethyl 4-bromocrotonate, 4,4-bis(4-hydroxyphenol)valeric acid, *N*-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), dimethyl sulfoxide (DMSO), BPA, phenolphthalein (PP), 4-CP, 4,4′-dihydroxybenzophenone (diOH-BP), hexestrol, bis(4-hydroxyphenyl)methane (BPF), 4,4′-sulfonyldiphenol (BPS), 4,4′-thiodiphenol, diethlstilbenstrol, 3,3′,5,5′-tetramethylbenzidine (TMB), and 4-hydroxybenzoic acid were purchased from Sigma-Aldrich (St Louis, USA). Dimethylformamide (DMF) and tetrahydrofuran (THF) are obtained from Ajax Finechem (Sydney, Australia). Bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), horseradish peroxidase (HRP), goat-anti-rabbit IgG, Tween 20, thiomersal, TiterMax Gold, and incomplete Freund's adjuvant were purchased from Sigma-Aldrich (St. Louis, USA). For the preparation of buffers, chemicals used were sourced from either BDH Chemicals (Melbourne, Australia) or Ajax Finechem (Sydney, Australia). Silica gel 60 (70–230) mesh was purchased from Merck (Darmstadt, Germany). Maxisorp polystyrene 96-well plates were obtained from Nunc (Thermo Fisher Scientific, Denmark). HiTrap™ protein-A HP column was purchased from GE Healthcare (Bellefonte, USA).

Instrumentation

^{1}H spectra and ^{13}C spectra were recorded with Bruker DPX 300 spectrometers using the residual protonated solvent peak as the internal reference (i.e., for chloroform-*d*, 7.26 ppm and for dimethyl sulfoxide-d_6 2.50 ppm). Thin-layer chromatography was performed on 0.2-mm thick pre-coated silica gel $60F_{254}$ on aluminium sheets from Merck (Darmstadt, Germany). Column chromatography was performed on silica gel (particle size, 63–200 μm; 70–230 mesh ASTM, Merck). ELISA plate reader (SpectraMax M2) was obtained from Molecular Devices (Sunnyvale, USA).

Hapten synthesis

Two approaches were employed in the hapten synthesis: (1) use of spacers with varying length, attached to a hydroxyl group of BPA and (2) use of a spacer attached to a methyl group of BPA (Fig. 1).

Synthesis of haptens (approach 1) a1–d1

(a1) BPA (1.00 g, 4.4 mmol) and K_2CO_3 (1.38 g, 9.75 mmol) were dissolved in 10 mL of anhydrous DMF. Subsequently, ethyl 4-bromobutanoate (0.95 g, 4.87 mmol) was added drop-wise into the mixture solution. The reaction was stirred overnight at 75 °C, then poured into water and extracted with ethyl acetate. After three washes with water, the organic phase was dried over Na_2SO_4 and evaporated to dryness under reduced pressure. The residue was chromatographed on silica [methanol (MeOH)/dichloromethane (DCM) (1:99)] to give

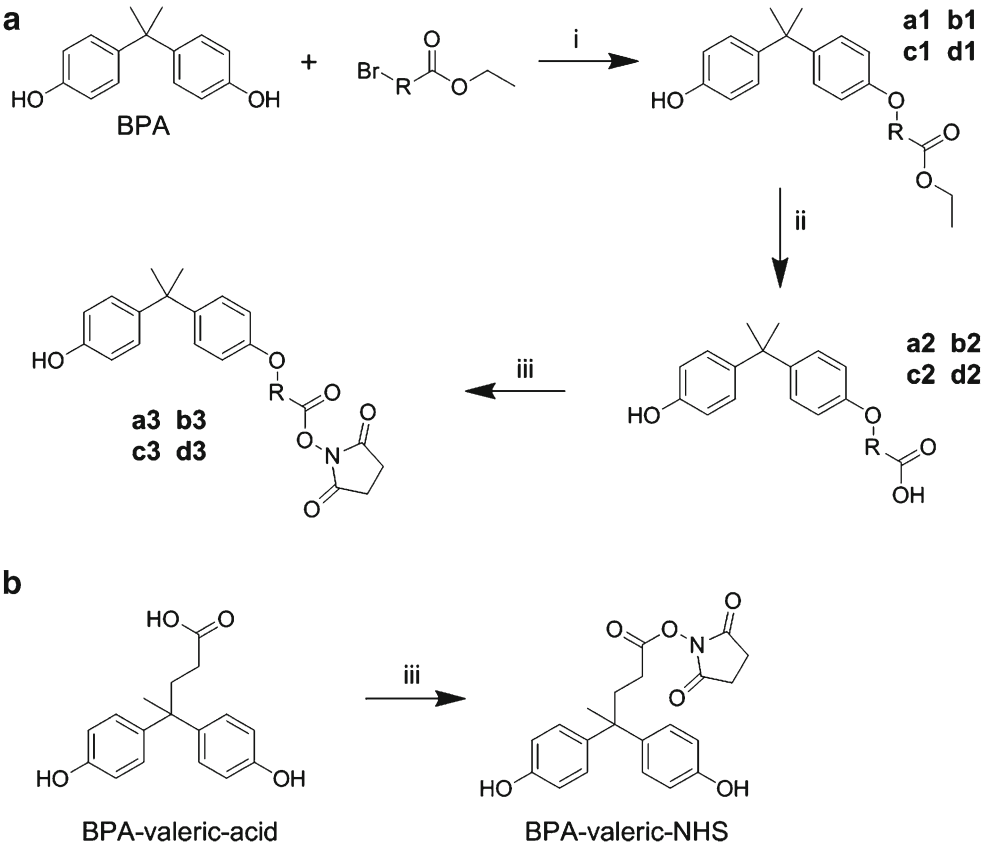


Fig. 1 (**a**) A reaction scheme for synthesis of BPA haptens with the spacer arm attachment at a hydroxyl group. *a1–3* R=$(CH_2)_3$ BPA-butyrate hapten, *b1–3* R=CH_2 BPA-acetate hapten, *c1–3* R=$(CH_2)_4$ BPA-2-valerate hapten, *d1–3* R=$CH_2(CH)_2$ BPA-crotonate hapten. (**b**) A reaction scheme for the synthesis of haptens with the spacer arm attachment at methyl group (BPA-valerate hapten). Refer to the method section for the compound names. (*i*) K_2CO_3, dry DMF, 75 °C, overnight, (*ii*) NaOH, MeOH/H_2O, rt, overnight, and (*iii*) DCC, NHS, dry THF, rt, overnight

