Talanta 121 (2014) 136–143

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Determination of sodium benzoate in food products by fluorescence polarization immunoassay

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ARTICLE INFO

Article history:
Received 26 September 2013
Received in revised form
12 December 2013
Accepted 17 December 2013
Available online 3 January 2014

Keywords:
Sodium benzoate
Fluorescence polarization immunoassay
Food additives
Polyclonal antibody

ABSTRACT

A rapid and sensitive fluorescence polarization immunoassay (FPIA), based on a polyclonal antibody, has been developed for the detection of sodium benzoate in spiked samples. The immunogen and fluorescein-labeled analyte conjugate were successfully synthesized, and the tracer was purified by TLC. Under the optimal assay conditions, the FPIA shows a detection range of 0.3–20.0 μg mL^{-1} for sodium benzoate with a detection limit of 0.26 μg mL^{-1} in the borate buffer. In addition, the IC_{50} value was 2.48 μg mL^{-1}, and the cross-reactivity of the antibodies with ten structurally and functionally related analogs were detected respectively. Four kinds of food samples (energy drink, candy, ice sucker, RIO™ cocktail) were selected to evaluate the application of FPIA in real systems. The recoveries were 96.68–106.55% in energy drink; 95.78–100.80% in candy, 86.97–102.70% in ice sucker, and 103.58–109.87% in benzoate contained sample RIO™ cocktail, and coefficients of variation of this method were all lower than 11.25%. Comparing with the detection results of HPLC, the developed FPIA has comparative performance in the real sample determination. The results suggest that the FPIA developed in this study is a rapid, convenient and simple method, which is suitable to be used as a screening tool for homogeneous detection of sodium benzoate in food products.

1. Introduction

Sodium benzoate, the sodium salt of benzoic acid, is generally used as a chemical preservative to prevent alteration or degradation caused by microorganisms during storage [1]. Both sodium benzoate and benzoic acid exhibited inhibitory activity against a wide range of fungi, yeasts, molds and bacteria [2,3]. Sodium benzoate is more widely used in a great variety of foods and beverages because of its good stability and excellent solubility in water. However, excessive intake of these preservatives might be potentially harmful to the consumers, because they have the tendency to induce allergic contact dermatitis, convulsion, hives and hepato cellular damage and others [4]. Moreover, with the growing use of the additives, the preservative residues can be considered as environmental contaminants [5]. For these reasons, benzoic acid and its salt are limitedly used as preservatives in some food products. In China, the maximum permitted utilization of benzoates in different types of food ranges from 0.2 to 2.0 g kg^{-1} (GB2760-2011). However, benzoates might be still excessively added in foods, because they are inexpensive and easily available. Therefore, developing a simple, rapid and economic analytical method for benzoates monitoring and controlling is important for food safety.

There are various techniques studied for benzoates determination. Traditionally, benzoates are analyzed mainly by thin layer chromatography (TLC) [6,7], gas chromatography (GC) [8,9], capillary electrophoresis (CE) [10–12], and micellar electro kinetic chromatography [13,14]. So far, the dominant way to detect benzoates is high-performance liquid chromatography (HPLC) [15–19]. Other methods are also reported for benzoates analysis, including second-order derivative spectrophotometry [20,21], chemometrics enhanced spectrophotometry [22,23], near-infrared reflectance spectroscopy [24], polarography [25] and enzymatic determination [26]. Nevertheless, these methods do not allow an easy analysis, because the instruments are expensive, the assays are relatively time-consuming and in some cases, require steps of extraction, laborious manipulation or sample pretreatment. It is necessary to develop a rapid, simple, economic and easy-to-use detection method as an alternative for benzoates determination.

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http://dx.doi.org/10.1016/j.talanta.2013.12.035

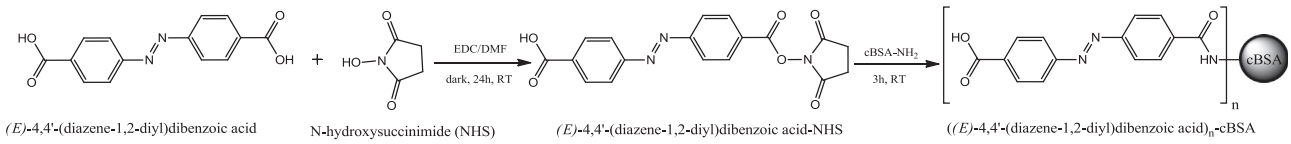


Fig. 1. Synthesis of immunogen ((*E*)-4,4′-(diazene-1,2-diyl)dibenzoic acid–cBSA).

Immunochemical methods, especially enzyme-linked immunosorbent assay (ELISA), provide simple, sensitive, specific and inexpensive tools for analysis of various targeted analytes [27]. However, ELISA is a heterogeneous method which involves repeated washing and a certain degree of reaction time (1–2 h). Fluorescence polarization immunoassay (FPIA) is a homogeneous technique (no separation or washing), which is an excellent screening tool in food and environmental analysis because of its rapidity, reliability and ease of use [28]. It bases on the different fluorescence polarization between antibody bound tracer (fluorescein-labeled analyte) and the nonbound form. If the sample contains free (unlabeled) analyte, it will compete with the tracer for antibody-binding sites, which will cause a decrease of the polarization signal. To our knowledge, development of FPIA for sodium benzoate or benzoic acid in real samples has not been reported yet. In this study, the polyclonal antibody against sodium benzoate was prepared by the well-designed immunogen. Based on that, an FPIA for sodium benzoate was developed and the accuracy, specificity and sensitivity of the method were studied.

