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# Simultaneous Detection of Eight Food Allergens Using Optical Thin-Film Biosensor Chips

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**ABSTRACT:** Food allergies are important food safety issues nowadays. To maintain the safety of people who experience allergic reactions, labeling is required in many countries and efficient and reliable detection methods are necessary. This paper reports a novel method for the rapid identification of food allergens through the use of a silicon-based optical thin-film biosensor chip with which color change results can be perceived by the naked eye without any extra equipment. The whole system can detect eight food allergens including soybean, wheat, peanut, cashew, shrimp, fish, beef, and chicken simultaneously. Sensitive and specific detection of the absolute detection limit of this method was 0.5 pg of cashew DNA, and the practical detection limit of 0.001%. The biosensor chip detection time was about 30 min after PCR amplification. The assay is proposed as a sensitive, specific, high-throughput, and ready-to-use analytical tool to detect the presence or confirm the absence of eight food allergens.

**KEYWORDS:** food allergen, optical thin-film biosensor chip, PCR, detection

## INTRODUCTION

Food allergy is considered to be an emergent public health problem, and it is estimated that prevalences range from 1 to 3% in adults and from 4 to 6% in children due to the consumption of hidden allergens in processed food.[1] Food allergic reactions involve numerous symptoms ranging from mild to life-threatening; anaphylactic shock and asthma are the most common causes of death in the occasional fatalities associated with true food allergies.[2,3] Common allergenic foods include cow's milk, crustaceans, eggs, fish, tree nuts, peanuts, soybean, and wheat.[4] To protect the health and safety of these allergic people, it is imperative to provide effective methods of analysis that are able to identify very low amounts of allergenic ingredients in processed food. To date, the enzyme-linked immunosorbent assay (ELISA) has been reported for species authentication in food allergen analysis.[4−11] However, the use of high-temperature and/or high-pressure industrial processing could result in the partial or complete denaturation of proteins in foods. Immunological methods based on direct detection of allergenic proteins have become complicated. Therefore, in recent years, significant attention has been turning toward DNA-based approaches, which have proven to be reliable, sensitive, and fast for many aspects of food authentication.[12,13] Among them, the polymerase chain reaction (PCR) has been proved to be an invaluable genetic technique used for tracing the species origin in food. Real-time PCR is the most commonly used technology for quantification of specific DNA fragments. The amount of product synthesized during the PCR is measured in real time by detection of fluorescent signals produced as a result of specific amplification. Numerous papers have been published on real-time PCR detection of different food allergens such as peanut, cereals with gluten, nuts, egg, soy, and milk.[14−17] Nevertheless, food products could contain many allergen combinations; if detected separately, the amount of isolated DNA may be insufficient to detect all possible allergens in a food sample. So far, very little work has been done to detect multiple allergens at the same time.

To provide rapid, accurate, efficient, and low-cost detection methods, we developed a highly efficient assay to simultaneously identify eight kinds of food allergens on the surface of optical thin-film biosensor chips on the basis of two tetraplex PCR systems that were developed and validated in this study. Compared with traditional biochips, aldehyde-labeled probes were arrayed and covalently linked to a hydrazine-derivatized biosensor chip surface, and biotinylated amplification products combining with the probes changed the interference pattern of light on the biosensor surface, producing a perceived color change. One advantage of this technology resides in that the optical thin-film biosensor chips are able to enzymatically transduce specific molecules into signals visualized by the unaided eye without any specific instruments, because chemicals deposited on the thin-film surface by enzymatic catalysis alter the interference pattern of light on the biosensor surface, producing a perceived color change on the surface (gold to blue/purple).[18−20] In general, this method reduces the cost of sophisticated equipment, shortens the experimental time, has better sensitivity and specificity, and remains very competitive with the other technologies.

