Research Article

A fluorescence polarization assay for bisphenol analogs in soybean oil using glucocorticoid receptor

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A fluorescence polarization (FP) assay based on receptor was developed for the determination of bisphenol analogs (BPs) in soybean oil. In the competitive binding system, dexamethasone fluorescein (Dex-fl) and glucocorticoid receptor (GR) were selected as probe and recognition element, respectively. Crucial factors that affect the precision and accuracy were optimized. After development, the method was characterized by IC_{50} values ranging from 1.8 to 66.9 μM of 11 BPs. The established method revealed a limit of detection (LOD) of 1.0 μM and a working range of 7.6–30 μM for BPA in soybean oil. Analysis of spiked soybean oil samples showed that acceptable recoveries ranged from 71.99 to 82.95% with coefficients of variation (CVs) less than 10%. Furthermore, the results detected by the FP assay showed good agreement with that of HPLC with a fit of $R^2 = 0.9171$. It is concluded that this method offers a reliable alternative for determination of BPs in soybean oil.

Practical applications: This work aims to develop FP assay for determination of BPs in soybean oil. Based on the recombinant GR, this method can detect multiple BPs simultaneously. It can potentially be used for high-throughput screening of BPs, which may serve as an assistant of chromatographic techniques.

Keywords: Bisphenol analogs / Fluorescence polarization / Glucocorticoid receptor / Soybean oil

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1 Introduction

Bisphenol A (BPA) is utilized in the manufacture of polycarbonate plastics and epoxy resins [1]. In recent years, global production of BPA is predicted to increase due to its diverse uses and the huge demand in consume products. When the BPA-based polycarbonate and epoxy resins are exposed to high temperatures or to acidic or basic substances, the monomer BPA may be released into the food sample following its formation via hydrolysis of ester bonds of the polymers [2]. From the toxicological point of view, BPA is a potential endocrine disruptor chemical (EDC) and several studies have been carried out to verify its adverse effects in humans and animal models [3]. The public concern and governmental regulations on BPA stimulated the development and production of alternative substances to replace BPA in a myriad of applications. Nowadays, many bisphenol analogs (BPs) such as BPS, BPF, and BPAF have already been used in the manufacture of consume products [4, 5]. Due to those chemicals share a common structure of two hydroxyphenyl functionalities and physicochemical properties, their effects in physiological system may be similar. Because of the toxicity of BPA and the limited availability, many bio-based resources have been tested as potential candidates for replacing BPA in epoxy resins [6].

Numerous of analytical methods have been reported for determination of BPs. Among which, high-performance liquid chromatography (HPLC) and gas chromatography (GC) were successfully applied for the detection of bisphenols in agricultural products [7, 8]. Although chromatographic techniques are accurate and reliable, they are expensive and time-consuming. By contrast,

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Abbreviations: BPs, bisphenol analogs; **CVs,** coefficients of variation; **Dex-fl,** dexamethasone fluorescein; **FP,** fluorescence polarization; **GR,** glucocorticoid receptor; **LOD,** limit of detection

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immunoassays offer many advantages that can complement instrumental analysis. ELISA methods are of low operating cost and more suitable for rapid detection [9–11]. However, antibody-based immunoassays can only detect individual molecule, which make it not applicable for multi-residue determination [12].

Compared with antibody, receptor protein displays its significant superiority of a broad specificity to various ligands [13]. A variety of studies confirmed that BPs can interact with estrogen receptor (ER), peroxisome proliferator-activated receptor (PPAR), and glucocorticoid receptor (GR), respectively [14–16]. In this study, we chose GR as recognition element for multi-residue determination of BPs. GR belongs to the family of nuclear receptors and shares a high degree of sequence and structural similarity with other members of the family [17]. GR also plays significant roles in metabolic, endocrine, immune, and nervous systems [18]. Recently, several studies demonstrated that GR was a potential target for endocrine disruptor chemicals [19]. BPA, like GR ligands such as dexamethasone, can bind to GR as an agonist and induce biological effects similar to that of glucocorticoids [20]. Like BPA, other BPs could interact with GR and showed varying degrees of antagonistic or agonistic properties [21, 22].