2. Materials and methods

2.1. Reagents and apparatus

Sodium benzoate, benzoic acid, 4-aminobenzoic acid, sodium salicylate, potassium sorbate, phenol, 4-aminobenzenesulfonic acid, sodium 2-hydroxypropanoate, calcium propionate, phenylalanine, phenol, *N*,*N*-dimethylformamide (DMF), methanol, trichloromethane, ethylenediamine, and triethylamine of analytical grade were supplied by Guangfu Fine Chemical Research Institute (Guangfu Chemical Co., Tianjin, China). 2-Phenylacetic acid, bovine serum albumin (BSA), Freund′s complete adjuvant (cFA), and Freund′s incomplete adjuvant (iFA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). *N*-hydroxysuccinimide (NHS) was from Academy of Military Medical Sciences (Military Medical Institute, Beijing, China). (*E*)-4,4′-(diazene-1,2-diyl)dibenzoic acid and 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (EDC HCl) were obtained from TCI (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan). 3′,6′-Dihydroxy-5-isothiocyanato-3H-spiro[isobenzofuran-1,9′-xanthen]-3-one (5-FITC) of ultrapure grade was from Amyjet Scientific Inc. (AAT Bioquest Inc., Sunnyvale, USA). Energy drink, candy, ice sucker, RIO^{TM} cocktail, redbull, and sports drink were purchased from a local supermarket (Tianjin, China). Thin-layer chromatography (TLC) plates (Silica gel GF254, 5×10 cm^2) were from SILIDA Science and Technology Co., Ltd. (Tianjin, China).

The buffers used in this work include: (1) borate buffer (BB, pH 8.5) consisted of 2.5 mM sodium tetraborate and 0.1% NaN_3; (2) phosphate-buffered saline (PBS, pH 7.4) composed of 138 mM NaCl, 1.5 mM KH_2PO_4, 7 mM Na_2HPO_4 and 2.7 mM KCl.

FPIA analyses were performed by the Sentry® 100 portable system (Diachemix Corporation, Milwaukee, WI, USA) using 10×75 mm^2 glass culture tubes (VWR Internationals.r.l., Milan, Italy) and excitation and emission wave lengths (λ_{ex}, λ_{em}) of 485 and 535 nm, respectively. UV–vis spectra of conjugates were obtained by SHIMADZU UV-1800 (SHIMADZU, Kyoto, Japan). Determination of molecular weight was acquired by Liquid Chromatograph Mass Spectrometer (LC/MS, Agilent 6520 Q-TOF, USA). Ultimate 3000 liquid chromatography system (Dionex, Sunnyvale, CA, USA) was used in real samples determination for comparison, with an Inert Sustain C_{18} column (150×4.6 mm^2, i.d., 5 μm, Shimadzu-GL, Shanghai, China) and a mobile phase of methanol – 0.02 mol L^{-1} ammonium acetate (5:95) at 1 mL min^{-1}. The samples were detected at 230 nm with an injection volume of 20 μL.

2.2. Preparation of immunogen

Firstly, carboxylic acid groups of the carrier protein of BSA were converted into primary amine groups with an excess of ethylenediamine as described previously [29]. Briefly, 30.0 mL of PBS (0.1 M, pH 7.4) containing 15.0 mL of ethylenediamine was neutralized by 35.0 mL HCl (12 M). One gram of BSA and 0.639 g EDC was added subsequently. The mixture was incubated for 4 h at room temperature and dialyzed against PBS as well as distilled water respectively. The solution was lyophilized and stored at −20 °C.

In order to expose sodium benzoate for antibody recognition, (*E*)-4,4′-(diazene-1,2-diyl)dibenzoic acid was used as the hapten (Fig. 1). The immunogen ((*E*)-4,4′-(diazene-1,2-diyl)dibenzoic acid–cBSA) was prepared by carbodiimide-modified active ester method [29,30] as follows. A solution of 3.58 mg of (*E*)-4,4′-(diazene-1,2-diyl)dibenzoic acid (13.25 μmol), 2.54 mg of EDC (13.25 μmol), and 1.52 mg of NHS (13.25 μmol) in 3.0 mL of DMF was incubated for 24 h at room temperature in dark. Then the mixture solution was added slowly into 3.0 mL of PBS (0.1 M, pH 7.4) containing 30.0 mg of cBSA (0.44 μmol), followed by incubation at room temperature for 3 h. At last, the reaction mixture was dialyzed under stirring against PBS (0.1 M, pH 7.4) as well as distilled water for 3 days respectively to remove the uncoupled free hapten. The purified resultant was lyophilized and then stored at −20 °C.

