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Development of an Enzyme-Linked Immunosorbent Assay for Bisphenol A Using Chicken Immunoglobulins

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Bisphenol A was coupled, after derivatization into a suitable hapten, to bovine serum albumin and ovalbumin in order to produce immunizing and coating antigens. The immunizing antigens were injected into chickens, which allowed the isolation of specific bisphenol A immunoglobulins from the egg yolk. These antibodies were used in an indirect competitive enzyme-linked immunosorbent assay for the determination of bisphenol A in aqueous solutions. Various parameters, influencing the assay sensitivity, were evaluated. The applicability of the assay for the determination of bisphenol A in milk was also studied. The assay was not as sensitive as other analytical techniques used in bisphenol A analysis, since typical I_{50} levels of 2.5 μM were reached in aqueous solutions. This study nevertheless illustrates the usefulness and the potency of chicken antibodies in the analysis of migration residues from packaging materials using immunochemical techniques. In addition, the assay showed to be quite specific for bisphenol A as well. Only for bisphenol A analogues, cross reactivities of about 40% were reached, enabling the use of the antibodies for the screening of bisphenol A and alike compounds.

KEYWORDS: Bisphenol A; immunoassay; IgY; ELISA; migration; chicken immunoglobulins; water; milk; cross reactivity; xeno-estrogens

INTRODUCTION

Bisphenol A (2,2-bis(4-hydroxyphenyl)propane) is an important monomer for the production of high quality food contact materials such as epoxy coatings (*1*) and polycarbonate (*2*). Because of incomplete polymerization or partial hydrolysis of the polymer, bisphenol A migration from a packaging material to the food occurs as revealed by several reports (*1*, *3*−*9*). Apart from being a food contaminant, it should be stressed that bisphenol A is considered an environmental contaminant as well, which, for example, is reported to be present in the sediment of rivers (*10*, *11*).

Because of the available toxicity data, it can be concluded that dietary exposure to bisphenol A is a food safety problem. Apart from an acute toxicity (*12*), the xeno-estrogenic effects, already known for a number of decades (*13*), are an important issue. Recent research revealed that the estrogenic activity is a multicause phenomenon. First of all, it has been proved that bisphenol A binds to the so-called estrogen receptors (*14*−*17*). The substance also enhances cell proliferation in general (*18*−*20*) and the cell proliferation of the female sexual organs in particular (*21*−*24*). Furthermore, bisphenol A is reported to induce prolactine secretion (*23*, *25*). Therefore, the influence of bisphenol A on all stages of development and in particular of the development of the male sexual organs has been studied in several animals such as mice, rats (*26*), and fish (*27*). Negative effects on sperm quality due to exposure to bisphenol A have been reported as well (*28*, *29*).

In addition to the xeno-estrogenic effects, the possible carcinogenicity of bsiphenol A was studied as well. Although bisphenol A is not a considered a mutagen (*30*) and although reports linking bisphenol A exposure to cancer incidence are limited (*31*, *32*), a number of observations illustrate that indeed bisphenol A exposure should be considered with some concern in this respect. The substance is reported to affect mitosis (*33*, *34*) and to induce aneuploidy (*34*−*36*). In addtion, bisphenol A metabolites have been shown to react with DNA inducing the production of DNA adducts (*35*−*38*). As already mentioned, bisphenol A may induce cell proliferation as well.

Because of its toxicity, the analysis of bisphenol A residues in foods has received some attention during the last 5 years as illustrated above. In these studies, classic instrumental analytical techniques have been used such as gas chromatography and high-performance liquid chromatography (HPLC) most frequently combined with mass spectrometric detection and extensive sample clean up techniques as reviewed by De Meulenaer and Huyghebaert (*39*). Recently, however, the use of immunochemical analytical techniques has been reported for bisphenol A analysis. Both monoclonal (*40*) and polyclonal (*41*) mammalian antibodies were used successfully in immunosorbent

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assays. The monoclonal antibodies were selected in such a way that a high resistance toward organic solvents was achieved, enabling the detection of bisphenol up to the 1 ppb range in solutions containing up to 50% of methanol (*40*). The polyclonal antibodies enabled the detection of bisphenol A in the 0.5−5 ppb range in urine samples (*41*). Although the use of immunochemical techniques in the analysis of food contaminants is well-established, the present paper and those mentioned above are the first to consider them for the analysis of migration residues from food contact materials. It should be stressed as well that immunochemical techniques are particularly useful for screening purposes. Such screening techniques are presently, however, not described for bisphenol A, which considering its possible presence in both food and in the environment, is a considerable disadvantage.

Although chicken immunoglobulins are less frequently used in immunochemical techniques in comparison with mammalian antibodies, they offer some distinct advantages as emphasized in a recent review (*42*). Therefore, the present paper considered the use of chicken immunoglobulins in an enzyme immunosorbent assay (ELISA) for bisphenol A analysis.

MATERIALS AND METHODS

Reagents and Buffers. 4,4′-Dihydroxybenzophenon 99%, 4,4′-ethylidenebisphenol 99%, 4-cumylphenol 99%, bis-(4-hydroxyphenyl)-methane 98%, *p*-cresol 99%, *m*-cresol 99%, 4-hydroxydiphenylmethane 99%, 4,4′-cyclohexylidenebisphenol 98%, 2,2-bis-(4-hydroxyphenyl)-perfluorpropane 97%, bis-(4-hydroxyphenyl)sulfone 98%, 4,4′-(1,4-phenylene-diisopropylidene)bisphenol 98%, 4,4′-isopropylidene bis(2,6-dimethylphenol) 98%, 3,4′-isopropylidene-diphenol 98%, 4,4′-(1,3-phenylenediisopropylidene)bisphenol 99%, 1,4-dihydroxybenzene, 4,4′-dihydroxybiphenyl 97%, butylbenzyl phthalate, 4-butylphenol, and 4,4′-(1-phenylethylidene) bisphenol 99% were from Aldrich Chemical Co., U.S.A. Benzoic acid, sodium hydrogen carbonate, methanol, hexane, and sodium hydroxide were obtained from Chem-Lab, Belgium. Butylhydroxyanisol was from Koch-light laboratories, England. BADGE was a generous gift from Ciba Specialty Chemicals, Belgium. Anhydrous disodium carbonate, 1,3-dihydroxybenzene, sodium chloride, hydrochloric acid 25%, DMF, THF, diethyl ether, chloroform, trisodium citrate dihydrate and gelatine were purchased from UCB, Belgium. Phenol 99%, 4-nonylphenol (mixture of isomers) 99%, bisphenol A 97%, potassium dihydrogen phosphate, disodium hydrogen phosphate dodecahydrate, dibutylphthalate, imidazole, tBCDS, anhydrous sodium sulfate, *N*-hydroxy succinimide, 4-(dimethylamino)-pyridine, glutaric anhydride, citric acid, TBAF, *N,N*′-dicyclohexylcarbodiimide, benzyl alcohol, anhydrous sodium sulfite, sodium tertaborate decahydrate, sodium fluoride, disodium ethylenediaminetetraacetic acid (EDTA), and ammonium sulfate were from Acros Organics, U.S.A. BSA (fraction V, 96%), OVA (Grade III), Freund's incomplete adjuvant, Freund's complete adjuvant, 98% ABTS, 95% TNBS, and Tween 20 were from Sigma Chemical.