Edible oils play a fundamental role in the human nutrition and are largely consumed worldwide. Therefore the quality monitoring of edible oils should be highlighted. Establishment and improvement of a method for the determination of BPs in oil matrix are important [23]. Nowadays, soybean oils are among the most popular and widely consumed edible oil in China. They are used as a relish or condiment. But soybean oils are also found to be contaminated by BPs, which may occur during manufacturing, storing, and packaging processes. The present work aims to set up a rapid method for the multi-residue determination of BPs in soybean oil using a fluorescence polarization (FP) competitive binding assay. For this purpose, full-length human GR protein was produced and the experimental parameters of FP assay were optimized. The IC_{50} value, the working range, the limit of detection (LOD), and the cross-reactivity (CR) were tested at best condition. The analytical performance with soybean oil was established by the spike and recovery studies. Finally, fortified soybean oil samples were validated by HPLC as the reference method.

2 Materials and methods

2.1 Materials

4,4′-Isopropylidenediphenol (BPA, >99%), 4,4′-(hexafluoroisopropylidene)diphenol (BPAF, >98%), 4,4′-(1-phenylethylidene)bisphenol (BPAP, >98%), 2,2-bis(4-hydroxyphenyl) butane (BPB, ≥98%), 2,2-bis(4-hydroxy-3-methylphenyl)propane (BPC, >98%), 1,1-bis(4-hydroxyphenyl)ethane (BPE, ≥98%), 4,4′-dihydroxydiphenylmethane (BPF, ≥99%), 4,4′-(1,3-phenylenediisopropylidene)bisphenol (BPM, >98%), 4,4′-(1,4-phenylenediisopropylidene)bisphenol (BPP, >98%), 4,4′-sulfonyldiphenol (BPS, 99%), and 4,4′-cyclohexylidenebisphenol (BPZ, ≥98%) were purchased from Aladdin (Shanghai, China) and Sigma–Aldrich (St. Louis, MO, USA). The structures of the BPs above are shown in Fig. 1. Dexamethasone fluorescein (Dex-fl) was purchased from Invitrogen Molecular Probes (Eugene, OR, USA). All other reagents used were of analytical grade.

2.2 Expression, purification, and identification of GR

Full-length human GR was expressed as contained six histidines at the N-terminus (6 × His tag) fusion protein from pET28a in *Escherichia coli* strain Rosetta (DE3). The whole expression and purification procedures were performed as described [17]. The full-length human GR was cleaved from the 6 × His tag by Thrombin protease. The final product was transferred into storage buffer containing 10 mM Hepes pH 7.4, 150 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.005% Tween-20.

2.3 FP assay procedure

In the procedure of FP assay, 100 μL of Dex-fl (5 nM) was preincubated with increasing concentration of BPs, followed by the additions of GR (10 nM) in a total volume of 200 μL. The FP signal was read after incubation at room temperature for 10, 20, 30, 40, 50, and 60 min. All the FP measurements were performed in black, nonbinding 96-well plates (Greiner bio one) and conducted through a microplate reader (TECAN, infinite F500, Austria) with excitation at 484 nm and emission at 520 nm, respectively. The FP value versus concentrations of BPs was plotted to obtain the kinetic curves, and the time corresponding to the stable FP response was chosen to be appropriate for the experiments follow-up experiments. Data analysis was performed using GraphPad Prism 5 (GraphPad Software, USA).