## MATERIALS AND METHODS

**Food Allergen Materials.** All samples, soybean (*Glycine max*), cashew (*Anacardium occidentale*), peanut (*Arachis hypogaea*), wheat (*Triticum aestivum*), beef (*Bos taurus*), chicken (*Gallus gallus*), fish (*Plecoglossus altivelis*), and shrimp (*Trachypenaeus curvirostris*), were

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**Table 1. Sequences of PCR Primers and Capture Probes for Detecting Eight Food Allergens**

| species | target gene | sequence[a] | fragment size (bp) | accession no. | ref |
|---|---|---|---|---|---|
| soybean | lectin | F, 5′GCCCTCTACTCCACCCCCA3′ | 118 | K00821 | 21 |
| | | R, 5′biotin-GCCCATCTGCAAGCCTTTTT3′ | | | |
| | | P, 5′ALD-aaaaaaaaaaAGCTTCGCCGCTTCCTTCAACTTCAC | | | |
| peanut | Ara h 3 | F, 5′CGCAAAGTCAGCCTAGACAA3′ | 78 | AF093541 | 14 |
| | | R, 5′biotin-CTTGTCCTGCTCGTTCTCT3′ | | | |
| | | P, 5′ALD-aaaaaaaaaaGAAGAGCGTGAATTTAGCCCTCGAGGACAGCA | | | |
| wheat | gliadin | F, 5′TGGTCTCATCCCTCTGGTCAA3′ | 96 | AF234648 | 22 |
| | | R, 5′biotin-GCTGCTGAGGAATCTGTGCTA3′ | | | |
| | | P, 5′ALD-aaaaaaaaaaTGGCCACAAAGCGATTGCCAAGTGATGA | | | |
| cashew | Ana o3 | F, 5′TGCCAGGAGTTGCAGGAAGT3′ | 67 | AY081853 | this study |
| | | R, 5′biotin-GCTGCCTCACCATTTGCTCTA3′ | | | |
| | | P, 5′ALD-aaaaaaaaaaACAGAAGGTGCCGCTGCCAGAA | | | |
| chicken | mitochondrion DNA | F, 5′CCCTCCTCCTTTCATCCTCAT3′ | 62 | AP003322 | 23 |
| | | R, 5′biotin-GTCATAGCGGAACCGTGGATA3′ | | | |
| | | P, 5′ALD-aaaaaaaaaaCTATGAATCCGGGCCTC | | | |
| beef | mitochondrion DNA | F, 5′GCCATATACTCTCCTTGGTGACA3′ | 271 | AY526085 | 24 |
| | | R, 5′biotin-GTAGGCTTGGGAATAGTACGA3′ | | | |
| | | P, 5′ALD-aaaaaaaaaaCACAACTTTTATCACAATCCAGAACTGACACCAAC | | | |
| fish | 16S rRNA | F, 5′ATA ACAGCGCAATCCTCTCCC3′ | 86 | EU621440 | 25 |
| | | R, 5′biotin-GCTGCACCATTAGGATGTCCT3′ | | | |
| | | P, 5′ALD-aaaaaaaaaaTTTACGACCTCGATGTTGGA | | | |
| shrimp | 16S rRNA | F, 5′AAGTCTAGCCTGCCCACTG3′ | 109 | AY264916 | this study |
| | | R, 5′biotin-GTCCAACCATTCATACAAGCC3′ | | | |
| | | P, 5′ALD-aaaaaaaaaaGACCGTGCGAAGGTAGCATAATCATTAGTCT | | | |

[a] F, forward PCR primer; R, reverse PCR primer; P, probe; ALD, aldehyde modification.

obtained from local markets in Beijing, China, and stored at room temperature in the dark. To dehydrate samples, all nut and seed materials were baked at 60 °C for 48 h.