yield of 37 %. R_f=0.34 [MeOH/DCM (1:99)]. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.25 (*t*, 3H, CH_3), 1.62 (*s*, 6H, 2×CH_3), 2.09 (*m*, 2H, CH_2), 2.50 (*t*, 2H, CH_2CO), 3.99 (*t*, 2H, OCH_2), 4.12 (*q*, 2 H, OCH_2), 4.69 (*s*, 1H, OH), 6.71–7.13 (*m*, 8H, 2×Ar). (b1) Ethyl 4-bromoacetate (0.83 g, 4.87 mmol); 53 % yield. R_f=0.27 [MeOH/DCM (1:99)], ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.29 (*t*, 3H, CH_3), 1.62 (*s*, 6H, 2×CH_3), 4.26 (*q*, 2H, CH_2COO), 4.58 (*s*, 2H, OCH_2), 5.30 (*s*, 1H, OH), 6.71–7.26 (*m*, 8H, 2×Ar). (c1) Ethyl 5-bromovalerate (1.00 g, 4.87 mmol); 43 % yield. R_f=0.27 [MeOH/DCM (1:99)], ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.25 (*t*, 3 H, CH_3), 1.62 (*s*, 6H, 2×CH_3), 1.78–1.83 (*m*, 4H, 2×CH_2), 2.37 (*m*, 2H, CH_2CO), 3.94 (*t*, 2H, OCH_2), 4.11 (*q*, 2H, OCH_2), 6.71–7.13 (*m*, 8H, 2×Ar). (d1) 4-bromocrotonate (0.93 g, 4.80 mmol); 28 % yield. R_f=0.30 [MeOH/DCM (1:99)], ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.26 (*m*, 3H, CH_3), 1.63 (*s*, 6H, 2×CH_3), 4.16 (*q*, 2H, OCH_2), 4.66 (*m*, 2H, OCH_2), 5.01 (*s*, 1H, OH), 6.20 (*s*, 1H, CHCO), 6.73 (*s*, 1H, CH), 6.48–7.17 (*m*, 8H, 2×Ar).

Synthesis of a2–d2

(a2) a1 (1.73 g, 5.05 mmol) dissolved in 5 mL MeOH was added into 3.3 mL of 4 M aqueous NaOH. After stirring overnight at room temperature, the mixture was washed with 30 mL of ethyl acetate. The aqueous phase was acidified to pH 3 with the concentrated HCl (32 %), and then extracted with ethyl acetate (3×30 mL). Subsequently, the organic phase was combined, dried over Na_2SO_4, and concentrated under reduced pressure to yield a2 (91 %). ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.53 (*s*, 6H, 2×CH_3), 2.34 (*t*, 2H, CH_2), 2.48 (*m*, 2H, CH_2CO), 3.90 (*t*, 2H, OCH_2), 6.60–7.08 (*m*, 8H, 2×Ar), 9.13 (*s*, 1H, OH), 12.04 (*s*, 1H, CO–OH). ^{13}C NMR (75 MHz, DMSO-d_6): δ 24.77 (CH_2), 30.62 (CH_3), 31.28 (CH_3), 41.49 (C–CH_3), 66.89 (CH_3–O), 114.21 (CH, Ar), 115.08 (CH, Ar), 127.76 (CH, Ar), 127.85 (CH, Ar), 141.26 (CH, Ar), 143.32 (CH, Ar), 155.42 (CH, Ar), 156.65 (CH, Ar), 174.59 (CO–OH). (b2) b1 (0.74 g, 2.34 mmol), 4 M NaOH (1.53 mL); 64 % yield. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.53 (*s*, 6H, 2×CH_3), 4.59 (*s*, 2H, OCH_2), 6.61–7.09 (*m*, 8H, 2×Ar), 9.14 (*s*, 1H, OH), 12.97 (*s*, 1H, CO–OH). (c2) c1 (0.26 g, 0.72 mmol), 4 M NaOH (0.47 mL); 80 % yield. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.63 (*s*, 6H, 2×CH_3), 1.58–1.68 (*m*, 4 H, 2×CH_2), 2.48 (*t*, 2H, OCH_2), 3.89 (*t*, 2H, OCH_2), 6.60–7.07 (*m*, 8H, 2×Ar), 9.15 (*s*, 1H, COOH), 11.92 (s, 1H, OH). (d2) d1 (0.28 g, 0.81 mmol), 4 M NaOH (0.53 mL); 67 % yield. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.63 (*s*, 6H, 2×CH_3), 6.30 (*q*, 2H, OCH_2), 4.60 (*m*, 2H, OCH_2), 6.73 (*s*, 1H, CH), 6.63–7.17 (*m*, 8H, 2×Ar), 9.17 (*s*, 1H, CO–OH).

Synthesis of hapten-protein conjugates

All of the conjugates of BPA derivatives with carrier proteins (KLH and BSA) and an enzyme probe (HRP) were produced by the NHS activated ester method [25].

Synthesis of active esters a3–d3

(a3) a2 (1.26 g, 4.14 mmol), DCC (1.10 g, 5.40 mmol) and NHS (0.69 g, 6.00 mmol) were dissolved in 20 mL of anhydrous THF. The mixture was stirred overnight at room temperature then filtered to remove the precipitate and chromatographed on silica [MeOH/DCM (5:95)] to afford a3 as a yellow oil (18 %). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.61 (*s*, 6H, 2×CH_3), 2.17 (*m*, 2H, CH_2), 2.31 (*s*, 2H, CH_2COO), 2.79 (*t*, 4H, 2×CH_2), 4.00 (*t*, 2H, OCH_2), 5.28 (*s*, 1H, OH), 6.71–7.26 (*m*, 8H, 2×Ar). (b3) Yield, 25 %. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.62 (*s*, 6H, 2×CH_3), 2.74 (*m*, 4H, 2×CH_2), 4.83 (*s*, 2H, CH_2), 6.69–7.09 (*m*, 8H, 2×Ar). (c3) Yield, 30 %. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.56 (*s*, 2H, CH_2), 1.91 (*s*, 2H, CH_2), 2.62 (*t*, 2H, OCH_2), 3.96 (*t*, 2H, OCH_2), 6.77–7.22 (*m*, 8H, 2×Ar). (d3) Yield, 23 %. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.65 (*s*, 6H, 2×CH_3), 2.79 (*t*, 4H, 2×CH_2), 4.60 (*m*, 2H, OCH_2), 5.0 (*s*, 1H, OH), 6.70 (*s*, 1H, CH), 6.63–7.17 (*m*, 8H, 2×Ar).