2.4 Determination of analytical parameters

The IC_{50} value, the working range, and the LOD were served as criteria for evaluating the FP assay. These characteristics represent the analyze concentrations that provide tracer-binding inhibitions in the FP assay of 50%, 20–80%, and 90%, respectively. The broad-specificity of the FP assay was determined under optimized FP assay conditions and evaluated as the CR. It is calculated according to the following equation:

$$CR\% = [IC_{50}(\text{Bisphenol A})/IC_{50}(\text{structurally related compounds})] \times 100\%$$

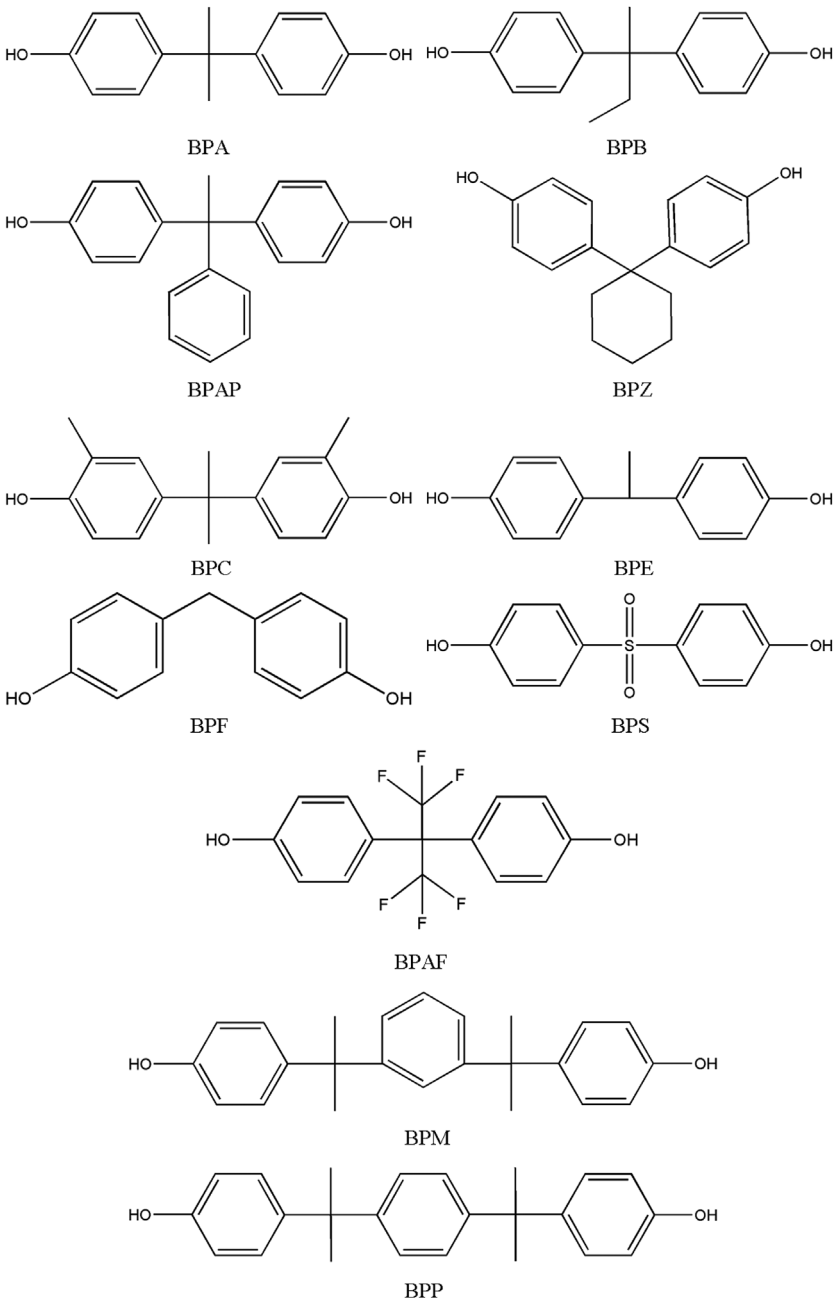

Figure 1. Chemical structures of BPA and its analogs.

2.5 Sample pretreatment

For matrix effect studies, 20 g soybean oil sample was added into a glass test tube and fortified with the appropriate BPA standard solution. The mixture of fortified sample and petroleum ether (80 mL) were transferred into a 250 mL separating funnel. After mixing with 110 mL methanol and water (50:50, v/v), the samples were allowed to stand at room temperature for 30 min. The methanol/water extracting solution was concentrated and diluted to 10 mL with buffer.