**DNA Extraction.** Extraction of DNA was performed using a CTAB method.[14] One hundred grams of sample was ground to 70 mesh using an IKAA11 basic (IKA, Germany). From the resulting homogenized sample, all DNA extractions were done in duplicate. Two portions of 100 mg each of the homogenized sample were used for DNA extraction. Clean instruments were used for each sample to prevent cross-contamination. One and a half milliliters of CTAB extraction buffer (CTAB 20 g/L, NaCl 1.4 mol/L, TRIS 0.1 mol/L, $Na_2EDTA$ 0.02 mol/L, pH 8.0) and 10 μL of Proteinase K solution (20 mg/mL) were added to the sample. After incubation at 60 °C overnight, samples were centrifuged at 13000*g* for 10 min, and the supernatant was transferred into a new 2 mL tube; 750 μL of chloroform was added and shaken vigorously. Following 5 min of centrifugation at 13000*g*, the upper phase was transferred into a new 2 mL tube, and the volume of the solution was determined. The amount of two volumes of the transferred solution of CTAB precipitation buffer (CTAB 5 g/L, NaCl 0.04 mol/L was added and incubated for 60 min at room temperature without agitation. The samples were centrifuged at 13000*g* for 15 min. After the supernatant had been discarded, the pellet was resuspended in 350 μL of NaCl solution (1.2 mol/L); 350 μL of chloroform was added, mixed well, and then centrifuged at 13000*g* for 10 min. The upper phase was transferred into a new 1.5 mL tube; 0.8 vol of isopropanol was added for nucleic acid precipitation. After incubation at room temperature for 20 min, the samples were centrifuged at 13000*g* for 10 min, and the supernatant was discarded. The pellet was washed with 500 μL of ethanol solution ($c$ = 70%) and resolved in 100 μL of TE buffer (TRIS 10 mmol/L, $Na_2EDTA$ 0.1 mmol/L, pH 8.0) for further use. The DNA was quantified with a DU 640 nucleic acid and protein analyzer (Beckman, Germany) and diluted to 100 ng/μL. DNA ladder markers (20 and 50 bp) (TaKaRa, Japan) were used for calibration.

**Primer and Probe Design and Synthesis.** Oligonucleotides were synthesized by Invitrogen (Shanghai, China). The primers and probes were taken from published single PCR systems or were established in this work (for details, see Table 1). To increase the specificity and sensitivity of the method, the sequences of primers and probes of Ana o3 gene from cashew and 16S rRNA gene from shrimp were designed according to the optimum principle of primer and probe design, using Oligo 6 Demo software. During the design, all primers and probes were successfully checked for relevant homologies by BLASTNr search within GenBank databases. The reverse primers for PCR were synthesized with biotin at their 5′ ends for subsequent detection. The probes have 10 deoxyadenosine residues that constitute a "spacer" with an aldehyde group modification at their 5′ ends for conjugating to amino groups on the chip surface, followed by about 30 nucleotides complementary to the corresponding target sequence.

**Simple PCR Amplification.** PCR reactions were carried out in 25 $\mu$L reaction mixtures containing 1× PCR buffer, 2 mmol/L $MgCl_2$, 0.1 mmol/L dNTPs, 0.2 $\mu$mol/L of each primer, and 1 U of HotMaster *Taq* DNA polymerase (Qiagen, Germany) on Mastercycler Gradient (Eppendorf, Germany) as follows: initial step of 15 min at 95 °C, 35 cycles of 30 s at 94 °C, 30 s at 58 °C, 20 s at 72 °C; and one step of 5 min at 72 °C. Amplification products were analyzed by electrophoresis on 3% (w/v) agarose gels and stained with ethidium bromide (EB). Gels were photographed with a Bio Imaging system (Gene Genius, Germany).

**Multiplex PCR Amplification.** To make the following biosensor detection more efficient, two tetraplex PCR systems were designed and named reaction A and reaction B, respectively. Reaction A simultaneously determines the contents of DNA from beef, shrimp, fish, and chicken, whereas reaction B is included to specifically amplify soybean, peanut, cashew, and wheat.

Reaction A was performed in a DNA thermal cycler in a 25 $\mu$L mixture containing 1 U of HotMaster *Taq* DNA polymerase, 0.1 mmol/L dNTPs, 5 $\mu$L of the template DNA, 80 nmol/L of each oligonucleotide primer of beef, 480 nmol/L of each oligonucleotide primer of shrimp, 80 nmol/L of each oligonucleotide primer of fish, and 64 nmol/L of each oligonucleotide primer of chicken in 1× PCR buffer with 2 mmol/L $MgCl_2$. The amplification profile included an initial denaturation step at 95 °C for 15 min and then 35 cycles with denaturation at 94 °C for 30 s, primer annealing at 58 °C for 30 s, and extension at 72 °C for 20 s. A final extension step at 72 °C for 5 min was performed. Amplification products were analyzed by electrophoresis on 4% (w/v) agarose gels, stained with EB, and observed using UV spectrometry.

