



Optical thin-film biochips for multiplex detection of eight allergens in food

Wei Wang^{a,b}, Yuanyuan Li^{a,b}, Fangyuan Zhao^{a,b}, Ying Chen^{a,*}, Yiqiang Ge^{b,c,**}

^a Institute of Food Safety, Chinese Academy of Inspection and Quarantine, No. 3 Gaobeidian North Road, Chaoyang District, Beijing 100123, China

^b College of Food Science & Nutritional Engineering, China Agricultural University, Beijing 100083, China

^c China Rural Technology Development Center, Beijing 100045, China

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ABSTRACT

An innovative method for the rapid detection of food allergens is developed and validated. Here we reported the development of silicon-based optical thin-film biochip technology that simultaneously permits visible detection of eight food allergens including celery, almond, oat, sesame, mustard, lupine, walnut and hazelnut on the basis of two tetraplex PCR systems. The biochip detection time was about 30 min after PCR amplification. Briefly, the optical thin-film biochip detects the presence of PCR fragment targets by enzymatically converting the formation of nucleic acid hybrids to molecular thin films. The mass contributed by the thin film alters the interference pattern of light on the biochip surface, resulting in a visible color change on the chip surface. Therefore, this assay permits sensitive, specific and high-throughput detection of allergens in food samples.

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1. Introduction

Allergens in food have become an emerging public health issue affecting around 4% of the general population and 8% of infants and children population respectively in recent years (Helm & Burks, 2000; Sicherer, Muñoz-Furlong, & Sampson, 2004). In order to protect the health and safety of these allergic people, ingredients that cause allergic reactions must be labeled on retail foods. Currently, the United States, the European Union, Canada, Australia, New Zealand, Japan, Korea and Hong Kong have promulgated legislations related to food allergen labeling for retail food samples. Thus, efficiency and appropriate methods are required for detecting traces of allergenic ingredients in processed food. Currently, immunoassays offer the necessary sensitivity and specificity for detecting traces of protein sources in foods (Chambers, Lodi, Nicoletti, & Brett, 2004; Monaghan et al., 2008; Roux, Teuber, Robotham, & Sathe, 2001; Scheibe, Weiss, Ruëff, Przybilla, & Görg, 2001; Wei, Sathe, Teuber, & Roux, 2003; Yamashita et al., 2001; Yeung & Collins, 1999). However, food processing methods like heat treatment or baking may cause modification, denaturation and degradation of food protein, so it is possible for some food that can lead to unsatisfied results with immunoassays. More recently, DNA-based assays as an indirect approach also has been used for allergen detection due to the higher stability than the proteins (Brzezinski, 2007; Ehlert, Hupfer, Demmel, Engel, & Busch, 2008; Hai et al., 2009; Köppel et al., 2001; Scaravelli, Brohée, Marchelli, & Hengel, 2008; Zeltner, Glomb, & Maede, 2009). Since

there are many different kinds of allergens in food products, it is necessary to establish the multiple detection methods. Among them, microarray method is one of the high-throughput methods for simultaneously detecting multiple proteins or DNA targets (Arenkov et al., 2000; Fukushima et al., 2003; Sergeev, Volokhov, Chizhikov, & Rasooly, 2004). Optical thin-film chip is one of microarray methods and has been used for detecting food-borne pathogens and genetically modified organisms (GMO) (Bai et al., 2010, 2007; Jenison, Rihaneck, & Polisky, 2001; Zhao, Bai, Huang, & Chen, 2008). Compared with traditional biochips, the procedure of optical thin-film biochip is easy and simple only heat denatures, and few minutes hybridization steps are needed. Moreover, the visualized signals are able to be caught by unaided eyes without any specific equipment because the biotinylated amplification products combining with aldehyde-labeled probes deposit on the thin-film surface and change the interference pattern of light on the biochip surface.

Here, we developed an optical thin-film microarray method on a silicon-based surface on the basis of two multiplex PCR systems for simultaneously detecting eight food allergens including celery, almond, oat, sesame, mustard, lupine, walnut and hazelnut. In general, this assay decreases the test cost, exhibits high sensitivity and specificity, and remains very competitive with the other methods.

2. Materials and methods

2.1. Materials

Nuts samples walnut (*Juglans regia*), almond (*Hodgsonia macrocarpa*), hazelnut (*Corylus* spp.), and seed samples sesame (*Sesamum indicum*), celery (*Apium graveolens*), oat (*Avena sativa*), mustard (*Sinapis alba*), lupine (*Lupinus polyphyllus*), and wheat (*Triticum aestivum*) were

* Corresponding author. Tel.: +86 10 85773355 2189; fax: +86 10 85783587.

** Correspondence to: China Rural Technology Development Center, Beijing 100045, China.

E-mail addresses: yqychen@yahoo.com.cn (Y. Chen), 68511009@163.com (Y. Ge).

obtained from local markets in Beijing, China. To dehydrate, all nut and seed materials were baked or roasted at 60 °C for 48 h in the lab.