Hydrogen peroxide 30%, OPD, silica gel G60, ethyl acetate, and potassium chloride were from Merck, Germany. HRP conjugated rabbit antichicken IgG was from ICN Biomedicals Inc., U.S.A. All of these reagents were of analytical grade unless otherwise mentioned.

Potassium caseinate and the skimmed milk powder were generous gifts of Rovita, Germany, and Belgomilk, Belgium, respectively. Sunflower oil was obtained from Vandemoortele (Belgium). Sephadex G25 was purchased from Pharmacia (Sweden) and was equilibrated for at least 16 h in an excess of PBS prior to use. Dried THF was obtained by continuous reflux of THF over sodium using benzophenon as an indicator.

PBS (pH 7.4) consisted of 0.135 M NaCl, 1.5 mM KH_2PO_4, 8 mM $Na_2HPO_4 \cdot 12H_2O$, and 2.7 mM KCl. The coating buffer (pH 9.6) was a 15 mM Na_2CO_3 and a 35 mM $NaHCO_3$ solution. The dilution buffer (PBS−Tween 20) consisted PBS with 0.05% (v/v) Tween 20. The wash solution was 0.05% (v/v) Tween 20 solution in 0.15 M NaCl. The blocking solution was PBS with 3% (w/v) K-caseinate. If OPD was used as a chromogen, the substrate buffer (pH 5.0) was a 40 mM citric acid and 35 mM $Na_2HPO_4 \cdot 12H_2O$ solution. The substrate solution consisted of 40 mg of OPD in 100 mL of substrate buffer to which just before use 5 mL of 0.03% (v/v) H_2O_2 was added. The stop solution was 2.5 M HCl. If ABTS was used as a chromogen, the substrate buffer (pH 4.0) consisted of 0.05 M tri-sodium citrate in water. The substrate solution consisted of 30 mg of ABTS in 100 mL of substrate buffer to which just before use 5 mL of 6% (v/v) H_2O_2 was added. The stop solution was a 0.1 M NaF, 0.008 M NaOH, and 0.001 M Na_2EDTA solution.

Aqueous solutions of bisphenol A and the substances for which cross reactivity was evaluated were prepared as follows. One gram of substance was dissolved in 50 mL of methanol, and this solution was diluted in water up to the desired concentration. Methanol concentration in the final solutions was considered to be negligible (constant concentration 1.5% v/v). For aqueous methanolic bisphenol A solutions, in which higher concentrations of methanol were used, methanol was added additionally until the desired concentration was reached.

Hapten Synthesis. *Synthesis of 4-[1-(4-{[tert-Butyl(dimethyl)silyl]-oxy}phenyl)methylethyl]phenol* ***2****.* To a solution of 2.28 g (10 mmol) of bisphenol A **1** in 50 mL of DMF, 1.70 g (25 mmol) of imidazole was added. Subsequently, a solution of 1.51 g (10 mmol) of tBCDS in 20 mL of DMF was added dropwise at RT. The reaction mixture was stirred for 1 h at RT and poured out in 100 mL of water. The mixture was extracted with hexane (2 × 50 mL and 2 × 25 mL) thus avoiding the coextraction of unreacted bisphenol A. The organic phase was dried over sodium sulfate and evaporated to dryness under reduced pressure. The white residue was dissolved in 5 mL of hexane−ethyl acetate 6:1 (v/v) and further purified by column chromatography. Therefore, a glass column (32 mm internal diameter) was filled with 60 g of silica gel using hexane−ethyl acetate 6:1 (v/v) as a mobile phase. Elution was accomplished by gravity. From the fraction eluting between 160 and 350 mL, 1.11 g (32%) of pure product could be obtained after evaporation to dryness under reduced pressure. ^{1}H NMR ($CDCl_3$): δ 0.08 (s, 6H, $SiCH_3$), 0.86 (s, 9H, *t*Bu), 1.49 (s, 6H, CH_3), 6.56 (d, 2H, aromatic), 6.63 (d, 2H, aromatic), 6.95 (d, 4H, aromatic). ^{13}C NMR ($CDCl_3$): δ −4.26 (2 × CH_3−Si), 18.29 (C_{quat}, *t*Bu), 25.82 (*t*Bu), 31.20 (2 × CH_3), 41.80 (*C*-CH_3), 114.82 (2 × CH, aromatic), 119.42 (2 × CH, aromatic), 127.80 and 128.05 (4 × CH, aromatic), 143.50 and 143.77 (2 × C aromatic), 153.20 and 153.30 (C_{quat}-OH and C_{quat}-OSi). MS *m*/*z* (%): 343 (41); 329 (27); 328 (80); 286 (21); 136 (18); 135 (100); 107 (11); 73 (20). IR (cm^{-1}): ν_{max} 3272 (OH); 1511 (Ph); 1252 ($Si(CH_3)_2$). Melting point: 79.5 °C.