PCR reaction parameters and results observation of reaction B were consistent with that of reaction A except the final concentration of each primer. In reaction B, the final primer concentrations of soybean and cashew were 80 nmol/L, whereas those for wheat and peanut were 160 nmol/L, respectively.

**Preparation of the Optical Thin-Film Biosensor Chips.** The biosensor chips were purchased from America Biostar and cut into individual element sizes of 7 mm × 7 mm by laser. The biosensors were prepared following the procedure described by Zhong et al.[19]

**Assay Protocol of Biosensor Chips.** The assay protocol of Bai et al.[18] was followed with some modifications. To improve the sensitivity of the chips, aldehyde-labeled probes from eight food allergens were spotted by an AD3200 (Biodot, America) using 50 nL per spot onto biosensor chips from 10 $\mu$mol/L stocks dissolved in 0.1 mol/L sodium phosphate buffer, pH 7.8. In addition, when the wash method of the chips was performed in two steps, 200 $\mu$L of $ddH_2O$ following 200 $\mu$L of 0.1× SSC for 3 times, the clarity of the chips was increased so that color reaction could be better observed with the naked eye. Digital images were taken with a Penguin pro 150ES (Pixera, America).

**Absolute Detection Limit and Practical Detection Limit.** To determine the sensitivity of the biosensor chips for detection food allergens, cashew with high fat and protein was chosen as a detected object. Cashew genomic DNA was quantified by UV spectrometry. For determining the absolute limit of detection (LOD), six levels of serial dilution samples containing 10, 1, 0.1, 0.01, 0.001, and 0.0001 ng of the cashew genomic DNA per microliter were prepared with $ddH_2O$. Five microliters of diluted sample was used for PCR, so that the amounts of cashew genomic DNA in the final 25 $\mu$L were 50, 5, 0.5, 0.05, 0.005, and 0.0005 ng, respectively. A series of mixtures with different percentages of cashew with maize powder, that is, 10, 1, 0.1, 0.01, and 0.001% (w/w), were used for determining the relative LOD and prepared as follows. The cashews were chopped in a blender and then defatted by washing several times with acetone; after drying at room temperature for 2 days, the defatted cashews were homogenized to a fine powder. A mixture with 10% (w/w) cashew content was made by mixing 1 g of cashew powder with 9 g of common maize powder; then, mixtures with 1% (w/w) cashew content were made by mixing 1 g of mixture with 10% (w/w) cashew content with 9 g of maize powder. In a similar manner, mixtures with 0.1, 0.01, and 0.001% (w/w) cashew content were prepared. DNA extraction was performed according to a CTAB protocol,[14] amplified by simple PCR, and detected by the thin-film biosensor chips assay as described above.

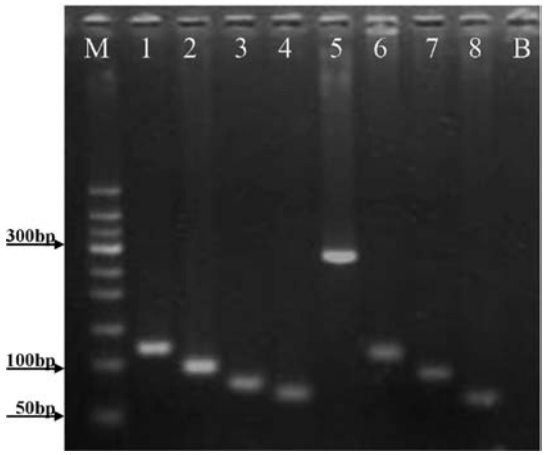


**Figure 1.** PCR amplification of target DNA fragments from eight food allergens. Lanes: M, 50 bp DNA Ladder marker; 1, soybean amplification with lectin gene (118 bp); 2, wheat amplification with gliadin gene (96 bp); 3, peanut amplification with Ara h 3 gene (78 bp); 4, cashew amplification with Ana o3 gene (67 bp); 5, beef amplification with mitochondrion DNA (271 bp); 6, shrimp amplification with 16S rRNA (109 bp); 7, fish amplification with 16S rRNA (86 bp); 8, chicken amplification with mitochondrion DNA (62 bp); B, blank control ($ddH_2O$).