2.3. Production of polyclonal antibodies

The polyclonal antibodies were obtained by immunizing two male New Zealand white rabbits (2.0 kg). They were subcutaneously immunized at multiple sites in the back with (*E*)-4,4′-(diazene-1,2-diyl)dibenzoic acid–cBSA conjugate. Before the immunization, 1.0 mL of blood was taken from the ear of each rabbit and used as the negative group. The initial immunization was subcutaneously injected with 0.5 mg of conjugate in 0.5 mL of NaCl (0.9%) emulsified with 0.5 mL of cFA. Subsequent booster injections (0.25 mg of conjugate in 0.5 mL of NaCl (0.9%) plus 0.5 mL of iFA) were performed 21 days later and then at 14-day intervals. At the fifth immunization, 0.25 mg of immunogen was dissolved in 1.0 mL of NaCl (0.9%) and injected. One week after the last immunization, the blood was collected and centrifuged. At last, the antiserum was obtained and stored at −20 °C until use.

2.4. Synthesis of the fluorescent conjugates

The fluorescent tracer should be prepared by conjugating sodium benzoate with fluorescein. However, sodium benzoate has no active groups to enable coupling reactions with commonly used fluorescein, FITC. Therefore, an analog of sodium benzoate,

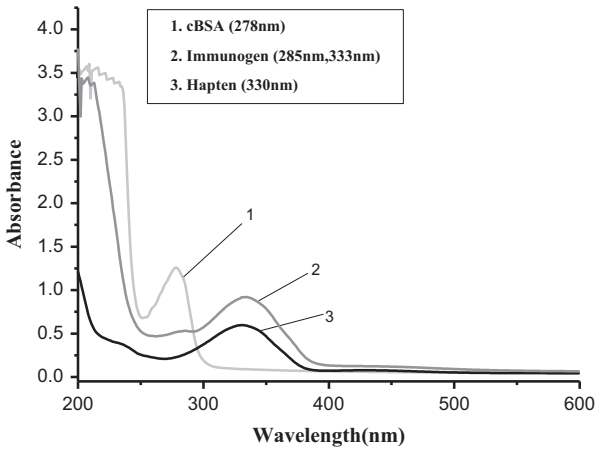


Fig. 2. UV–vis spectra of cationized bovine serum albumin (cBSA) (light gray; no. 1), immunogen, (*E*)-4,4′-(diazene-1,2-diyl)dibenzoic acid–cBSA (dark gray; no. 2) and hapten, (*E*)-4,4′-(diazene-1,2-diyl)dibenzoic acid (black, no. 3).

4-aminobenzoic acid, was chosen to synthesize the tracer (Fig. 2). One hundred microliter of methanol containing 10.0 μmol 4-aminobenzoic acid was added dropwise to 1.0 mL of methanol containing 3.9 mg FITC (10.0 μmol) and 50 μL of triethylamine. The reaction mixture was stirred overnight in dark at room temperature. Small portions of the reaction mixture (about 20–50 μL) were firstly separated by a preparative TLC using trichloromethane–methanol (4:1, v/v) as the eluent. The yellow bands at varying R_f were scraped off, eluted with 0.2 mL methanol, purified and stored at 4 °C in dark until use. The target product was identified by LC/MS.

2.5. Optimization of the assay conditions

(a) The tracers with various dilutions were prepared in the borate buffer (50 mM, pH 8.5) and the optimal dilution was selected by the fluorescent intensity signals. The fluorescence intensity of the targeted tracer dilution should be equal to about 5-fold the signal of borate buffer. (b) The optimal dilution of antibodies was chosen based on the titer determination. The antibodies were double diluted from 1/10 to 1/5120. The working solution of tracer (500 μL) and each antibody solution (500 μL) were mixed and fluorescent intensity signals were measured. The dilution of antibodies which corresponded to approximately 70% of the maximal signal was chosen as the optimum. (c) The tracer working solution, optimized antibody solution and sodium benzoate standard were mixed to give final concentration of sodium benzoate at 0, 0.01, 0.1, 1.0, 10, 100 μg mL^{-1} and the fluorescence polarization was measured at 2-min intervals. The equilibration time was optimized by the stable fluorescence signals.

2.6. Fluorescence polarization immunoassay (FPIA)

Different concentration of sodium benzoate standard was prepared in the borate buffer with a final concentration of 200,000, 20,000, 5000, 1000, 200, 80, 20, 8, 2, 0.4, 0.1 μg mL^{-1}. Ten microliter of the standard, 495 μL of the tracer and 495 μL of appropriately diluted antibody were mixed in the glass culture tube. The mixture was incubated, stirred and the fluorescence polarization was measured. Ten microliter of borate buffer (pH 8.5) was used in place of the standard as the blank control. The result is expressed as follows: %inhibition = %mP/mP_0, where mP is the value of the fluorescence polarization containing different concentrations of sodium benzoate as competitor, and mP_0 is the fluorescence polarization value of the control.

The IC_{50} value is calculated as a 50% reduction of the analytical signal in the competitive curve. Three concentrations of sodium benzoate solutions were selected to test the accuracy and precision. The experiments were repeated five times a day and continued for three days to acquire the intra-assay variation (%) and inter-assay variation (%).