2.6 Analysis of spiked samples

Soybean oil samples, which had not been exposed to polycarbonate plastics and epoxy resins, were applied to perform a recovery test. Samples were fortified with BPA at their corresponding 1/2, 1, and 2 specific migration limits (SMLs). Then the selective extractions were analyzed by the FP assay under the optimized experimental condition. Each sample was evaluated with three replicates to verify repeatability.

2.7 Evaluation of authentic sample by FP assay and HPLC

Comparative studies of soybean oil samples contaminated with BPA were performed by the FP assay and the reference methods of HPLC. Correlation studies between methods were carried out on the same spiked solutions. HPLC was carried out as follows: chromatography was performed on LC-20A HPLC instrument with a fluorometric detector. The mobile phase is methanol and water (70:30, v/v). The excitation wavelength and emission wavelength were 227 and 313 nm, respectively. A C18 column (250 × 4.6 mm, 5 μm particle size) was used as the stationary phase. The flow rate was 1 mL/min, and the column temperature was room temperature. The measured results were compared with the FP assay results.

3 Results and discussion

3.1 Principle of FP assay

FP assay is a competitive homogeneous assay in solution phase, which is receiving attention as a screening tool in environmental monitoring and food-safety. When exciting the sample with a polarized light, a fluorescent ligand freely diffusing in the medium with an important molecular mobility will emit a non-polarized fluorescence, whereas upon binding a receptor, the molecular mobility of the fluorophore within the receptor–fluorescent ligand complex will frequently decrease, and a polarized fluorescence emission will be observed [24]. In such a method, Dex-fl and BPs compete for binding to the GR. When there are no competing compounds present, the Dex-fl will be bound by the GR, resulting in a high FP value. However, in the presence of BPs, the Dex-fl is displaced from the GR, resulting in a decreased FP value. The basic principle of FP assay is illustrated in Fig. 2.

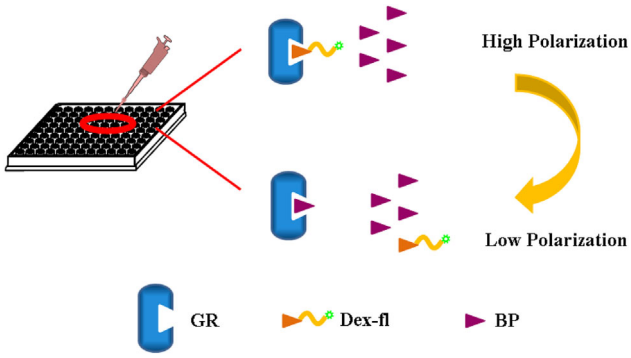

Figure 2. The basic principle of FP assay.

3.2 Optimization of the experimental conditions

In aiming for a satisfactory sensitivity, the concentration of tracer and receptor are primarily optimized. The lowest possible tracer concentration, which allows the reliable detection of label and effectively minimizes the interference to competition, is desirable for the highest sensitivity. In general, the optimal tracer concentration was determined by its fluorescence intensity at least ten times higher than the background signal [25]. The working concentrations of the corresponding GR were optimized at a fixed concentration of tracer according to a series of GR concentration gradient. In order to obtain a wider analytical range, the amount of the receptor is greater than or equal to the K_d value so that the FP value before adding a competitor is at 50% or more of the maximal mP shift [26]. Therefore, 5 nM Dex-fl and 10 nM GR were selected as the optimal couple for the tracer and receptor protein on the basis of higher sensitivity. As reported previously, the time given to the competitive reaction might have a direct effect on the sensitivity of the assay. Thus we allowed sufficient incubation time to reach equilibrium before measuring the response. As shown in Fig. 3, the signals showed a significant difference at 0–30 min and then decreased slightly at 30–60 min, which indicates that 30 min is recordable for the FP assay.