**Analysis of Retail Samples.** Five food samples of different product groups were obtained from local markets in Beijing, China, including cereal bar (product components included nuts, gluten, and milk), chocolate chips (product components included gluten, soy, and milk), wheat biscuit (product components included gluten and milk), dark chocolate (product components included soy, egg, and milk), and fried mud carps with fermented soybean (product components included gluten, soy, and fish). DNA extraction of these five samples were performed according to the CTAB protocol,[14] amplified by two tetraplex PCR systems (reactions A and B) and analyzed by the biosensor chips assay as above-mentioned.

## RESULTS AND ANALYSIS

**Amplification of Eight Target Fragments by Simple PCR.** The PCR products for the eight species are shown in Figure 1. The sizes of PCR products are the same as predicted (Table 1). The results also showed that no EB staining was observed in the blank lane, indicating that no contamination occurred during PCR. From lanes 1−8, only one clear-cut band with intensive staining and molecular size as expected was found for each allergen, implying that the eight PCR systems were specific and reliable.

**Amplification of Eight Target Fragments by Two Tetraplex PCR.** On the basis of the single-factor and orthogonal experiments, different concentrations of primers from reactions A and B were optimized, and the best multiplex PCR results were obtained. The results showed that PCR amplification of the mixed genomic DNA from the multiple allergens with each set of oligonucleotide primers produced a simple DNA fragment of expected molecular weight (Figure 2). The results from reactions A and B yielded specific amplification of eight targets, beef,

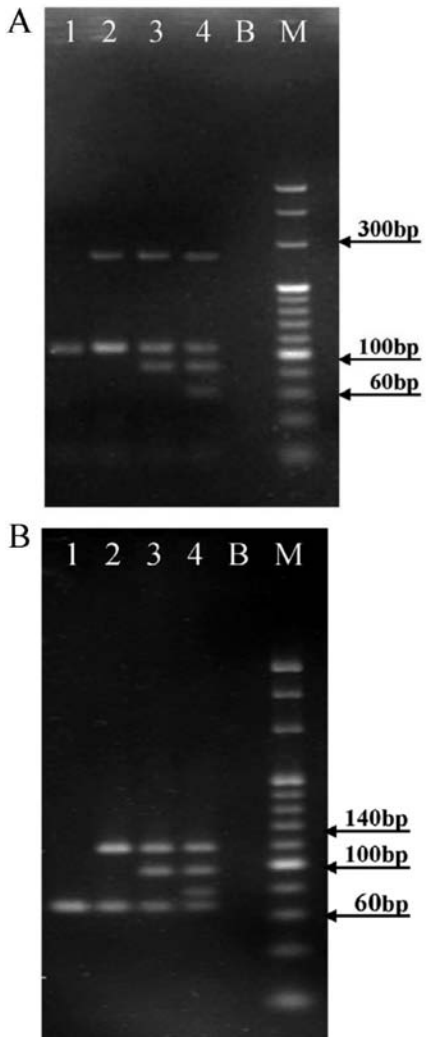


**Figure 2.** Agarose gel electrophoresis of tetraplex PCR system amplification of different samples. (A) Agarose gel electrophoresis of reaction A amplification products. Lanes: M, 20 bp DNA ladder marker; 1, shrimp (109 bp); 2, beef (271 bp) and shrimp (109 bp); 3, beef (271 bp), shrimp (109 bp), and fish (86 bp); 4, beef (271 bp), shrimp (109 bp), fish (86 bp), and chicken (62 bp); B, blank control ($ddH_2O$). (B) Agarose gel electrophoresis of reaction B amplification products. Lanes: M, 20 bp DNA ladder marker; 1, cashew (67 bp); 2, soybean (118 bp) and cashew (67 bp); 3, soybean (118 bp), wheat (96 bp), and cashew (67 bp); 4, soybean (118 bp), wheat (96 bp), peanut (78 bp), and cashew (67 bp); B, blank control ($ddH_2O$).