2.7. Specificity

The specificity of the polyclonal antibody was investigated by cross-reactivity (CR) with ten functional and structural analogs related to benzoic acid as the competitors, including benzoic acid, 4-aminobenzoic acid, sodium salicylate, potassium sorbate, 2-phenylacetic acid, phenylalanine, phenol, 4-aminobenzenesulfonic acid, sodium 2-hydroxypropanoate, and calcium propionate. The cross reactivity studies were performed by FPIA by adding each free competitor at different concentrations to compete with tracer for the binding of the antibody. The cross-reactivity values were calculated according to the formula:

$$CR(\%) = IC_{50\ \text{benzoic acid}} / IC_{50\ \text{competitor}} \times 100\%$$

2.8. Application in food products

Sodium benzoate-free and benzoate-contained samples (scream lemon taste energy drink, Oishi lychee taste candy, pineapple and apple taste ice sucker, and RIO™ cocktail) were purchased from a local supermarket. For the preparation of samples for FPIA, energy drink was diluted 10-fold with the borate buffer (pH 8.5) in order to determine the sodium benzoate spiked. For candy and ice sucker, 1.0 g of sample was dissolved in 10 mL of the borate buffer (pH 8.5) and centrifuged to remove the undissolved substance. RIO™ cocktail is a blend of spirit and juice, peach plus brandy, which is a benzoate-contained sample available in the local market. It was boiled to remove the alcohol and then diluted. Before analysis, the samples were filtered using 0.22 μm microporous filtering film. The well treated samples were spiked with sodium benzoate at levels of 0.50, 5.00 and 10.00 μg mL^{-1} and the recoveries were determined by FPIA. Blank samples were prepared as described above without adding sodium benzoate. In addition, the content of benzoate in three real samples (redbull, orange honey sports drink and RIO™ cocktail) was detected by the developed FPIA. The results were compared to that of HPLC method.

3. Results and discussion

3.1. Synthesis of immunogen

Sodium benzoate is a small molecule, and it only has one carboxyl group in its benzene ring, which forms its specific structure. In order to keep its characteristic group for antibody recognition, the carboxyl group cannot be used as the connect point in the design of the immunogen synthesis. However, it is impossible to find else suitable moiety to link with carrier proteins. Based on the above consideration, an analog of sodium benzoate, (*E*)-4,4′-(diazene-1,2-diyl)dibenzoic acid, was chosen as the hapten for the immunogen synthesis. The selected hapten, (*E*)-4,4′-(diazene-1,2-diyl)dibenzoic acid has a cis-isomer, and the cis- and trans-isomer might switch at certain condition. More stable trans-isomer was used in this study, and the immunogen preparation was performed in dark and at room temperature to reduce this cis–trans-tautomerism as far as possible. One of the carboxylic acid groups of (*E*)-4,4′-(diazene-1,2-diyl)dibenzoic acid was selected as the site to link with cBSA in order to expose the feature of benzoic acid to improve the specificity of the antibody.

There are two carboxylic acid groups in the structure of (*E*)-4,4′-(diazene-1,2-diyl)dibenzoic acid and theoretically, they could equally react with the primary amino group of the cBSA. However, the raw ratio (hapten:cBSA=30:1 mol/mol) and steric hindrance limit the connection of cBSA to both carboxylic acid groups in each hapten. The performance of the antibody will also prove that the structure of the reaction product is the same as shown in Fig. 1. The effectiveness of the conjugation reaction was determined by UV–vis spectrometry. The UV–vis spectra of cBSA, (*E*)-4,4′-(diazene-1,2-diyl)dibenzoic acid, and (*E*)-4,4′-(diazene-1,2-diyl)dibenzoic acid–cBSA are shown in Fig. 2. The characteristic absorption peak of cBSA locates at 278 nm, and that of (*E*)-4,4′-(diazene-1,2-diyl)dibenzoic acid appears at 330 nm. The absorbance of (*E*)-4,4′-(diazene-1,2-diyl)dibenzoic acid–cBSA is observed at 285 and 333 nm, which gives a red-shifted peak compared with the 278 nm peak for cBSA and the 330 nm peak for (*E*)-4,4′-(diazene-1,2-diyl)dibenzoic acid. It probably indicates the successful conjugation of the immunogen.

3.2. Selection of the tracer and verification for the fluorescein-labeled conjugate

The primary amino group in 4-aminobenzoic acid is active, and it can directly react with fluorescein, FITC. There are two possible react products, which are shown in Fig. 3(a) and (b). After the reaction, the tracers were isolated from the product mixture by thin-layer chromatography. There are two bands (R_f=0.1, 0.3) resulted on the TLC plate. These two tracers were extracted with methanol (0.2 mL) and checked for antiserum binding. It shows that the extract obtained from R_f=0.1 exhibits well affinity to the anti-benzoate antibodies. For further validation, LC/MS was used for the molecular weight determination of the extract obtained. As shown in Fig. 4, the value of $(M+H)^+$ (m/z) is 527.0910, and $(M+Na)^+$ is 549.0718, which corresponds with the structure of Fig. 3(a). The above results indicate a successful conjugate of 4-aminobenzoic acid–FITC and the structure of the tracer (R_f=0.1) is verified as shown in Fig. 4.