Synthesis of 5-{4-[1-(4-{[*tert*-Butyl(dimethyl)silyl]oxy}phenyl)-1-methylethyl]phenoxy}-5-oxopentanoic Acid 3. To a solution of 1368 mg (4 mmol) of **2** in 30 mL of dry THF, 13.68 g (120 mmol) of glutaric anhydride and 489 mg of 4-(dimethylamino)pyridine (4 mmol) were added. The mixture was heated to reflux for 2 h, poured out in 50 mL of water, acidified with 10 N HCl to pH < 2, and extracted with chloroform (2 × 50 mL and 2 × 25 mL). The organic phase was dried over sodium sulfate and evaporated to dryness under reduced pressure. The residue was dissolved in 20 mL of diethyl ether and cooled for 2 h at −18 °C in order to precipitate part of the glutaric anhydride in excess, which was removed by filtration. After it was evaporated to dryness under reduced pressure, the residue was dissolved in 2 mL of hexane−ethyl acetate 4:1 (v/v) and further purified using column chromatography. Therefore, a glass column (10 mm internal diameter) was filled with 5 g of silica gel and hexane. Elution was accomplished with 40 mL of hexane−ethyl acetate (4:1) (v/v). After it was evaporated to dryness under reduced pressure, the residue was dissolved in 2 mL of hexane−ethyl acetate 4:1 (v/v) and again purified using a similar chromatographic setup. From the fraction eluting between 5 and 55 mL, 967 mg (53%) of pure product could be obtained after evaporation to dryness under reduced pressure. ^{1}H NMR ($CDCl_3$): δ 0.08 (s, 6H, $SiCH_3$), 0.86 (s, 9H, *t*Bu), 1.54 (s, 6H, CH_3), 1.96 (quint, 2H, C$H_2$$CH_2$COOH), 2.41 (t, 2H, C$H_2$COOH), 2.55 (t, 2H, C$H_2$COOPh), 6.62 (d, 2H, aromatic), 6.85 (d, 2H, aromatic), 6.96 and 7.08 (d, 2 × 2H, aromatic). ^{13}C NMR ($CDCl_3$): δ −4.23 (2 × CH_3−Si), 18.08 (CH_2CH_2COOH), 19.65 (C_{quat}, *t*Bu), 25.59 (*t*Bu), 30.91 (2 × CH_3), 32.64 (CH_2COOPh), 33.11 (CH_2COOH), 42.02 (*C*-CH_3), 119.23 (2 × CH aromatic), 120.62 (2 × CH aromatic), 127.62 and

Table 1. Characteristics of Immunizing and Coating Bisphenol A Antigens

antigen	bisphenol A/protein ratio during synthesis (mol/mol)	actual bisphenol A/protein ratio (mol/mol)
immunizing antigen	138.0	26.0 ± 0.26
coating antigen A	90.0	4.86 ± 0.10
coating antigen B	45.0	3.80 ± 0.11
coating antigen C	22.5	2.95 ± 0.03

127.71 (4 × CH aromatic), 142.88 and 148.17 (2 × C aromatic), 148.57 (C_{quat}-OSi), 153.34 (C_{quat}-OCO), 171.43 (COOPh), 178.80 (COOH). MS *m*/*z* (%): 88 (24); 86 (90); 84 (100); 49 (26); 47 (30). IR (cm^{-1}): ν_{max} 3416 (OH); 1749 (*CO*OPh); 1704 (*CO*OH); 1510 (Ph); 1226 (Si-$(CH_3)_2$).

Synthesis of 5-{4-[1-(4-Hydroxyphenyl)-1-methylethyl]phenoxy}-5-oxopentanoic Acid 4. To an ice-cooled solution of 790 mg (2.5 mmol) of TBAF in 15 mL of THF, an ice-cooled solution of 1140 mg (2.5 mmol) of **3** in 15 mL of THF was added dropwise. After 1 h at 0 °C, the reaction mixture was poured out in 30 mL of water, acidifed with 10 N HCl to pH < 2, and extracted with chloroform (3 × 20 mL). The combined organic fractions were washed with 25% HCl (10 × 10 mL), dried over sodium sulfate, and evaporated to dryness under reduced pressure, yielding 633 mg of pure hapten **4** (74%). ^{1}H NMR ($CDCl_3$): δ 1.63 (s, 6H, CH_3), 2.06 (quint, 2H, C*H*$_2$CH$_2$COOH), 2.49 (t, 2H, C*H*$_2$COOH), 2.67 (t, 2H, C*H*$_2$COOPh), 6.75 (d, 2H, aromatic), 6.95 (d, 2H, aromatic), 7.07 (d, 2H, aromatic), 7.22 (d, 2H, aromatic).^{13}C NMR ($CDCl_3$): δ 20.36 (*C*H$_2$CH$_2$COOH), 31.58 (C-*C*H$_3$), 33.43 (*C*H$_2$-COOPh), 34.08 (*C*H$_2$COOH), 42.65 (*C*-CH$_3$), 115.39 (2 × CH aromatic), 121.33 (2 × CH aromatic), 128.46 and 128.52 (CH aromatic), 143.36 (C aromatic), 149.00 (C aromatic), 149.33 (C_{quat}-OH), 154.22 (C_{quat}-OCO), 172.65 (COOPh), 179.43 (COOH). MS *m*/*z* (%): 228 (37); 213 (100); 86 (24); 84 (35). IR (cm^{-1}): ν_{max} 3416 (OH); 1749 (*CO*OPh); 1704 (*CO*OH); 1510 (pH); 1226 (Si$(CH_3)_2$).

Preparation of Immunizing and Coating Conjugates. Haptens were covalently attached to BSA and OVA, respectively, using the method originally described by Cuatrecasas and Parikh (*43*). For the synthesis of the immunizing conjugates, 102 mg of hapten **4** (0.3 mmol) in 3 mL of DMF was mixed with 48 mg (0.4 mmol) of *N*-hydroxy succinimide and 62 mg (0.3 mmol) of *N*,*N*′-dicyclohexylcarbodiimide. The solution was left for 16 h at RT, and the crystals formed were removed by decantation of the supernatant, which was added dropwise to 10 mL of a protein solution (15 mg BSA/mL in 50 mM sodium carbonate at pH 9.6). This solution was stirred for 4 h at RT, and finally, the conjugates were purified by gel filtration on Sephadex G25 using PBS as mobile phase.

Three different coating conjugates were produced using OVA, with a varying bisphenol A to protein ratio as summarized in **Table 1**. Similar reaction conditions as for the production of BSA−bisphenol conjugates were used except for the volume of protein solution, since the concentration in the carbonate buffer was kept constant at 15 mg OVA/mL.

The extent of coupling for each conjugate was determined using the TNBS method as described by Fields (*44*). Keeping into account the number of available amino groups in BSA and OVA as reported by Habeeb (*45*), the bisphenol A load of the immunizing and coating antigen was estimated as summarized in **Table 1**.

Chicken Immunization and Immunoglobulin Isolation. Three Isa Brown chickens of 40 weeks old were injected intramuscularly with 1 mL of a 1:1 (v/v) mixture of Freund's complete adjuvant and PBS containing 500 μg of immunizing antigen (BSA−bisphenol A conjugate). After 3 weeks, a supplementary injection of 1 mL of a 1:1 (v/v) mixture of Freund's incomplete adjuvant and PBS containing 500 μg of immunizing antigen was given. Afterward, boaster injections of 500 μg of immunizing conjugate in PBS were repeated every 3 weeks during a 70 day period, after which the immunization procedure was stopped. Eggs were collected daily and individually identified. The immunoglobulins were isolated from the egg yolk using a modified aqueous dilution method described by Akita and Nakai (*46*). Briefly, υ mL of egg yolk was separated from the egg and diluted with 8 × υ mL of water and pH was set with 1 N HCl between 5.0 and 5.2. After they were incubated for 16 h at 4 °C and centrifuged (10 000*g*, 1 h, 4 °C), the supernatant was filtered. After 72 g of ammonium sulfate and about 170 mL of water was added in order to achieve a 60% saturated solution of ammonium sulfate, the mixture was incubated for 1 h at RT and centrifuged (10 000*g*, 20 min, RT). The residue was dissolved in a 19% (w/v) sodium sulfate solution. After it was incubated for 20 min at RT and centrifuged (2000*g*, 20 min, RT), the residue was dissolved in a 14% (w/v) sodium sulfate solution. After it was incubated for 20 min at RT and centrifuged (2000*g*, 20 min, RT), the residue was dissolved in υ/6 mL of PBS and stored in small aliquots at −18 °C. For further use, they were diluted from this final solution.