Synthesis of BPA-valerate hapten active ester

BPA-valerate (1.02 g, 3.49 mmol), DCC (0.96 g, 4.54 mmol), and NHS (0.53 g, 4.54 mmol) were dissolved in 16 mL of anhydrous THF. Subsequently, the mixture was stirred overnight at room temperature, filtered to remove the precipitate and concentrated under reduced pressure. The crude product was recrystallized from THF/*n*-hexane (1:40, *v*/*v*). The yield was 24 %. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.61 (*s*, 6H, 2×CH_3), 2.17 (*m*, 2H, CH_2), 2.31 (*s*, 2H, CH_2COO), 2.79 (*t*, 4H, 2×CH_2), 4.00 (*t*, 2H, OCH_2), 5.28 (*s*, 1H, OH), 6.71–7.26 (*m*, 8H, 2×Ar). ^{13}C NMR (75 MHz, $CDCl_3$): a2 δ, 21.06 (2×CH_2), 24.84 (CH_2), 25.59 (CH_3), 33.75 (CH_2), 41.06 (C–CH_3), 115.06 (2×CH, Ar), 128.36 (2×CH, Ar), 140.49 (2×CH, Ar), 153.27 (2×CH, Ar), 168.05 (2×OC–CH_2).

The active ester dissolved in DMF was added slowly in droplets into a protein solution in 50-mM sodium phosphate buffer (pH 8.5). The reaction was allowed to proceed for 16–24 h. Finally, the crude conjugate was dialyzed with phosphate-buffered saline (PBS) for 3 days with six times buffer changes. The hapten-BSA conjugate and hapten-HRP conjugate were stored at 4 °C until use. The hapten-KLH conjugates, which were used as immunogens, were stored at −20 °C.

Polyclonal antibody production

Two New Zealand white rabbits were immunized with each of BPA-butyrate-KLH and BPA-valerate-KLH. Briefly, the KLH conjugates were emulsified with an equal volume of Titer Max Gold adjuvant. The immunogen was injected subcutaneously in multiple sites into the rabbits. Following the booster injections given at 4-week intervals, each containing an immunogen emulsified with an incomplete Freund's adjuvant, blood was collected from the marginal ear vein 8–10 days after the injection. The serum containing polyclonal antibodies was collected after centrifuging at 3,500 rpm.

Preparation of BPA standard

Standard stock solutions (1×10^5 μg L^{-1} and 1×10^6 μg L^{-1}) of BPA and its analogues (4-CP, BPF, 4,4′-thiodiphenol, BPS, PP, diOH-BP, DES, hexestrol, BPA-butyrate, BPA-valerate, and 4-hydroxybenzoic acid) were prepared by dissolving an appropriate amount of each chemical in MeOH. The individual stock solutions were stored at 4 °C in amber glass vials. Working standards were prepared by diluting the stock solution in assay buffer just before use.

ELISA procedures

Indirect ELISA

A hapten-BSA conjugate was immobilized with a carbonate buffer (100 μL/well) on a microwell plate at 1×10^3–1×10^4 μg L^{-1}. After washing the plate three times with the washing solution (0.05 % Tween 20), 3 % of the skim milk powder (SMP) in the PBS (200 μL/well) solution was incubated for 1 h, as a blocking step. After another washing of the microwell plate, the calibration solutions (100 μL/well) and the antiserum in 1 % BSA-PBS were incubated (100 μL per well) in the respective microwells for 1 h. The plate was washed 4 times with the washing solution and the anti-rabbit IgG-HRP conjugate in 1 % BSA-PBS containing 0.05 % Tween 20 (1 % BSA-PBS/T) was incubated (100 μL/well) for 30 min. The plate was washed five times before the TMB substrate solution was incubated (150 μL/well) in all the microwells for 20 min to develop the color. The stop solution (1.25 M sulfuric acid) was added (50 μL/well) to the wells to stop further color reaction and measurement of the absorbance was conducted using a microplate reader with a dual wavelength mode at 450 and 650 nm.

Direct ELISA

Wells were immobilized with an antibody at 1×10^4 μg L^{-1} in carbonate buffer (100 μL/well). After washing three times with washing solution, the SMP-PBS was incubated (200 μL/well) in all the wells for 1 h. Following the blocking step, the plate was washed three times with the washing solution. The BPA standard solutions were incubated (100 μL/well) together with a hapten-HRP conjugate (100 μL/well) in their respective

wells for 1 h. Thereafter, the plate was washed four times and the TMB substrate solution (150 μL/well) was added to develop the color for 30 min. The absorbance values were determined at 450 and 650 nm using a microplate reader after the color reaction was stopped.

Cross-reactivity

The specificity of the ELISAs was evaluated using selected structurally related BPA analogues in the concentration range $0.05–1\times10^{3}$ μg L^{-1}. These were 4-CP, BPF, 4,4′-thiodiphenol, BPS, PP, diOH-BP, DES, and hexestrol. Percent cross-reactivity (%CR) was calculated at IC_{50} (midpoint in the standard curve) and IC_{20} values (as the limit of detection (LOD)):

$$\text{Cross}-\text{reactivity}(\%)\ \text{at}\ IC_{50(20)} = \left(\frac{IC_{60(20)}\text{BPA}}{IC_{60(20)}\ \text{test compound}}\right)\times 100$$

An IC_{50} is defined as a concentration of a test compound at which the corresponding absorbance is equal to a half of the maximum absorbance. LOD is defined as a concentration of a test compound at which the corresponding absorbance is equal to 20 % of the maximum absorbance (within the most linear part of the curve).

Sample extraction and spiking method

Fresh corn and glass bottle packing carbonated drink were obtained from the local supermarket and were assumed to be BPA-free as during processing and packaging neither was likely to be exposed to BPA. For the bottled water study, reverse osmosis (RO) water or purified water (Millipore Academic), which was assumed to be free of BPA, was used as a control.

Corn

In a typical spiking study, corn samples were steamed for 10 min to simulate canning of corn, and then homogenized using a coffee grinder. Corn mince (5 g) in the glass jar was spiked with BPA dissolved in MeOH to the final concentrations at 0.1, 0.5, 1.0, 5.0, and 10.0 μg L^{-1}. The spiked samples were allowed to stand at room temperature for 60 min prior to the extraction. For the immunoassay, BPA was extracted by shaking 5 mL 80 % MeOH in 5 g of corn mince for 30 s using a vortex machine. The samples were centrifuged to separate the supernatant. The clear supernatant was transferred into a new glass tube, and further diluted 100-fold with PBS.