3.3. Fluorescence polarization immunoassay (FPIA) procedure

The FPIA instrument used in this study Sentry® 100 portable system, gives two parameters simultaneously for one test, mP value and intensity. The mP value of borate buffer is around zero and the intensity of blank buffer is about 2000. When the tracer is diluted by 20,000 times, the intensity is around 5-fold the signal of the borate buffer, and it is used as the optimal dilution for the tracer. If the tracer has effective binding with antibody, the fluorescence polarization signals would decrease significantly with the dilution of antibodies, for example, tracer of R_f=0.1 and anti-benzoate antibody. However, the mP values have no remarkable change when the tracer cannot bind to the antibody (Fig. S1). Based on the results of ELISA, the titer of the antibodies is 1/12,80,000, before using, the antibodies must be diluted properly. To optimize the dilution of the antibody further, inhibition curves of sodium benzoate with three dilutions of antibody (1/50, 1/100, 1/200) were evaluated (Fig. S2). Compared with the antibody in 50 and 200 dilutions, the antibody which was diluted 100 times, exhibits a higher sensitivity and a wider linear range, and it is selected as the optimum. The equilibration time of the assay has no significant difference when various levels of competitors are added. The solution containing higher level of competitors equilibrates slightly faster, at the optimal assay conditions (1/20,000 tracer; 1/100 antibody). However, the fluorescence polarization signals of all solutions are stable in 5 min and equilibration time of the assay is chosen at 5 min for the further study (Fig. S3).

The FPIA is a homogeneous assay technique, and it involves the competition between free analyte and the tracer for binding to a specific antibody. Under the optimal assay conditions, a competitive inhibition curve was constructed (n=6), and the sensitivity of the FPIA was detected and expressed by IC_{50} value. As shown in Fig. 5, the antibody shows significant binding with sodium benzoate, and the concentration of sodium benzoate leading to 50% binding decrease (IC_{50}) is calculated to be 2.48 μg mL^{-1}. In addition, the limit of detection (LOD, IC_{20}) is 0.26 μg mL^{-1}. The detection standard curve for sodium benzoate is shown in Fig. 6, which exhibits good linearity (R^2=0.9972, n=4) with the concentration of

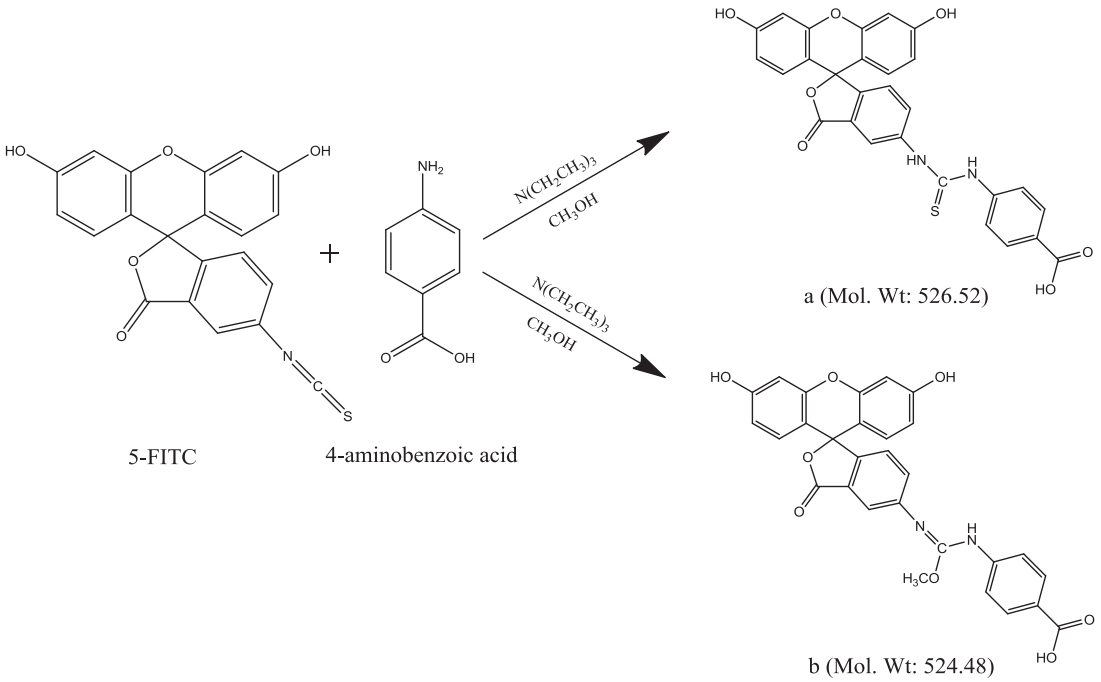


Fig. 3. Synthesis of the tracer, fluorescein-labeled 4-aminobenzoic acid conjugate. Two possible products (a) and (b) have different molecular weight (MWa=526.52 and MW_b=524.48).