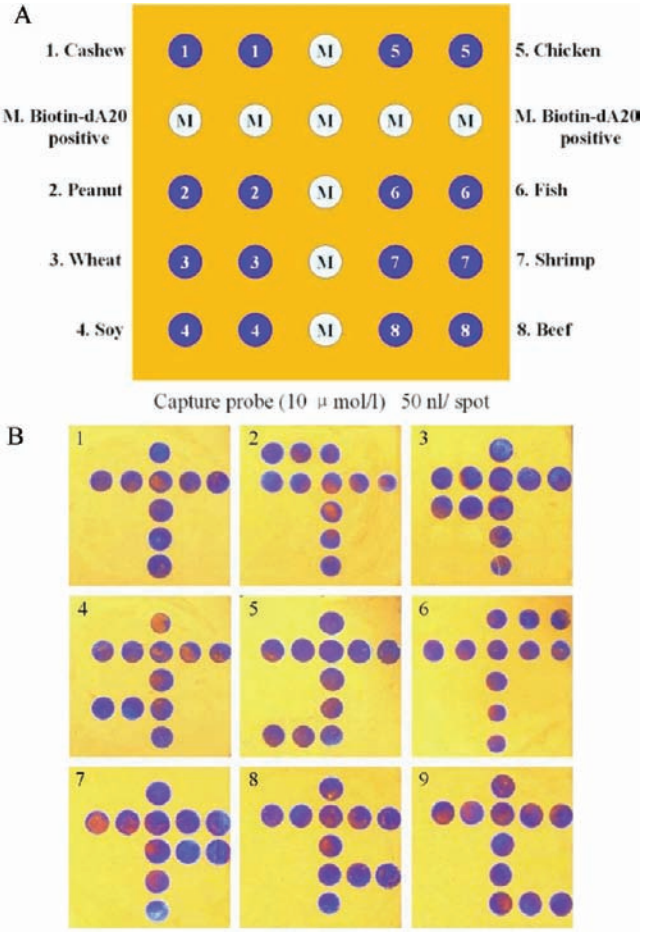


**Figure 3.** Food allergen detection on a chip with capture probes spotted by a computer-controlled dispenser. Each spot comprised 50 nL of 10 $\mu$mol/L probe solution and was printed in the order shown in (A). M, biotin−dA20 (positive control and marker); spot 1, Ana o3 gene (cashew); spot 2, Ara h 3 gene (peanut); spot 3, gliadin gene (wheat); spot 4, lectin gene (soybean); spot 5, mitochondrion DNA gene (chicken); spot 6, 16S rRNA (fish); spot 7, 16S rRNA (shrimp); spot 8, mitochondrion DNA (beef). (B) Detection of the food allergen-specific genes on thin-film biosensor chips: 1, blank control ($ddH_2O$); 2, cashew; 3, peanut; 4, wheat; 5, soybean; 6, chicken; 7, fish; 8, shrimp; 9, beef.

shrimp, fish, chicken, soybean, wheat, peanut, and cashew, with comparable band intensities using the PCR cycling parameters and an annealing temperature of 58 °C. The amplicon sizes of beef, shrimp, fish, and chicken were 271, 109, 86, and 62 bp, respectively, whereas those of soybean, wheat, peanut, and cashew were 118, 96, 78, and 67 bp, respectively. There were no cross-reactions within reactions A and B, which ensured that biosensor chip detection could be performed.