Immunoassays. *General Conditions.* Ninety-six well F 96 Maxisorp Nunc immunoplates from Nunc (Denmark) were coated with coating antigen A solution (12.5 μg/mL coating buffer, 100 μL/well) by overnight incubation at 4 °C in the dark. Plates were washed three times (200 μL wash solution/well) and blocked (200 μL blocking solution/well) for 2 h at RT in the dark. Afterward, the plates were washed twice as previously. To study the immune response of the chickens or to obtain competition curves, primary antibodies were added as described elsewhere. Afterward, the plates were washed as described above (three times).

For the detection reaction, the HRP-conjugated secondary antibody was added (100 μL/well, 3.4 μ/mL dilution buffer). After it was incubated for 1 h at 37 °C and the plates were washed (three times), 100 μL/well of substrate solution was added followed by an additional incubation at 37 °C for 1 h. Finally, 25 μL/well of the appropriate stop solution was added before measuring the absorbance at the appropriate wavelength (for OPD 492 nm; for ABTS 405 nm). Absorbances were corrected for blank readings obtained by using immunoglobulins isolated from the eggs of nonimmunized chickens. These conditions were followed unless otherwise stated.

Indirect ELISA. To study the immune response of the chickens, the appropriate primary antibody dilution was added to the coated wells (100 μL/well in dilution buffer) and the plates were incubated for 1 h at 37 °C. OPD was used as chromogen.

Indirect Competitive ELISA. For the competition step, 50 μL of the appropriate bisphenol A dilution and 50 μL of the primary antibody solution were added to each well. Primary antibodies were diluted as follows: 20 μL of the original primary antibody solution in PBS was further diluted to 7.24 mL with PBS. Subsequently, 26.64 mL of PBS containing 0.3% (w/v) BSA, 520 μL of NaOH 0.1 N, and 5.6 mL of a 4 M NaCl solution were added, obtaining a final dilution of the primary antibody of 1/2000, a pH of 8.0, a BSA concentration of 0.2% (w/v), and a calculated ionic strength of 700 mM. This dilution is referred to as the competition buffer. The plates were incubated for 1 h at 37 °C. Both OPD and ABTS were used as chromogens.

Studies on the Composition of the Competition Buffer. Characteristics of competition buffer were evaluated as follows: ionic strength, presence of surface active agents, and pH.

For the ionic strength studies, the amount of 4 M NaCl added to the competition buffer was adjusted in order to vary its ionic strength, reducing the amount of PBS accordingly, thus keeping the primary antibody concentration constant for all experiments. If necessary, the addition of NaCl solution was replaced by the addition of water. The ionic strength was calculated using the following formula

$$I = 0.5\sum_i c_i z_i^2$$

where I is the ionic strength, c is the concentration of each ion, and z is its charge. Ionic strengths reported refer to the ionic strength of the diluted IgY solution before it is applied in the assay. BSA itself was not present in the competition buffer for the reported experiment.

The influence of Tween 20 in the competition buffer was evaluated as a function of the ionic strength and its concentration. Therefore, BSA was replaced in the competition buffer by Tween 20 (0−0.4% v/v). In addition to Tween 20, however, BSA (0.2% w/v) and potassium caseinate (0.2% w/v) were also evaluated.

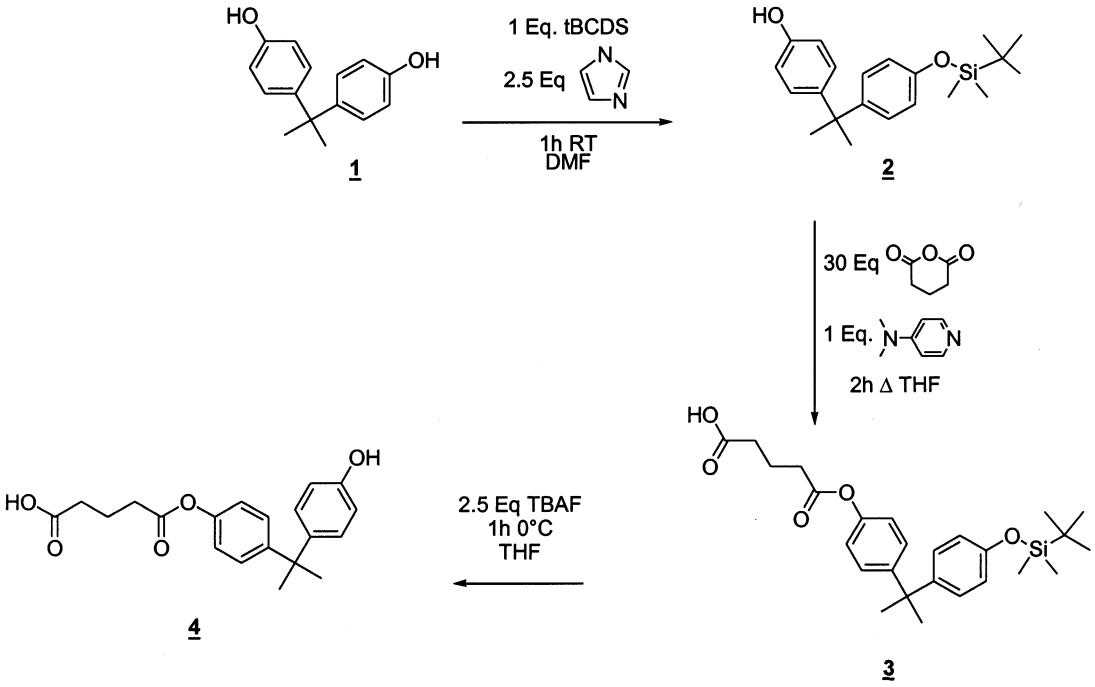


Figure 1. Bisphenol A hapten synthesis.