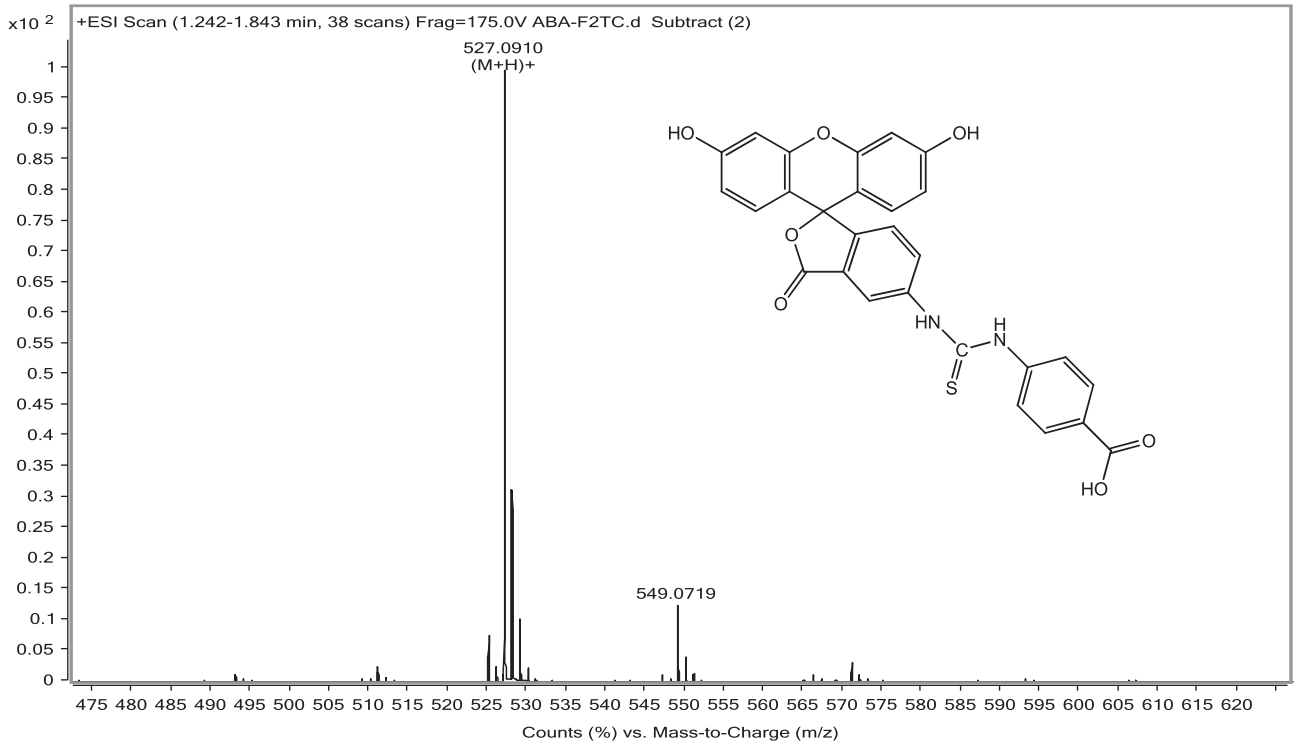


Fig. 4. LC/MS of the tracer, fluorescein-labeled 4-aminobenzoic acid conjugate (R_f=0.1).

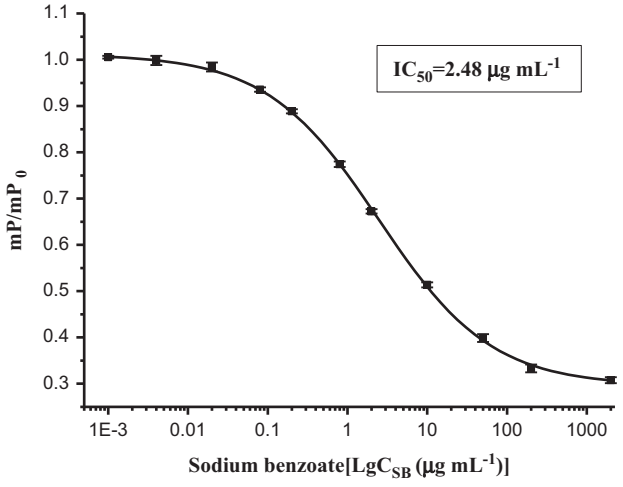


Fig. 5. Inhibition curve of the FPIA for sodium benzoate analysis (n=6).

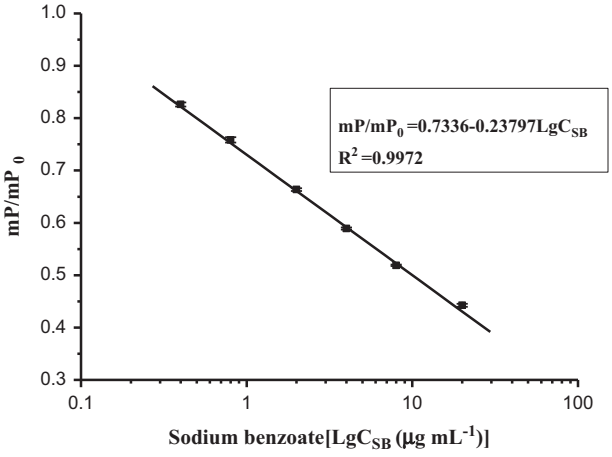


Fig. 6. The standard calibration curve of FPIA. Each data point represents the mean ± SD of 4 determinations.

sodium benzoate from 0.3 to 20 μg mL^{-1}. For precision study, three levels of sodium benzoate standard solutions (0.40, 2.00 and 10.00 μg mL^{-1}) were measured five times within one day for the calculation of intra-assay variations and three different days for the inter-assay variations. As shown in Table 1, the intra-assay coefficient of variation (CV) ranges from 0.17 to 5.76%, and the inter-assay CV is no more than 4.04%. The results certify that the developed FPIA is stable and credible for determining the concentration of sodium benzoate.