Bottled water

Bottled water samples were spiked with BPA at five different levels (0.1, 0.5, 1.0, 5.0, and 10.0 μg L^{-1}). The survey of bottled water from 10 commonly known brands showed the pH to range between 4.6 and 7.4. Thus, pH of the bottle water samples (30 mL) was adjusted by adding PBS (1 mL) to maintain the pH around 7.4.

Carbonated drinks

Carbonated drinks were spiked with BPA at five different levels (0.1, 0.5, 1.0, 5.0, and 10.0 μg L^{-1}). After degassing for 30 min, each sample was diluted 1:5 with PBS and immediately assayed by ELISA.

Result and discussion

Production of hapten and immunoreagents

Two basic approaches were used for design and synthesis of BPA haptens to raise antibodies with high specificity for BPA. The strategy of these approaches was aimed to preserve the two phenolic groups as the potential immunodominant epitope, and utilized various spacer attachment points and lengths of the linkers to generate antibodies with sufficiently different specificities. In the first approach, four linkers of varying carbon number in saturated and unsaturated form were attached to BPA at one of the hydroxyl groups. Ethyl 4-bromobutyrate represents a flexible linker with four-carbon chain, ethyl bromoacetate represent a linker with two carbons, ethyl 5-bromovalerate represent a linker with five carbons and ethyl 4-bromocrotonate a more rigid linker with four carbons. Then, the hapten intermediates were subjected to hydrolysis to yield BPA derivatives with terminal carboxyl groups for subsequent coupling to carrier proteins. It was expected that antibodies raised against these haptens recognize the remaining phenolic group and the methyl groups that are distal from the point of protein conjugation. In this study, a shorter BPA-butyrate-hapten was used as an immunoreagent to minimize any folding back of hapten into the carrier protein [26]. Simultaneously, using haptens of differing chain lengths as competing haptens in a competitive format would be expected to improve assay sensitivity by increasing antibody affinity towards the analyte over the competitor [26]. In the second approach where the commercial 4,4-bis(4-hydroxyphenyl) valeric acid was used as a hapten, protein conjugation was achieved via the methyl group, leaving the two phenol groups free for the immune recognition. The antibodies raised against this hapten were expected to recognize the two phenolic groups, thus resulting in different antibody binding epitopes on the target analyte. Furthermore, the ethyl 4-bromocrotonate was used as a spacer for the first time in the synthesis of BPA hapten. This was introduced to give more rigidity and to give rise to greater affinity differentiation between an analyte and a competing hapten by the antibody.

Screening the antibody

The antibodies against BPA-butyrate-KLH conjugate were denoted as Ab∝BPA-B1, Ab∝BPA-B2 and those against BPA-valerate-KLH conjugate as Ab∝BPA-V1 and Ab∝BPA-V2.

The two groups of antibodies from each bleed were determined using an indirect homologous assay. The assay sensitivity increased with the successive booster immunization, indicating the successful immunization and antibody affinity maturation. These results also confirmed the stability of immunogens during the storage and immunization. The 5th bleed of Ab∝BPA-butyric-hapten (denoted as Ab∝BPA-B1#5, Ab∝BPA-B2#5) and 4th bleed of Ab∝BPA-valerate-hapten (denoted as Ab∝BPA-V1#4 and Ab∝BPA-V2#4) gave the best sensitivity in the optimized conditions.

Screening the antibody/enzyme conjugate combinations

The four antibodies (Ab∝BPA-B1#5, Ab∝BPA-B2#5, Ab∝BPA-V1#4, and Ab∝BPA-V2#4) were selected for further characterization and optimization with five competing haptens by determining the sensitivity and limits of detection.

Sensitivity of indirect ELISAs

The generated antibodies were of high affinity for their respective immunizing haptens and did not interact with each other despite the structural similarity between them (Table 1). For example, the antibodies against the BPA-valerate conjugate did not interact with the BPA-butyrate conjugate.

The antibodies against the BPA-butyrate-hapten displayed better sensitivity than those raised against the BPA-valerate-hapten in a homologous system. Evaluating the trend of cross-reactivity suggests that the two methyl groups may be the immunodominant epitope of BPA.

In our study, the heterogeneous systems proved to yield better sensitivity than the homologous system. Of 40 antibody/enzyme conjugate combinations evaluated, four assays displaying good sensitivity were chosen for future characterization and validation of assay performance. These were Ab∝BPA-B1#5/BPA-acetate-BSA pair, Ab∝BPA-B2#5/BPA-crotonate-BSA pair, Ab∝BPA-V1#4/BPA-acetate-BSA pair, and Ab∝BPA-V2#4/BPA-acetate-BSA pair. The best sensitivity for BPA analysis was achieved by the Ab∝BPA-V2#4 and BPA-acetate-BSA pair. The IC_{50} of this assay was 0.78±0.01 μg L^{-1} and the detection range was 0.10±0.03–10.00±0.01 μg L^{-1}.

Sensitivity of direct ELISAs

Similarly, the antibodies against BPA-valerate hapten only interact specifically with BPA-valerate hapten conjugate in a direct competitive assay format. Conversely, the antibodies against BPA-butyrate hapten interacted with all of the four haptens except for BPA-valerate hapten (Table 1).

Four antibody/enzyme combinations were selected on the basis of sensitivity: Ab∝BPA-B1#5/BPA-crotonate-HRP pair, Ab∝BPA-B2#5/BPA-crotonate-HRP pair, Ab∝BPA-V1#4/BPA-valerate-HRP pair, and Ab∝BPA-V2#4/BPA-valerate-HRP pair. The best sensitivity was given by the Ab∝BPA-B1#5/BPA-crotonate-HRP pair, with an IC_{50} value of 1.05±0.05 μg L^{-1} and the detection range of 0.20±0.04–10.00±0.01 μg L^{-1}.

Assay specificity

According to the thermal degradation pathway of BPA polycarbonate and the environmental fate of BPA, 4-CP, BPA, and phenols were the most suspected residues in canned foods [27–30]. Therefore, the specificity of the antibody and of the ELISA was determined by the cross-reactivity studies with 11 structural analogues of BPA including the degradation products: PP, 4-CP, diOH-BP, hexestrol, DES, BPF, BPS, 4,4′-thiodiphenol, BPA-butyrate, BPA-valerate, and 4-hydroxybenzoic acid (Table 2). The cross-reactivity of the assays for all of the tested analogues was much lower than the published work [20–22,24], in particular, the cross-reactivity of 4-CP. The direct format displayed higher cross-reactivity than the indirect format.