The amount of NaOH (0.1 N) or HCl (0.1 N) added to the competition buffer was adjusted together with the amount NaCl 4 M and PBS in such a way that the desired pH was reached keeping the ionic strength constant. Initially, these studies were performed using Tween 20, but apart from this surfactant, potassium caseinate (0.2% w/v) and BSA (0.2%) were also used, respectively.

Coating Antigen Studies. Three different coating antigens, as indicated in **Table 1**, were used at varying concentrations (0.4−12.5 μg/mL) during the coating of the multiwell plates.

Cross Reactivity. Competitive assays using coating antigen C (0.8 μg/mL in coating buffer) were performed using various structural bisphenol A analogues in order to determine their respective I_{50} values (micromolar). I_{50} is the concentration of the analyte at which half of the maximal signal intensity is reached. Cross reactivity was calculated as

$$\text{cross reactivity (\%)} = (I_{50,\text{bisphenol A}})/(I_{50,\text{compound}}) \times 100$$

Solvent Effect. Competitive immunoassays using coating antigen C (0.8 μg/mL in coating buffer) and ABTS as a chromogen were accomplished using aqueous methanol bisphenol A solutions at the appropriate methanol and bisphenol A concentrations.

Assay Performance in Milk. Competitive immunoassays using coating antigen C (0.8 μg/mL in coating buffer) and ABTS as a chromogen were accomplished using milk samples that were spiked with bisphenol A at the appropriate concentration. Reconstituted milk was prepared as follows: 10 g of skimmed milk powder was dissolved in 60 mL of distilled water. For the addition of bisphenol A, the appropriate aqueous solution was added at this stage as well. If necessary, sunflower oil at the appropriate concentration was emulsified in the dispersion using a Ultraturrax mixer at moderate speed. Afterward, the mixture was diluted until a final volume of 100 mL was reached. Pasteurized milk samples at various fat contents were obtained from retail shops. These samples were spiked with a concentrated methanolic bisphenol A solution, keeping the methanol concentration constant at 1.5% v/v.

Data Processing. Competition curves were obtained in 4-fold. When required, curves were normalized by expressing the experimental absorbance levels (B) as (B/B_0), where B_0 is the absorbance in absence of analyte. Absolute or normalized signals were fitted to a four parameter logistic equation using a commercial software package (SPSS 10.0).

Instruments. The Titertek multiskan plus MK II (U.S.A.) and the Organon Teknika (The Netherlands) multireaders were used throughout this research. NMR spectra were obtained using a JEOL PMX 270 SI (270 MHz) instrument by dissolving the samples in deuterated chloroform, using tetramethylsilane as a reference. For the mass spectra, a Varian MAT 112 mass spectrometer (U.S.A.) (70 eV) was used, which was coupled with a Varian aerograph 2700 gas chromatograph (U.S.A.). The gas chromatograph was equipped with a CPSil 5CB column (Chrompack, The Netherlands) (internal diameter 0.32 mm, film thickness 0.25 μm, length 30 m). A Perkin-Elmer model 1310 infrared spectrometer was used to obtain IR spectra. For the determination of the melting point, a Büchi 535 was used.

RESULTS AND DISCUSSION

Bisphenol A Hapten and Antigen Synthesis. To produce suitable protein conjugates for immunization and coating, bisphenol A **1** was transformed into hapten **4** according to the reaction scheme outlined in **Figure 1**. Therefore, bisphenol A **1** was primarily derivatized with tBCDS to compound **2** in order to protect one of the hydroxyl groups as described by Corey and Venkateswarlu (*47*). Of course, reaction at the second hydroxyl group of bisphenol A could not be prevented. So, consequently, compound **2** was isolated from the reaction mixture by preparative column chromatography. The free hydroxyl group in compound **2** reacted with an excess of glutaric anhydride yielding derivative **3** after various purification steps (selective partial precipitation of glutaric anhydride; column chromatography). Removal of the protecting group was achieved using TBAF at low temperatures, similarly as described by Sinha et al. (*48*), yielding hapten **4**.

This hapten, carrying a five carbon spacer arm, was used for the production of immunizing and coating antigens. Therefore, BSA and OVA were used, respectively. Three different coating antigens were obtained by variation of the bisphenol A to protein ratio during synthesis as indicated in **Table 1**.

Chicken Immunization. Response of the chickens toward the immunization procedure was evaluated by using an indirect ELISA as described. Two of the immunized chickens reacted toward the applied immunization procedure. Because one of

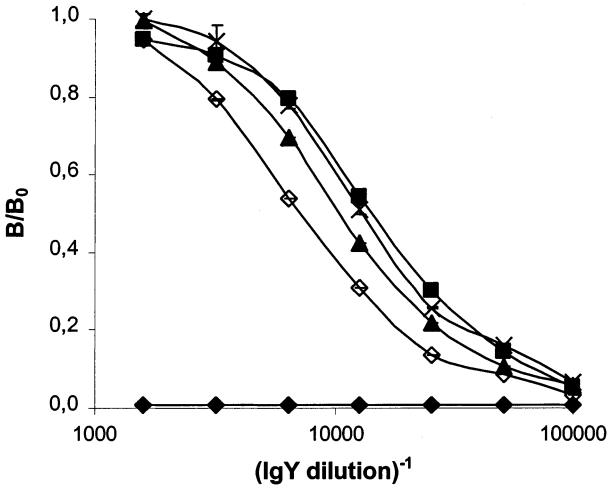


Figure 2. Response of a chicken immunized with bisphenol A–BSA conjugate at various days after start of the immunization (♦ = 7 days; ■ = 28 days; ▲ = 42 days; ◊ = 45 days; × = 70 days. Response was normalized to the maximal absorbance observed, B_0).

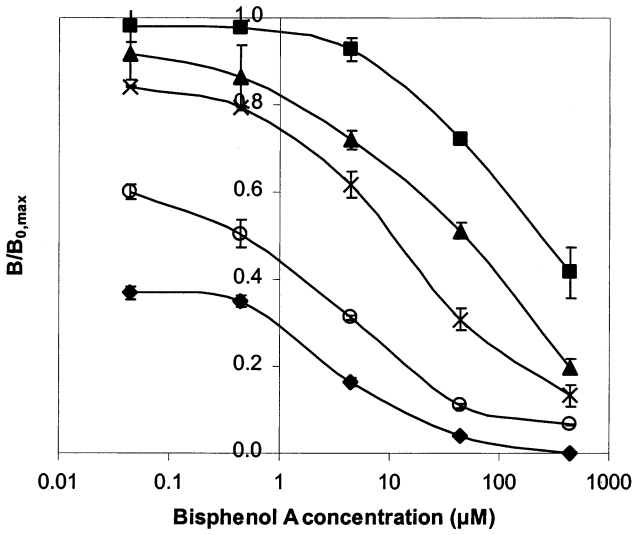


Figure 3. Competition curves with a varying ionic strength of the competition buffer (■ = 200 mM; ▲ = 300 mM; × = 400 mM; ○ = 800 mM; ♦ = 3000 mM. Responses were normalized to the highest maximal absorbance observed, $B_{0,max}$).