3.3 Study of analytical parameters

As the assay principle described above, the affinities of the tracer to receptor were assessed by performing competitive assays under the optimized conditions. The compounds that have structures similar to that of BPA were selected for the study. The IC_{50} and LOD values of the assay for 11 BPs are summarized in Table 1. The IC_{50} values of the BPs ranged from 1.8 to 66.9 μM, and the lowest value was achieved by

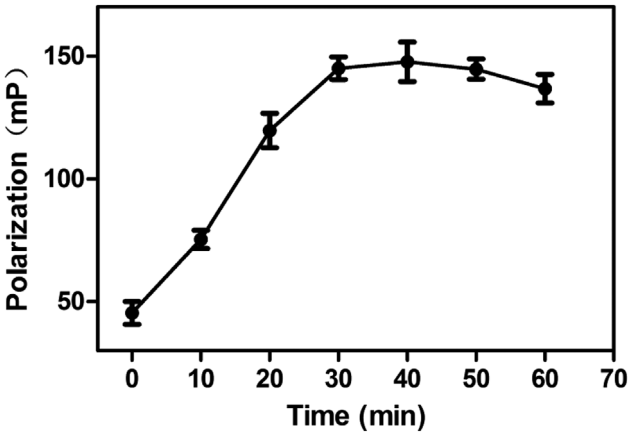

Figure 3. Kinetic curve of competitive binding assay.

Table 1. IC_{50} values, LODs, working ranges, and CRs for 11 BPs in FP assay

Compound	IC_{50} (μM)	LOD (μM)	Working range (μM)	CR (%)
BPA	18.8	1.0	7.6–30.0	100.0
BPAF	66.9	43.5	52.1–81.8	28.1
BPAP	7.1	4.8	5.7–8.6	264.3
BPB	14.8	6.2	9.4–20.3	127.0
BPC	6.9	3.7	4.9–8.9	272.0
BPE	31.7	15.0	21.2–42.2	59.5
BPF	61.8	10.5	29.4–94.2	30.5
BPM	2.8	0.1	1.1–4.6	669.8
BPP	1.8	0.2	0.8–2.8	1040.4
BPS	25.2	13.2	17.6–32.8	74.7
BPZ	2.9	0.1	1.7–4.2	640.1

BPP. The LOD of the BPs ranged from 0.1 to 43.5 μM. Among which, most of the LODs were below 15 μM, except BPAF. As shown in Table 1, the working ranges for FP assay of analytes were varying, indicating the binding abilities of BPs to GR are different.

It can be noted that the CR of the 11 compounds could be divided into two groups. BPE, BPS, and BPB formed the first group and the others were in second group. The first group had a similar CR to BPA, which was about 59.5, 74.7, and 127%. The three compounds each consist of two phenolic rings which are quite similar with that of BPA. Although the second group has a large rangeability in CR level, the CRs are all above 28%. The main reason about the difference between two groups is that the change of characteristic group could greatly influence the space conformation, and further causing the different CR levels. Conclusively, these results show that the method developed had an expected broad-specificity for BPs.

3.4 Matrix influence determination

Matrix effects are one of the most common challenges in performing FP assays on different samples. Therefore, sample extraction and a cleanup process are necessary for the FP assay [27]. In order to further reduce matrix effects brought by sample extracts, dilution has been adopted after selective extraction and considered to be a common and optimal way for sample preparation in FP assay. For the purpose of acquiring information on matrix effects of soybean oil samples, calibration curve generated by diluted sample extracts was compared with standard solutions of BPA in buffer. As shown in Fig. 4, these two calibration curves were superposed to a great extent, implying that the matrix interference could be basically eliminated by sample pretreatment procedures.