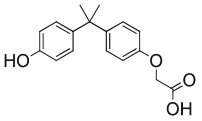
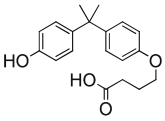
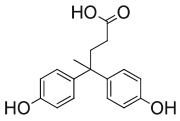
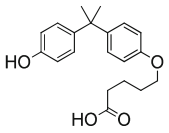
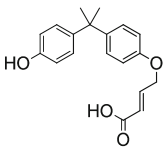
Specificity of the indirect ELISA

All of the generated antibodies were highly specific to BPA in the indirect assay format with less than 1 % cross-reactivity for the test compounds, except for 4-CP. The cross-reactivity-IC_{50} (CR-IC_{50}) of 4-CP was slightly higher (3–7.8 %), but was within the acceptable criterion of 10 %. Considering, 4-CP and BPF differed from BPA by the absence of one methyl group and two methyl groups respectively, the low cross-reactivity displayed by these compounds strongly suggested the immunodominance of the two methyl groups of BPA.

Specificity of the direct ELISA

Overall, the %CR of the same antibody in a direct ELISA format was slightly higher than that in an indirect ELISA format, even though the general cross-reactivity pattern remained similar. As in the indirect ELISA assay, the cross-reactivity of 4-CP by the direct ELISA was higher than those of the other test compounds: 26 % in Ab∝BPA B1#5/BPA-crotonate-HRP pair, 10 % in Ab∝BPA B2#5/BPA-crotonate-HRP pair, 12 % in Ab∝BPA V1#4/BPA-acetate-BSA pair, and 40 % in Ab∝BPA-V2#4/BPA-acetate-BSA pair. Since the amount of 4-CP which was observed during the thermal

Table 1 The IC_{50} (in micrograms per liter) values of the indirect and direct ELISAs

Antibody	Enzyme conjugate				
					
	BPA-acetate	BPA-butyrate	BPA-valerate	BPA-2-valerate	BPA-crotonate
Indirect Assays					
Ab∝ BPA-B1#5	1.20 ± 0.26	3.00 ± 0.01	>1000	2.00 ± 0.01	1.40 ± 0.21
Ab∝ BPA-B2#5	300.00 ± 0.07	8.00 ± 0.01	>1000	11.00 ± 0.02	7.00 ± 0.70
Ab∝ BPA-V1#4	8.75 ± 0.01	>1000	21.00 ± 0.02	>1000	>1000
Ab∝ BPA-V2#4	0.78 ± 0.01	>1000	30.00 ± 0.01	3.00 ± 0.03	2.00 ± 0.27
Direct Assays					
Ab∝ BPA-B1#5	44.00 ± 0.02	1.00 ± 0.02	>1000	3.00 ± 0.03	1.05 ± 0.05
Ab∝ BPA-B2#5	>1000	3.00 ± 0.04	>1000	20.00 ± 0.07	2.00 ± 0.05
Ab∝ BPA-V1#4	>1000	>1000	7.00 ± 0.01	7.00 ± 0.40	>1000
Ab∝ BPA-V2#4	>1000	>1000	8.00 ± 0.01	>1000	>1000

degradation was around 15 % of BPA [28], the cross-reactivity of this metabolite can be considered not significant in all of the assays. Nevertheless, the assays with lower cross-reactivity of 4-CP, such as Ab∝BPA B2#5/BPA-crotonate-HRP pair and Ab∝BPA V1#4/BPA-acetate-BSA pair, would be preferred for food analysis.

The cross-reactivity of 4-CP was also tested at pH 5.5 and 9.5 to investigate the effects of pH on antibody specificity, as many fruit and vegetable based foods are acidic. Significant increases in the percent cross-reaction of 4-CP at both pH tested were observed. For example, the %CR of 4-CP of the BPA-B1Ab#5/BPA-acetate-BSA assay increased from 2.9 to 33.3 and 36.0 %, respectively, for pH 5.5 and 9.5. Therefore adjustment of sample pH would be critical to maintain the low cross-reaction of 4-CP in these assays.

The overall cross-reactivity pattern displayed by an antibody in either assay format is expected to be similar, although the previous works suggest cross-reactivity in an indirect assay is generally higher than that in a direct assay. The degree of cross-reactivity for each cross-reacting compound can be influenced by the assay format, assay conditions and competing hapten used, and relative concentrations of an antibody and a conjugate in an assay [20,25,31], and this was also evident from this study, e.g., 4-CP. Thus cross-reactivity of an immunoassay could be manipulated to some degree by assay format and assay conditions.

Optimization of the indirect and direct ELISAs

Two indirect ELISAs, based on Ab∝BPA B1#5/BPA-acetate-BSA pair and Ab∝BPA-V2#4/BPA-acetate-BSA pair, and Ab∝BPA B1#5/BPA-crotonate-HRP pair in the direct ELISA were chosen for further development. To evaluate the variability of the assays due to matrix effects, the assay performance was evaluated by varying the ionic strength, inorganic ions, pH, and extracting solvents.

Effects of inorganic ions and ionic strength

Inorganic ions and ionic strength could affect antibody-antigen interaction and stability of a reporter enzyme, thus affecting both color development and assay sensitivity. Common ionic salt in spring water and foods (such as Na^+, Cl^-, K^+, Ca^{2+}, Mg^{2+} SO_4^{2-}, and Fe_2^{3+}) were tested at a wide range of molar concentrations (0.01 M, 0.1 M and 1 M using NaCl, KCl, $CaCl_2$ and $MgSO_4$, and 0.001, 0.01, and 0.1 M for $Fe_2(SO_4)_3$) (Table S1 in the Electronic supplementary material). In regards to the antibody-antigen interaction, the sensitivity of all three assays was not affected by NaCl, while the higher ionic strength (e.g., 0.1 and 1 M NaCl) inhibited the enzyme activity which caused significant reduction of absorbance value. Increasing the ionic strength of KCl from 0.01 to 1 M led to the same effect as NaCl, and the absorbance value decreases more sharply. In the Ab∝BPA B1#5/BPA-crotonate-HRP assay, $CaCl_2$ did not affect enzyme activity, but the antigen-antibody binding was interfered at 1 M $CaCl_2$. However, excluding the lower absorbance values at 0.1 and 1 M, $CaCl_2$ did not interfere with the antigen-antibody binding in the indirect assays. Increasing the ionic strength of $MgSO_4$ from 0.01 to 1 M, the binding between Ab∝BPA-B1#5 and the antigen (BPA) was not hindered while the enzyme activity was inhibited at 0.01 M in both assay formats. Simultaneously, $MgSO_4$ showed the same inhibition of enzyme activity in the Ab∝BPA-V2#4/BPA-acetate-BSA assay and interfered the Ab∝BPA-V2#4 even at 0.01 M $MgSO_4$. $Fe_2(SO_4)_3$ at 0.001 M in the sample did not interfere with the antibody-antigen binding in all the