**Simultaneous Detection of Eight Allergens with Biosensor Chips.** The biosensor chips spotted by robotic pipetting (50 nL per spot) were used to detect eight food allergens (Figure 3A). M represented the positive control biotin−dA20 that always showed signals if the chip detection system worked. In this detection system, all other genes were negative controls for the detected gene. Results showed that specific signals were detected from eight food allergens (Figure 3B). For example, $ddH_2O$ instead of PCR-amplified products was used for hybridization in a 100 $\mu$L reaction, and the assay showed that only positive signals were observed. The Ana o3 gene targets amplified from cashew sample were used for hybridization to a biosensor chip, and the assay resulted in one set of colored dots. The other seven gene targets showed their own specific sets of colored dots (Figure 3B, 3−9). No false-positive signals were observed among these tests. Our results indicated that this chip method could be readily applied to analyze the presence of these eight food allergens and may be further modified to accommodate more needs for the detection of commercialized food allergens.

**Sensitivity and Limitations of Biosensor Chips.** Six concentrations of cashew genomic DNA in serially diluted samples were used to confirm the absolute LOD of the chips. The results showed that all six levels produced the hybridization signals except the blank control, and the change in hybridization signals

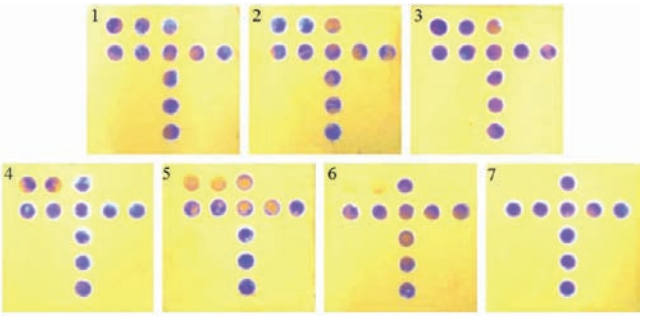


**Figure 4.** Absolute sensitivity of the biosensor chip method. The results indicated the change in hybridized signals of different cashew genomic DNA levels: 1, 50 ng; 2, 5 ng; 3, 0.5 ng; 4, 0.05 ng; 5, 0.005 ng; 6, 0.0005 ng; 7, blank control ($ddH_2O$).

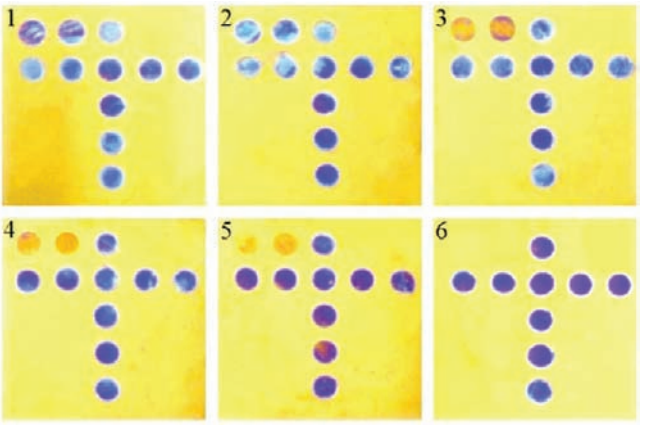


**Figure 5.** Practical detection limit of the biosensor chip method. The hybridized signals were obtained from different concentrations of cashew in maize powder: 1, 10%; 2, 1%; 3, 0.1%; 4, 0.01%; 5, 0.001%; 6, blank control ($ddH_2O$).

was consistent with the decrease in cashew genomic DNA concentration; when cashew genomic DNA content was reduced to 0.0005 ng, weak signals could also be observed (Figure 4), which means that the absolute LOD of the biosensor chips was 0.0005 ng. The practical LOD of the biosensor chips was determined by hybridizing different-percentage cashew mixtures including 10, 1, 0.1, 0.01, and 0.001% (w/w) cashew. As indicated in Figure 5, the hybridization signal could be observed from blended samples with a cashew concentration down to 0.001%, which means that the practical LOD of this method was 0.001%.