3.5 Test reproducibility of the FP assay

To further evaluate the utility of the FP assay for actual sample analysis, we determined the recovery values of the method for two representatives BPs in soybean oil sample. Among the BPs we used, BPA is typical due to its widespread use and representative toxic effects. The specific migration limit of BPA is set 600 μg/kg by the European Commission [28]. The accuracy and precision of the assay, represented by recovery and coefficient of variation, were assessed by BPA for which SML had been set in oil matrix. Three duplicates of spiked samples at a given concentration were measured using the developed FP assay. The recovery of BPA from fortified soybean oil ranged from 71.99 to 82.95%. In addition, the precision between analyses were below 10%, ranging from 5.59 to 9.79% (Table 2). These

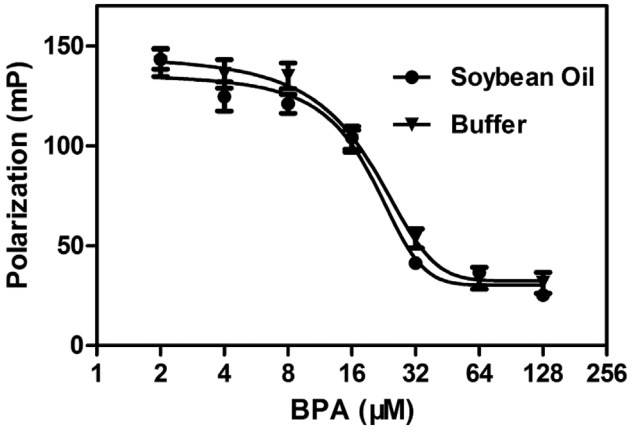

Figure 4. Competitive binding assay of BPA with GR in buffer and soybean oil.

Table 2. Recovery of BPA from soybean oil samples detected by FP assay ($n = 3$)

Spiked (μM)	Detected (μM)	Recovery (%)	CV (%)
1.21	0.86 ± 0.08	71.99	9.79
2.42	1.91 ± 0.18	78.83	9.37
4.84	4.01 ± 0.22	82.95	5.59

results indicated that the developed FP assay was reliable for the detecting analysis of the BPs in soybean oil.

3.6 Correlation of FP assay with HPLC

Comparative analyses of soybean oil samples contaminated with BPs were performed by both the developed FP assay and the reference methods of HPLC. As shown in Fig. 5, both methods were well correlated, further indicating the feasibility of FP assay in practical application. A good correlation coefficient ($R^2 = 0.9171$) was obtained. The result confirmed that the developed FP assay was reliable for detection of BPs in soybean oil.

4 Conclusions

A fluorescence polarization assay was developed for the monitoring of BPs in soybean oil. In the competitive binding system, Dex-fl and GR were selected as probe and recognition element, respectively. The experimental conditions were optimized and the analytical parameters were determined. Soybean oil spiked with BPA could be positively detected in combination with simple pretreatment. All the results suggest that the proposed method could be a potential tool for the high-throughput screening of BPs. In the future work, the receptor protein may be reconstructed to improve the sensitivity of the FP assay.

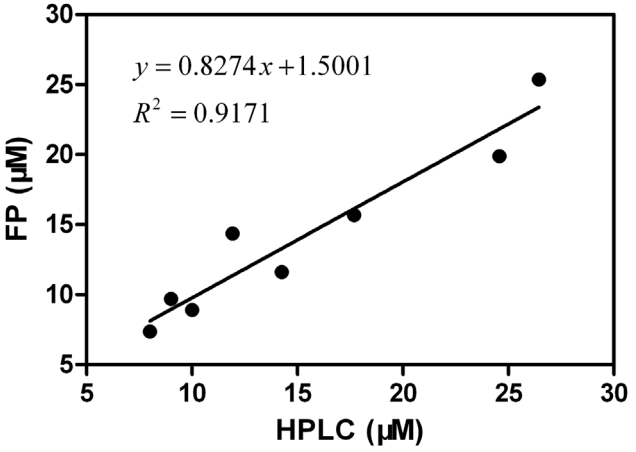

Figure 5. Correlation between FP assay and HPLC for spiked soybean oil samples.

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The authors have declared no conflicts of interest.

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