Table 2 Cross-reactivity of the indirect and direct ELISAs, at pH 7.2, calculated at IC_{50} and IC_{20} values

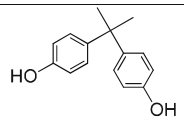
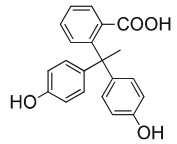
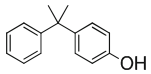
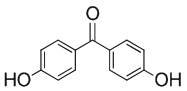
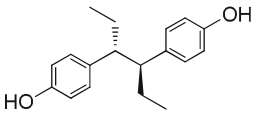
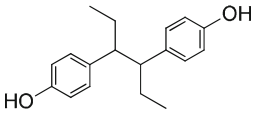
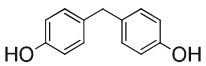
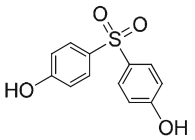
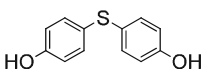
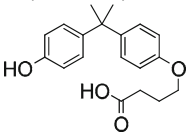
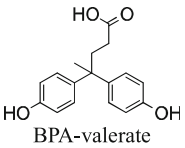
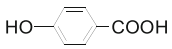
Compound	%Cross-Reactivity at IC_{50} and IC_{20}		
	Indirect ELISA format		Direct ELISA format
	Ab∝ BPA-B1#5/ BPA-acetate-BSA	Ab∝ BPA-V2#4 / BPA-acetate-BSA	Ab∝ BPA-B1#5/ BPA-crotonate-HRP
 Bisphenol-A (BPA)	100/100	100/100	100/100
 Phenolphthalein (PP)	<0.1/<0.1	<0.1/<0.1	<0.1/<0.1
 4-Cumylphenol (4-CP)	2.9/1.0	7.8/12.5	26.3/40
 4,4'-Dihydroxybenzophenone (diOH-BP)	<0.15/<0.1	<0.15/<0.1	<0.15/0.15
 Hexestrol	<0.15/<0.1	<0.15/<0.1	<0.15/<0.1
 Diethylstilbestrol (DES)	<0.15/<0.1	<0.15/0.25	<0.15/<0.1
 Bis(4-Hydroxyphenyl)methane (BPF)	0.12/0.3	0.4/2.0	0.1/0.2
 4,4'-Sulfonyldiphenol (BPS)	<0.15/<0.1	<0.15/0.25	<0.15/<0.1
 4,4'-Thiodiphenol	<0.15/0.1	0.75/8.3	<0.15/<0.1

Table 2 (continued)

 BPA-butyrate	-/-	<0.1/<0.1	-/-
 BPA-valerate	<0.1/<0.1	-/-	<0.1/<0.1
 4-Hydroxybenzoic acid	<0.1/<0.1	<0.1/<0.1	<0.1/<0.1

assays, but when it was increased to 0.1 M, the enzyme activity was completely inhibited and there was almost no observable antigen-antibody binding. Considering the molar concentration of the ions tested are much higher than normally present in food and beverages, the assay performance would not be compromised by these common ions for food analysis in most cases.

Effects of pH

The effects of pH were investigated using the RO water with pH adjusted to 3.5, 5.5, 9.5, and 7.4. In the indirect ELISAs, the color development inhibited slightly at pH 5.5 while at pH 3.5, it was significantly affected. The assay sensitivity in the pH range of 5.5 and 9.5 was unaffected (Fig. 2). Therefore, it would be essential that the sample pH be maintained between 5.5 and 9.5 to ensure acceptable performance. Surprisingly, the direct ELISA was slightly more tolerant to pH than the indirect ELISAs, as shown by no significant changes in the assay sensitivity and absorbance in the range of 3.5 to 9.5. In all, it can be concluded that these antibodies were relatively stable to pH change.

Effects of organic solvent

Since MeOH and acetone are commonly used extracting solvents for BPA, their effects were evaluated on ELISA. MeOH showed a concentration-dependent decrease in color development affecting assay sensitivity (Fig. 3). The three selected assays can tolerate up to 10 % (*v*/*v*) MeOH. While inhibition of the enzyme activity and antigen-antibody binding at 5 %, acetone was observed. This was evident by the low color development and loss of sensitivity (higher IC_{20}) in all three assays. Therefore, acetone was not recommended as an extraction solution in these ELISA systems.

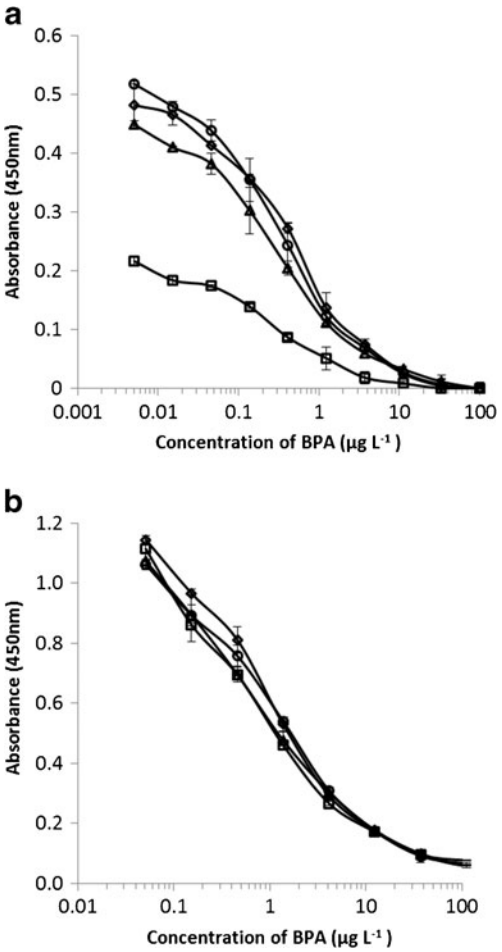


Fig. 2 Effects of pH. Standard curves of the assay based on Ab∝BPA-V2#4/BPA-acetate-BSA (**a**) in % inhibition (*filled symbols*) and absorbance (*open symbols*) and of the assay based on Ab∝BPA-B1#5/BPA-crotonate-HRP (**b**)—*open and filled diamonds* (pH=7.4), *open and filled squares* (pH=3.5), *open and filled triangles* (pH=5.5), and *open and filled circles* (pH=9.5)