3.4. Specificity

Cross-reactivity was measured by comparing the IC_{50} of the analogs with that of benzoic acid. In this work, ten functionally

Table 1
Accuracy and precision of sodium benzoate detection in borate buffer system.

Added level (μg mL^{-1})	Day	Measured level (μg mL^{-1})	Recovery (%)	Intra-assay CV (%)	Inter-assay CV (%)
0.40	1	0.42 ± 0.01	103.98	1.89	3.28
	2	0.43 ± 0.02	106.27	5.76	
	3	0.42 ± 0.02	104.89	4.79	
2.00	1	1.98 ± 0.06	99.00	3.02	4.04
	2	1.83 ± 0.01	91.41	0.63	
	3	1.87 ± 0.01	93.74	0.65	
10.00	1	10.28 ± 0.02	102.75	0.17	3.03
	2	9.99 ± 0.15	99.91	1.54	
	3	10.20 ± 0.29	101.98	2.81	

Table 2
Cross-reactivity (CR) of FPIA for sodium benzoate and related compounds.

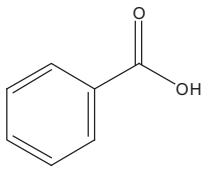
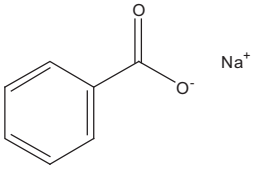
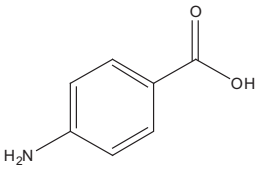
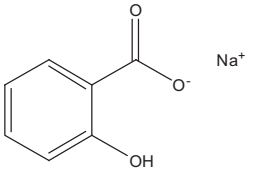
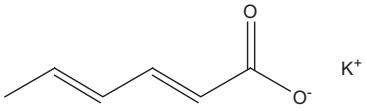
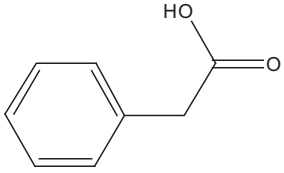
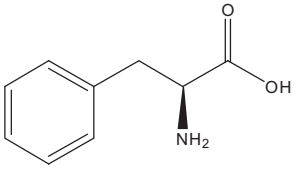
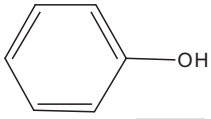
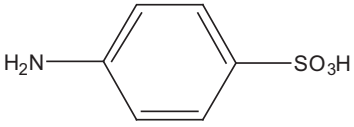
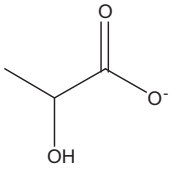
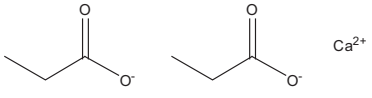
Analyte	Structure	IC_{50} (μg mL^{-1})	Cross-reactivity (%)
Benzoic acid		2.44	100
Sodium benzoate		2.48	98
4-Amino benzoic acid		3.85	63
Sodium salicylate		12.78	19
Potassium sorbate		18.27	13
2-Phenylacetic acid		> 50	< 5
Phenylalanine		> 50	< 5
Phenol		> 100	< 3
4-Aminobenzenesulfonic acid		> 100	< 3
Sodium 2-hydroxypropanoate		> 1000	< 1
Calcium propionate		> 1000	< 1

Table 3
Recovery and coefficient of variation (CV) for sodium benzoate determination in food products by FPIA.

Sample	Spiked ($\mu g\ mL^{-1}$)	Intra-assay			Inter-assay	
		Detected ($\mu g\ mL^{-1}$)	Recovery (%)	CV (%)	Detected ($\mu g\ mL^{-1}$)	CV (%)
Energy drink	0.50	0.49 ± 0.01	97.98	2.97	0.51 ± 0.04	7.29
	5.00	4.83 ± 0.04	96.68	0.82	4.63 ± 0.30	6.54
	10.00	10.65 ± 0.33	106.55	3.11	9.92 ± 0.57	5.76
Candy	0.50	0.48 ± 0.03	95.78	6.43	0.53 ± 0.06	11.25
	5.00	5.04 ± 0.06	100.80	1.21	5.08 ± 0.18	3.48
	10.00	10.06 ± 0.05	100.64	0.51	10.07 ± 0.11	1.08
Ice sucker	0.50	0.43 ± 0.01	86.97	1.22	0.41 ± 0.02	5.08
	5.00	4.98 ± 0.05	99.61	0.97	4.84 ± 0.16	3.24
	10.00	10.27 ± 0.19	102.70	1.82	10.23 ± 0.23	2.23
RIO^{TM} cocktail	0.50	0.52 ± 0.02	103.88	3.77	0.50 ± 0.02	3.62
	5.00	5.49 ± 0.07	109.87	1.30	5.85 ± 0.32	5.39
	10.00	10.36 ± 0.11	103.58	1.02	11.03 ± 0.58	5.27