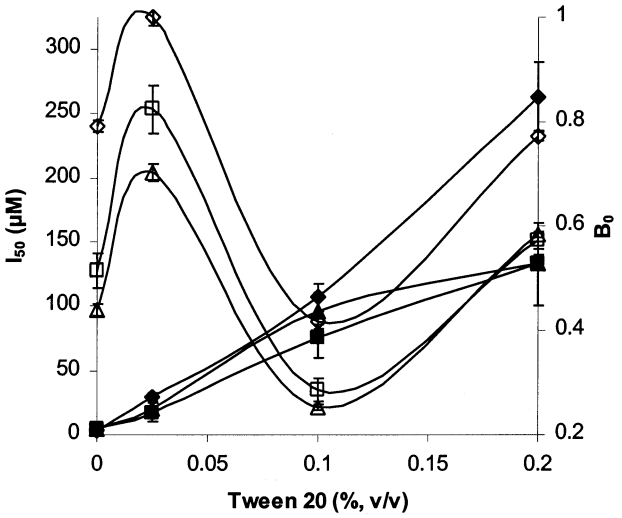


Figure 4. Influence of Tween 20 concentration at various ionic strengths on the I_{50} (filled symbols) and B_0 (open symbols) values (♦,◊ = 700 mM; ▲,△ = 1600 mM; ■,□ = 2000 mM).

these chickens, however, stopped egg production 35 days after the start of the immunization procedure, a small amount of useful eggs could be collected for further use. Therefore, only the eggs from one single chicken were used throughout further experiments. As can be seen from **Figure 2**, appreciable response was observed about 1 month after the immunization procedure started. In addition, this response could be maintained until at least 70 days, after which the immunization experiments were aborted because of the large number of useful eggs already collected. To the authors knowledge, this is the first reported successful chicken immunization with plastic monomer−protein conjugates in general and with bisphenol A−protein conjugates in particular. Moreover, it should be noted that the use of chicken antibodies in the analysis of food contaminants is not so well-documented as compared to the use of other antibodies, despite the ease of immunization and antibody isolation and purification. In addition, large quantities of antibodies can be collected because of the daily egg production and the relatively high concentration of antibodies in the egg yolk (*42*).

Optimization of the Competitive Indirect Bisphenol A Assay. The isolated antibodies from the chickens were used to evaluate their use in a competitive indirect ELISA. Therefore, in the first place, the parameters having an influence on the assay performance were evaluated. In the preliminary experiments, it was revealed that the composition of the competition buffer had an important effect on the assay characteristics.

Ionic Strength Effect. As indicated in **Figure 3**, ionic strength of the competition buffer was of prime importance with regard to the assay performance. Within the range of 400−800 mM, assay performance was considered to be acceptable within the achievable sensitivity range. At lower levels, the I_{50} values were unacceptably high, while for higher ionic strengths, overall absorbance became too low. Therefore, an ionic strength of 700 mM was selected as an optimal level. Previously, Abad and Montoya (*49*) observed a strong influence of the ionic strength on the performance of an assay for carbaryl using monoclonal mice antibodies as well. They suggested that the interaction between antibodies and hydrophobic analytes is highly influenced by the polarity of the buffers used.

From **Figure 3**, it was revealed as well that the overall sensitivity of the assay was rather poor, as compared to other competitive immunoassays. This was unexpected; therefore, further attempts to optimize the sensitivity of the assay were performed.

Influence of Surface Active Compounds. Surface active compounds such as Tween 20 are frequently applied in immunoassays in order to reduce nonspecific interactions. As can be seen from **Figure 4**, I_{50} values increased with Tween 20 concentration at several ionic strengths tested. Consequently, Tween 20 concentration should be as low as possible in order to achieve better assay sensitivity. If no Tween 20 was present, however, assay reproducibility was rather poor. Similar observations were made previously by Abad and Montoya (*49*). Apart from the effects on assay sensitivity, Tween 20 affected the maximum absorbance B_0 as well: B_0 values became maximal at relatively low Tween 20 concentrations (0.025% v/v) at all ionic strengths tested. These observations are not in complete agreement with those previously reported by Abad and Montoya (*49*), who found a constant decrease of the maximal absorbance as a function of the Tween 20 concentration. For the sake of completeness, it should be noted that at lower ionic strengths

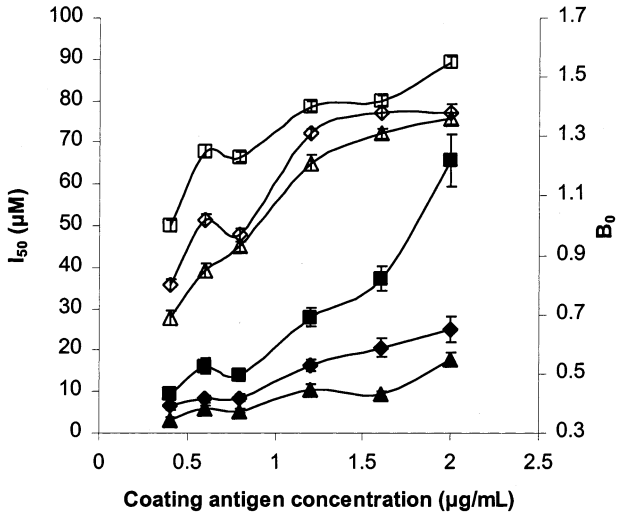


Figure 5. Influence of the kind of coating antigen and its concentration on I_{50} (filled symbols) and B_0 (open symbols) values (♦,◇ = coating antigen A; ■,□ = coating antigen B; ▲,△ = coating antigen C; for the characteristics of these coating antigens, see **Table 1**).

than those reported in **Figure 4**, even higher maximal absorbance levels were observed, which was in correspondence with the data shown in **Figure 3**.