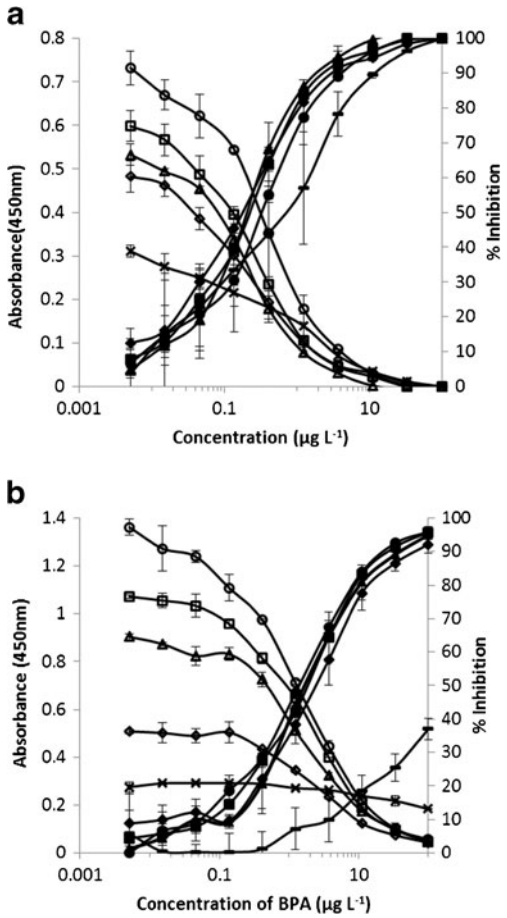


Fig. 3 Effects of extraction solvents. Standard curves of the assay based on Ab∝BPA-V2#4/BPA-acetate-BSA (**a**) in % inhibition (*filled symbols*) and absorbance (*open symbols*) and of the assay based on Ab∝BPA-B1#5/BPA-crotonate-HRP (**b**)—*open and filled circles* 0 % MeOH in PBS, *open and filled squares* 5 % MeOH in PBS, *open and filled triangles* 10 % MeOH in PBS, *open and filled diamonds* 20 % MeOH in PBS, and *error marks* 40 % MeOH in PBS

Matrix effects and their removal in food samples

Matrix interferences such as pigment, fat, and excess salt or sugar in foods can cause false positives by either lowering the color development or interfering with the antigen-antibody interaction. Matrix effects are observed when the interaction between the antigen/analyte and the antibody is hindered, or the enzyme activity is inhibited or both of these phenomena have occurred concurrently in an immunoassay [32].

For canned corn which contains sugar and minerals (e.g., Ca, Fe, Mg, P, K, and Na), the matrix interference was minimized by diluting the sample extract with PBS. The standard curve prepared from the diluted corn extract superimposed with the control standard curve which was prepared in PBS (Fig. 4). For bottled water and carbonated drinks, simply adding 5×PBS into bottled water and diluting the carbonated drinks 1:5 with PBS to adjust pH to 7.4 respectively could overcome their matrix interference.

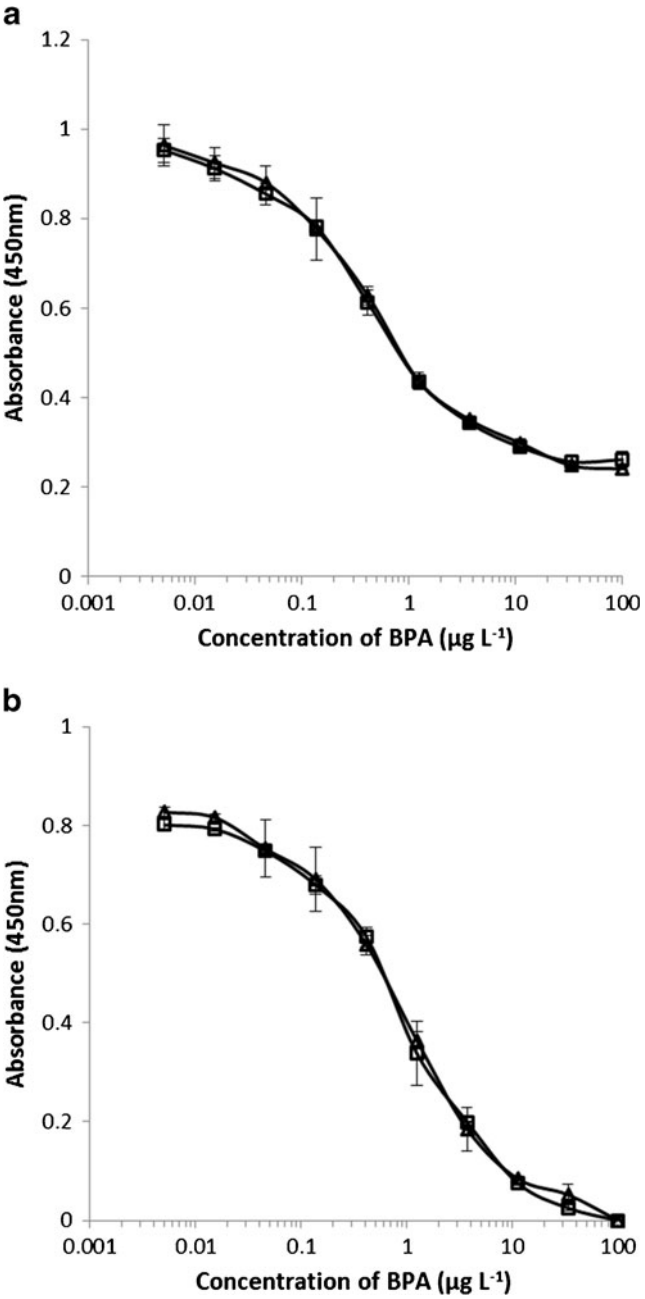


Fig. 4 Effects of sample matrix. Standard curves of the assay based on Ab∝BPA-V2#4/BPA-acetate-BSA (**a**) in % inhibition (*filled symbols*) and absorbance (*open symbols*) and of the assay based on Ab∝BPA-B1#5/BPA-crotonate-HRP (**b**)—*open and filled triangles* standard curve in PBS and *open and filled squares* standard curve in corn sample

Recovery studies of food samples

Analytical performance of the assays was evaluated by determining percent recovery of BPA in a spike and recovery study. Each sample type was spiked to 0.1, 0.5, 1.0, 5.0, and 10.0 μg L^{-1} BPA. The data shown in Table 3 represent the recoveries of BPA—66.7–113.3, 102.5–126.7, and 75.0–115.0 % for canned corn, bottled water, and carbonated