**Detection of Eight Allergens in Retail Samples by Biosensor Chips.** To verify the veracity and reliability of biosensor chips, five different retail samples were chosen as detected objects. Chips were spotted by robotic pipetting as indicated in Figure 3A. The DNA targets amplified by reactions A and B were hybridized to the chips to detect eight food allergens in retail samples. The results of examined foods showed that food allergens declared on the label could be detected by the chip method (Figure 6). No false positives were observed among these tests. This assay may be a valuable tool both for the enforcement of labeling requirements concerning potentially allergenic ingredients in food and for tracking and localization of sources of contaminations with eight food allergens in industrial production environments.

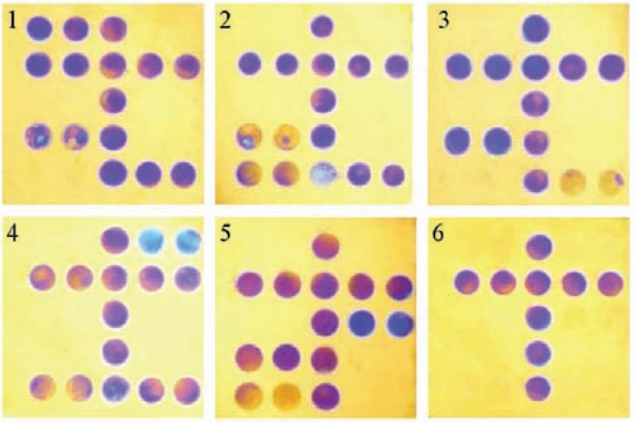


**Figure 6.** Results of commercial food products analyzed by the biosensor chip method. The results showed the detection of allergens present in commercial food products on thin-film biosensor chips: 1, cereal bar (cashew, wheat, and beef); 2, chocolate chips (wheat, soy, and beef); 3, wheat biscuit (wheat and beef); 4, dark chocolate (soy, chicken, and beef); 5, fried mud carps with fermented soybean (wheat, soy, and fish); 6, blank control ($ddH_2O$).

**Conclusion and Discussion.** Currently, 20 allergens are listed by European Union and Swiss legislation, comprising cereals with gluten, crustaceans, eggs, fish, milk, molluscs, soy, nuts, sesame, celery, mustard, lupine, and sulfites.[16] However, PCR together with ELISA cannot be applied to the analysis of all possible allergens in a food sample. Therefore, it is urgently necessary to adopt a high-throughput chip technique for simultaneous detection of many allergen combinations in foods. The assay we developed here, biosensor chips for the analysis of food allergens, has both high-throughput and sight advantages, which allow signals to be observed directly by the naked eye. At present, this assay is primarily used in the detection of foodborne pathogens and genetically modified organisms (GMO).[18,20,26] In this study, based on specific primers and probes from eight allergens, two tetraplex PCR systems were developed and validated, which greatly shortened the experimental time and laid the foundations for the optical thin-film biosensor chip technology applied in the detection of food allergens. To ensure the equivalent efficiency of hybridization signals in a uniform condition, hybridization conditions of probes from eight allergens were optimized. The change as described above increased detection sensitivity of cashew, which had been lower during simple PCR. The absolute detection limit of this method was 0.5 pg of cashew DNA, and the practical detection limit of 0.001% (w/w) was determined by a series of mixtures with different percentages of cashew. Because of differences in primers and probes, the biosensor chip had a much greater sensitivity than reported conclusions.[27,28] Practical applicability of the biosensor chip method was tested by the analysis of five different products. For all food samples, results obtained conformed to the labeling. Taken together, the biosensor chip method in this study has both zero-cross and high-throughput characteristics, which is suitable for the detection and surveillance of different ingredients in processed foodstuffs.

## AUTHOR INFORMATION

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## ■ ABBREVIATIONS USED

EB, ethidium bromide; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; IgG, antibiotin immunoglobulin G; ALD, aldehyde modification; LOD, limit of detection; CTAB, cetyl trimethyl ammonium bromide; TRIS, tris(hydroxymethyl)aminomethane; SSC, saline sodium citrate/standard saline citrate.

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