Apart from Tween 20, caseinate and BSA were also considered. The use of the caseinate resulted in a slightly improved assay sensitivity in combination with lower maximal absorbance levels as compared to Tween 20 (0.1%, w/v). For BSA, no significant differences as compared to the use of Tween 20 (0.1%, w/v) were observed. This is in contrast to the results obtained by Abad and Montoya (*49*), who observed improved assay characteristics by replacing Tween 20 with BSA.

pH Effect. Because bisphenol A can be considered a weak organic acid, the pH of the competition buffer was tested together with the use of various surface active agents (Tween 20, caseinate, BSA). Generally, at pH levels lower than five and higher than ten, very low absorbance levels were obtained ($B_0 < 0.2$). Within this range, however, the influence of the pH on the assay performance was not significant for all surfactants tested (not shown). This was again surprising since pH dependence of both signal intensity and sensitivity of ELISAs have been reported (*49*−*51*). Remarkably, however, it was observed that assay reproducibility increased if BSA was used instead of Tween 20, in a pH-dependent manner (starting from pH 8 to pH 10). Again, no explanation could be given for these phenomena.

Coating Antigens. The influence of the coating antigen on the assay performance was evaluated as well. In the initial experiments using coating antigen A, it was observed that lower antigen concentrations resulted in decreasing I_{50} values. Unfortunately, however, B_0 values decreased as well. Because of these observations, other coating antigens were produced with a lower bisphenol A load and similar experiments were performed. A similar trend as in the preliminary experiments could be observed (**Figure 5**). It should be noted, however, that for the antigen with the lowest bisphenol A load tested (coating antigen C), the decrease in I_{50} values was less pronounced as compared to the other antigens. It should also be noted that although the bisphenol A load of the coating antigen B was lower as compared to coating antigen A, higher I_{50} values were observed at all concentration levels tested. This was surprising because of the earlier observations.

Other Relevant Parameters. To obtain good assay characteristics of a competitive ELISA, limiting concentrations of immunoreagents were required. Therefore, the effect of the antibody and the HRP-labeled antibody concentration in combination with the incubation time of the competition step and the detection reaction were evaluated as well. In summary, lowering the concentration of the primary antibody and the HRP-labeled antibody resulted both in lower maximal absorbances and lower I_{50} values. Similar observations could be made if the incubation times were restricted. Also, the following blocking reagents were evaluated for their capability to reduce a-specific binding: gelatine (0.1−3%, w/v), ovalbumine (3%, w/v), skimmed milk powder (5%, w/v), and potassium caseinate (3%, w/v). Milk powder and potassium caseinate proved to be the best. Potassium caseinate was preferred because of its more simple chemical composition as compared to skimmed milk powder.

The use of ABTS as an alternative detecting reagent instead of OPD was evaluated as well. Maximal absorbance levels decreased together, however, with the blank signal. In addition, a significant increase in assay sensitivity was observed as well (I_{50} (OPD): 11.36 μM ± 0.67; I_{50} (ABTS): 2.40 μM ± 0.19).

None of the optimization steps reported changed the assay sensitivity by orders of magnitude. Therefore, the use of the assay is currently limited to solutions in which the bisphenol A concentration is relatively high (hundreds of ppbs). Despite this fact, the specificity and the applicability of the assay to relevant samples was evaluated.

Specificity of the Assay. Cross reactivity in aqueous solutions of various structural analogues (phenols) was evaluated. Most compounds were selected because they can be present in food contact materials as well, apart from bisphenol A. Some of these are known to exhibit an estrogenic effect (BADGE, phthalates) as well (**Table 2**).

Compounds with a similar structure as bisphenol A showed some varied cross reactivity. Removal of central methyl groups of the bisphenol A molecule resulted in a fairly strong reduction in cross reactivity, while removal of one of the hydroxyl groups resulted in a cross reactivity of about 40%. This high cross reactivity for these particular compounds can probably be explained by the type of antigen used for immunization, since one of the hydroxyl groups of the bisphenol A molecule was used to couple it to the carrier protein. More complex bisphenol A analogues could not be recognized by the isolated immunoglobulins. Curiously, simple phenolic compounds such as 4-butylphenol and butylhydroxyanisol showed appreciable cross reactivity (10%). Phthalates did not show cross reactivity at all, while BADGE was slightly cross-reactive. This indicates that there is no link between the estrogenic character of these compounds and their immunochemical recognition. For the sake of completeness, it should be mentioned that no significant variation was observed between the cross reactivity of several eggs.

Influence of Methanol on the Assay. A draft CEN procedure was prepared for the quantification of bisphenol A in oil. This method is based on an extraction of bisphenol A from the oil using an aqueous methanol solution and subsequent HPLC−UV analysis (*52*). Therefore, the assay characteristics were evaluated as a function of the methanol concentration in the competition buffer. The presence of methanol resulted in a significant concentration-dependent decrease in maximum signal and assay sensitivity (**Figure 6**). At the highest methanol concentration tested, high assay variability was observed as well. This is in correspondence with the results obtained by Abad

Table 2. Cross Reactivity of the Assay

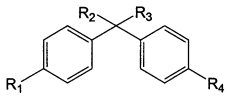
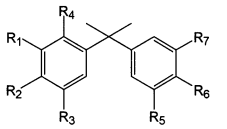
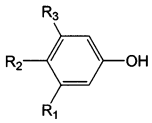
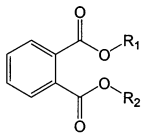
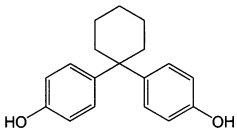
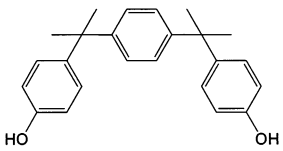
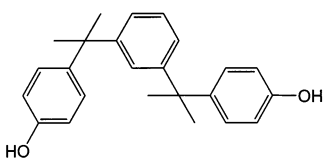
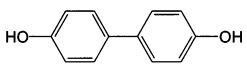
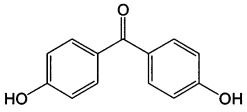
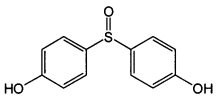
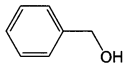
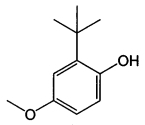
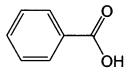
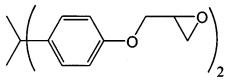
Chemical structure		Name	Cross reactivity (%)
	R_2,R_3 = CH_3, R_1,R_4 = OH	bisphenol A	100
	R_2,R_3 = H, R_1,R_4 = OH	bis-(4-hydroxyphenyl)-methane	5
	R_2= CH_3, R_3 = H, R_1,R_4 = OH	4, 4'-ethylidenebisphenol	18
	R_1,R_2,R_3 = CH_3, R_4 = OH	4-hydroxydiphenylmethane	3
	R_1=H, R_2,R_3 = CH_3, R_4 = OH	4-cumylphenol	43
	R_2,R_3 = CF_3, R_1,R_4 = OH	2,2-bis-(4-hydroxyphenyl)-perfluorpropane	3
	R_2= CH_3, R_3 = C_6H_5, R_1,R_4 = OH	4,4'-(1-phenylethylidene)bisphenol	<0.1
	R_1, R_3,R_5,R_7 = CH_3, R_2,R_6 = OH, R_4 = H	2,2-bis-(4-hydroxy-3-methyl-phenyl)-propane	<0.1
	R_1, R_2,R_3,R_5,R_7 = H, R_4,R_6 = OH,	3,4'-isopropylidene-diphenol	32
	R_1,R_2,R_3 = H	phenol	<0.1
	R_1, R_3 = H, R_2 = CH_3	p-cresol	0.5
	R_2, R_3 = H, R_1 = CH_3	m-cresol	0.5
	R_1, R_3 = H, R_2 = *sec* butyl	4-butylphenol	9
	R_1, R_3 = H, R_2 = C_9H_{19}	4-nonylphenol	2
	R_1, R_3 = H, R_2 = OH	1,4-dihydroxybenzene	<0.1
	R_2, R_3 = H, R_1 = OH	1,3-dihydroxybenzene	<0.1
	R_1, R_2 = C_4H_9	dibutyl phthalate	<0.1
	R_1= C_4H_9, R_2 = C_6H_5	butylbenzyl phthalate	<0.1
		4,4'-cyclohexylidenebisphenol	3
		4,4'-(1,4-phenylene-diisopropylidene)-bisphenol	2
		4,4'-(1,3-phenylenediisopropylidene)-bisphenol	0.5
		4,4'-dihydroxybiphenyl	<0.1