Table 3 Spike and recovery of BPA by indirect and direct ELISAs

Assay type	Antibody/conjugate	Spike level (μg kg^{-1})	Mean±95 % CI (μg kg^{-1}; n=4)	% RSD	% Recovery±95 % CI (n=4)
Bottled water samples					
Indirect	Ab∝ BPA-B1#5and BPA-acetate-BSA	0.1	0.10±0.01	4.9	102.5±0.2
		0.5	0.60±0.01	10.5	110.0±0.5
		1	1.20	0	120.0
		5	6.30±0.03	9.1	126.7±0.5
		10	10.80±0.02	4.7	107.5±0.2
	Ab∝ BPA-V2#4 and BPA-acetate-BSA	0.1	0.12±0.01	16.7	110.0±0.6
		0.5	0.58±0.01	8.7	115.0±0.4
		1	1.30±0.01	15.3	125.0±0.8
		5	5.70±0.04	17.4	114.0±0.9
		10	11.00±0.06	12.9	110.0±0.6
Direct	Ab∝ BPA-B1#5 and BPA-crotonate-HRP	0.1	0.07	0	70.0
		0.5	0.45±0.01	15.7	90.0±0.6
		1	0.80	0	80.0
		5	6.00	0	120.0
		10	11.50±0.03	6.2	115.0±0.3
Carbonated drink samples					
Indirect	Ab∝ BPA-B1#5and BPA-acetate-BSA	0.1	0.08±0.01	13.3	75.0±0.4
		0.5	0.45±0.02	15.7	90.0±0.6
		1	0.90±0.04	19.2	90.0±0.8
		5	5.75±0.25	8.7	115.0±0.4
		10	9.67±0.43	15.8	96.7±0.7
	Ab∝ BPA-V2#4 and BPA-acetate-BSA	0.1	0.09±0.01	9.1	90.0±0.4
		0.5	0.43±0.02	13.3	86.7±0.5
		1	0.88±0.04	17.1	87.5±0.7
		5	3.70±0.16	9.4	74.0±0.3
		10	7.67±0.34	15.1	76.7±0.5
Direct	Ab∝ BPA-B1#5 and BPA-crotonate-HRP	0.1	0.09±0.01	8.3	85.0±0.3
		0.5	0.50	0	100.0
		1	1.00±0.01	7.4	95.0±0.3
		5	4.10±0.01	3.4	82.0±0.1
		10	11.00±0.06	12.9	110.0±0.6
Corn samples					
Indirect	Ab∝ BPA-B1#5and BPA-acetate-BSA	0.1	0.07±0.01	8.7	66.7±0.2
		0.5	0.50±0.01	10.2	113.3±0.4
		1	0.70±0.01	8.7	66.7±0.2
		5	3.80±0.01	9.1	76.0±0.3
		10	10.30±0.02	5.6	103.3±0.2
	Ab∝ BPA-V2#4 and BPA-acetate-BSA	0.1	0.07	0	70.0
		0.5	0.50±0.01	25.4	100.0±0.9
		1	0.70±0.01	2.0	100.0±0.1
		5	4.50±0.03	20.7	80.0±0.7
		10	9.50±0.03	7.4	80.0±0.3
Direct	Ab∝ BPA-B1#5 and BPA-crotonate-HRP	0.1	0.07±0.01	20.2	70.0±0.6
		0.5	0.40±0.01	22.3	76.0±0.8
		1	1.10±0.01	12.9	110.0±0.6
		5	4.60±0.04	18.4	92.0±0.8
		10	10.00	0	100.0

drinks respectively by the Ab∝BPA-B1#5/BPA-acetate-BSA assay; 70.0–100.0, 110.0–125.0, and 86.7–90.0 % by the Ab∝BPA-V2#4/BPA-acetate-BSA assay; and 70.0–110.0, 70.0–120.0, and 82.0–110.0 % by the direct ELISA format of Ab∝BPA-B1#5/BPA-crotonate-HRP assay. In general, most of the recovery of BPA was within the acceptable range

of 80–120 % with a few exceptions (bottled water: 0.1 μg L^{-1} in Ab∝BPA-B1#5/BPA-crotonate-HRP assay; carbonated drinks: 0.1 μg L^{-1} in Ab∝BPA-B1#5/BPA-acetate-BSA assay, 5.0 and 10.0 μg L^{-1} in Ab∝BPA-V2#4/BPA-acetate-BSA assay; canned corn—0.1, 1.0, and 5.0 μg L^{-1} in Ab∝BPA-B1#5/BPA-acetate-BSA assay, 0.1 μg L^{-1} in Ab∝BPA-V2#4/BPA-acetate-BSA assay and 0.1 and 0.5 μg L^{-1} in Ab∝BPA-B1#5/BPA-crotonate-HRP assay) indicating the assays quantified BPA with good recoveries. The % recovery of the bottled water analyses was typically 20–45 % higher in the low spike levels (0.1, 0.5, and 1.0 μg L^{-1}) in the indirect format when comparing with the direct format. Additionally, at the low spiking level (0.1 μg L^{-1}) the recoveries were also low, especially in canned corn sample using Ab∝BPA-B1#5/BPA-acetate-BSA assay (66.7±0.2 %), could be due to lower extraction efficiency *via* adsorption of BPA by the food constituents such as protein and lipids.

Conclusions

Some immunoassays reported previously for the detection of BPA, although they exhibited good sensitivity for BPA, displayed significant cross-reactivity against 4-CP (>20 %) which is one of the degradation products that could be present in canned foods. For monitoring the BPA residues in foods and beverages, we developed a series of highly sensitive ELISAs in direct and indirect assay formats with high specificity (<10 %). Five BPA haptens with varying chain length and points of attachment to carrier proteins were synthesized. Of the 40 assays evaluated with diverse antibody/antigen combination, two indirect and one direct ELISAs were fully characterized and optimized. The assays were highly specific to BPA with less than 10 % cross-reactivity for 4-CP, BPF, and 4,4′-thiodiphenol, and no cross-reactivity with other similarly structured compounds. The most sensitive assay was based on Ab∝BPA-V2#4 and BPA-acetate-BSA in an indirect format with an IC_{50} of 0.78± 0.01 μg L^{-1}, and a LOD of 0.10±0.03 μg L^{-1} for BPA. The sensitivity was higher than most of the previous studies [20–24]. Additionally, all three selected assays were optimized to quantify BPA residues in three food and beverage sample types. The validation conducted using the spike and recovery study demonstrated that these three assays would be valuable analytical tests in quantifying BPA residue in food and beverages that complements instrumental analyses.

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