and structurally similar analogs related to benzoic acid were tested with the FPIA to characterize the specificity of the antibody. As shown in Table 2, sodium benzoate and benzoic acid have comparable IC_{50} values. It is obvious because the analog of benzoic acid, (*E*)-4,4′-(diazene-1,2-diyl)dibenzoic acid, has been used as the hapten to link to carrier protein cBSA for immunogen preparation. The salt form of benzoic acid, sodium benzoate has no significant difference in structure and function with benzoic acid. However, sodium benzoate is well soluble in water and widely used in food product, which is studied as the analyte in this study. In addition, it is well-known that antibodies exhibit a preferential recognition to the part of the molecule that is the farthest from the conjugate site of the immunogen [31]. In this case, the carboxyl group in benzene ring is the mentioned farthest group which the anti-benzoate will recognize prior to other groups. Furthermore, the presence of a para-positioned group will not alter the antibody recognition or binding [32]. Thus, the antibody shows certain specificity to 4-amino benzoic acid (CR=63%). However, 4-amino benzoic acid, as a UV filter in sunscreen formulations commonly used after the world war II, is rarely used in cosmetics because of the dermatological side effects nowadays [33]. It can also be used as the intermediate of certain dyes and drugs, and it is not allowed to be added in foods. There is very little possibility of interfering in the analysis of sodium benzoate by FPIA. Additionally, FPIA, as a rapid, simple and homogeneous screening method, is especially suitable for field utilization, and the results could be confirmed by other methods in lab for the suspected samples. Based on the CR values of potassium sorbate (13%), sodium 2-hydroxypropanoate ($<1\%$) and calcium propionate ($<1\%$), it could be concluded that loss of the benzene ring results in a sharp decrease in antibody binding. In addition, if the ortho-position of the carboxyl group is substituted, the specificity of the antibody also decrease significantly (for sodium salicylate, CR=19%). The length and the types of the substituents also have considerable influence on the antibody specificity, for example, the CR values of 2-phenylacetic acid, phenylalanine, phenol, 4-aminobenzenesulfonic acid are extremely low ($<5\%$).

3.5. FPIA performance in food products

Sodium benzoate-free and benzoate-contained samples (scream lemon taste energy drink, Oishi lychee taste candy, pineapple and apple taste ice sucker, and RIO^{TM} cocktail) purchased from local supermarkets were used as the matrices. It is well known that various substances existing in complex food systems will affect antigen–antibody interaction in immunoassays. In order to reduce matrix effect, the simplest method, dilution was performed for sample treating. The matrices were spiked with sodium benzoate at the final levels of 0.50, 5.00, and 10.00 $\mu g\ mL^{-1}$ and then measured five times for three days for calculation of intra-assay and inter-assay variations. As shown in Table 3, analytical recoveries of sodium benzoate are 96.68–106.55% in energy drink; 95.78–100.80% in candy, 86.97–102.70% in ice sucker, and 103.58–109.87% in benzoate contained sample RIO^{TM} cocktail, while CV values range from 0.51 to 11.25% in these foods. It indicates that the developed FPIA method for sodium benzoate is stable and satisfactory in reproducibility. In addition, the detection results of benzoate-contained samples (redbull energy drink, orange and honey sports drink and RIO^{TM} cocktail) by the developed FPIA are listed in Table 4. The results are also compared to that of HPLC (Table 4). The results of these two methods do not exhibit significant difference. The FPIA for benzoate studied in this work provides an alternative for benzoate analysis in real food samples.

Table 4
Determination of sodium benzoate in authentic samples.

Samples	Detected by FPIA ($mg\ kg^{-1}$)	Detected by HPLC ($mg\ kg^{-1}$)
Redbull (energy drink)	165.02 ± 2.22	167.87 ± 2.81
Orange and honey sports drink (carbonated beverage)	155.90 ± 8.58	151.73 ± 4.61
RIO^{TM} cocktail	100.31 ± 5.27	100.03 ± 1.34

4. Conclusion

In this study, an FPIA based on the polyclonal antibody for the detection of sodium benzoate in spiked samples was developed and optimized. The immunogen and fluorescein-labeled conjugate were successfully synthesized. The antibodies obtained in this method, have good sensitivity with an IC_{50} value of 2.48 $\mu g\ mL^{-1}$ and specificity to various structural analogs except 4-amino benzoic acid which will have scarcely any interference in food preservative monitoring. Finally, the performance of the developed FPIA method in food product was investigated, and the recovery rate in food samples ranges from 86.97% to 109.87%. Additionally, the detection results of FPIA in real samples are comparative to that of HPLC. Overall, the FPIA provides a method, which is suitable for simply and rapidly screening a large number of samples for field test of sodium benzoate abuse.

Acknowledgements

This work was supported by the financial support of National Natural Science Foundation (No. 81173017, No. 31101277), Tianjin Science and Technology Program (No. 11ZCGHHZ01200, No. 12ZXCXSY08400, No. 12JCQNJC08900), the Tianjin Research Program of Application Foundation (13JCZDJC29700) and the Fundamental Research Funds for the Central Universities (No. 65011751).

Appendix. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.12.035.

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