Table 2 (Continued)

Chemical structure	Name	Cross reactivity (%)
	4, 4'-dihydroxybenzophenon	0.1
	bis-(4-hydroxyphenyl)-sulphone	<0.1
	benzylalcohol	<0.1
	butylhydroxyanisol	10
	benzoic acid	<0.1
	bisphenol A diglycidyl ether (BADGE)	2

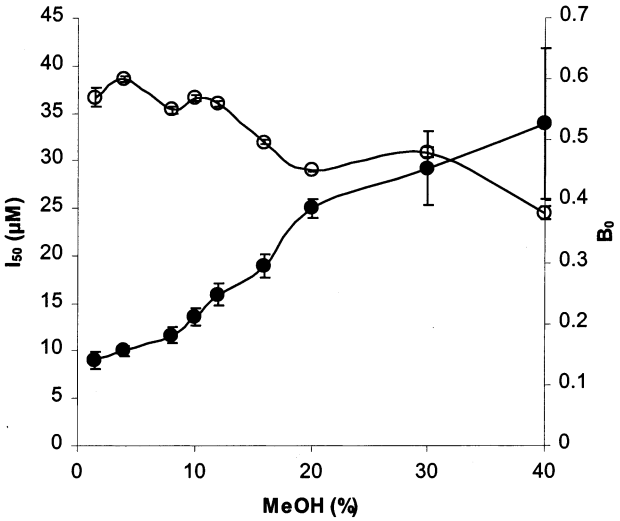


Figure 6. Influence on methanol during the competition on the I_{50} (filled symbols) and B_0 (open symbols).

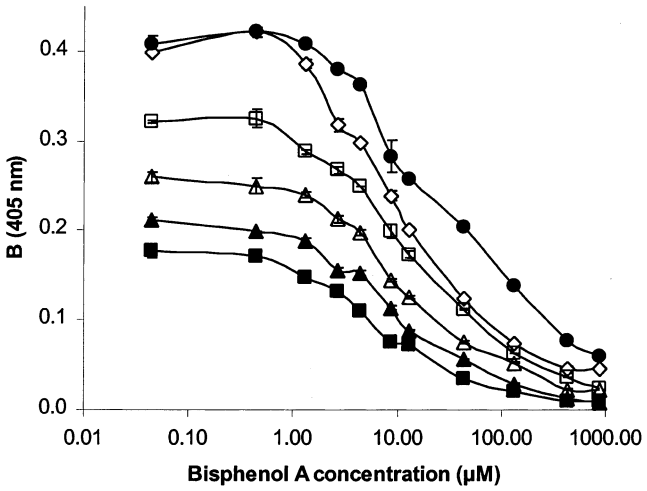


Figure 7. Competition curves obtained in skimmed milk at selected ionic strengths of the competition buffer (aqueous bisphenol A solution: ● = 700 mM; bisphenol A in milk: ◇ = 140 mM; □ = 220 mM; △ = 300 mM; ▲ = 400 mM; ■ = 600 mM).

and Montoya (*49*), who investigated the influence of several organic solvents on the characteristics of a carbaryl assay. The presence of 10% (v/v) methanol could be tolerated in order to apply the assay for the quantification of bisphenol A in oil. Therefore, the aqueous methanolic extract of the oil could be applied without significant dilution, illustrating the potency of the presented assay.

Assay Performance in Milk. Because dairy products are frequently contacted with polycarbonate, application of the assay for milk could be of practical interest. Therefore, the performance of the assay was evaluated for milk at various fat contents. Initial experiments on whole reconstituted milk revealed a strong reduction in assay quality (increased I_{50} and reduced B_0). Because similar results were obtained for skimmed reconstituted milk, however, the reduced assay performance could not be solely due to an absorption of bisphenol A by the fat globules rendering it unavailable for immunochemical reactions (results not shown).

Because milk contains an appreciable amount of salts as well, the ionic strength of the competition buffer was adjusted because of the earlier observations with regard to the influence of ionic strength on the assay performance. By changing the ionic strength of the competition buffer, the assay performance could be influenced in such a way that the inhibition curves obtained in milk were similar to those obtained in water (**Figure 7**). A complete match of the competition curves obtained in water and milk, respectively, is difficult to achieve because of complex sample matrix. Especially the presence of milk proteins seem

to be of importance in this regard because similar competition curves were obtained for skimmed, semiskimmed, or whole milk (results not shown). This indicates that the fat content, within the range (0−3.5%) tested, is of no importance with regard to the assay performance. This result was quite surprising because bisphenol A is probably partially absorbed by the fat globule indicating that the antibodies used are able to penetrate within the interface of the fat globules and the aqueous phase thus inducing immunochemical reactions.

ABBREVIATIONS USED

ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); BADGE, bisphenol A diglycidyl ether; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; CEN, Centre Européen de Normalization, European Centre for Normalization; HRP, horseradish peroxidase; IgY, chicken egg yolk immunoglobulins; OPD, orthophenylenediamine; OVA, ovalbumin; PBS, phosphate-buffered saline; RT, room temperature; THF, tetrahydrofurane; DMF, dimethylformamide; TBAF, *N*,*N*,*N*-tributyl-1-butylammonium fluoride; tBDCDS, *tert*-butyl-(chloro)dimethylsilane; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

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