2.2. DNA template preparation

In order to improve the yield of DNA extraction, five samples with high fat (walnut, almond, sesame and hazelnut) were pretreated with a blender and defatted three times with 4 volumes of ice-cold acetone and then air-dried at room temperature for two days before start extraction. DNA extraction was performed according to a CTAB protocol (Scaravelli et al., 2008). One hundred grams of sample was ground to 70 mesh using an IKA®A11 basic (IKA, Germany). From the resulting homogenized sample, all DNA extractions were done in duplicates. 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. Each sample was mixed with 1.5 ml of CTAB extraction buffer (2% CTAB, 1.4 mol/l NaCl, 0.1 mol/l Tris/HCl, 20 mmol/l Na₂EDTA, pH 8.0) and 10 µl of proteinase K solution (20 mg/ml) in a 2 ml tube and incubated at 60 °C overnight. The samples were centrifuged for 10 min at 13,000 g; the supernatant was transferred to a new 2 ml tube, mixed with 750 µl of chloroform, vortexed and centrifuged again for 10 min at 13,000 g. The upper phase was transferred into a new 2 ml tube, and the volume of the solution was determined. Two volumes of precipitation buffer (0.5% CTAB, 0.04 mol/l NaCl, pH 8.0) were added and incubated for 60 min at room temperature without agitation. The samples were centrifuged for 15 min at 13,000 g, the supernatant was discarded and the pellets were redissolved in 350 µl of NaCl solution (1.2 mol/l), vortexed and transferred to a new 2 ml tube and mixed with 350 µl of chloroform. After centrifugation for 10 min at 13,000 g, the upper phase was transferred into a new 1.5 ml tube; 0.8 volumes of isopropanol were added for nucleic acid precipitation. After incubation at room temperature for 20 min, the samples were centrifuged at 13,000 g for 10 min, and the supernatant was discarded. The pellet was washed with 500 µl of 70% ethanol and resolved in 100 µl of TE buffer (10 mmol/l Tris, 1 mmol/l Na₂EDTA, 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 marker (50 bp) (TaKaRa, Japan) was used for calibration.

2.3. Specific primers and probes design and synthesis

Oligonucleotides were purchased from Invitrogen (Shanghai, China). The primers and probes were established in this work or were taken from published single PCR systems (for details, see Table 1). To increase the specificity and sensitivity of the method, the sequences of primers and probes of Pru du1 gene from almond, Avenin gene from oat, Sin a I gene from mustard, ITS (18S–26S) gene from lupine and Oleosin gene from hazelnut 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 BLASTN 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.

2.4. Multiplex PCR amplification

In order to improve the detection efficiency of biochip, two tetra-plex PCR systems were designed and named reaction A and reaction B, respectively. Reaction A simultaneously determines the contents of DNA from celery, almond, oat and sesame, whereas reaction B is included to specifically amplify mustard, lupine, walnut and hazelnut. To obtain the optimized conditions of multiplex PCR, the reaction conditions of different annealing temperatures (50 °C, 52 °C, 54 °C, 56 °C, 58 °C, and 60 °C) and final primer concentrations (40 nmol/l, 80 nmol/l, 160 nmol/l, 320 nmol/l, 640 nmol/l, and 1280 nmol/l) were designed, and the optimized condition were applied in the following experiments.

Table 1
Sequences of PCR primers and capture probes for the detection of eight food allergens.

Species	Target gene	Sequence	Size (bp)	Accession number	Reference
Mustard	Sin a I	F ^a : 5'TGAGTTTGATTTTGAAGACGATATGG3' R ^b : 5'biotin-TGTTTAACGGCTTTGGATGCTC3' P ^c : 5'ALD ^d -aaaaaaaaCAGGGACCACAGCAGAGGCCACC3'	147	S54101	This study
Lupine	ITS (18S–26S)	F: 5'CCTCACAAGCAGTGCGA3' R: 5'biotin-TTGTTATTAGGCCAGGAGGA3' P: 5'ALD-aaaaaaaaCCTGAATTTGTTTACTACTCAGGGTGGCT3'	129	GU058035	This study
Walnut	Jug r 2	F: 5'CGCGCAGAGAAAGCAGAG3' R: 5'biotin-GACTCATGTCTCGACCTAATGCT3' P: 5'ALD-aaaaaaaaGGGAAGAGGAGCAACAGAGGCACAA3'	91	AF066055	Hai et al. (2009)
Hazelnut	Oleosin	F: 5'CCCCGTGTTGTGATAT3' R: 5'biotin-ATGATAATAAGCGATACTGTGAT3' P: 5'ALD-aaaaaaaaTCCCGTTCTCGTCCCTGCGGT3'	67	AY224599	This study
Celery	Mtd	F: 5'TTTGATCCACCGACTTACAGCC3' R: 5'biotin-ACAGATAACGCTGACTCATCAC3' P: 5'ALD-aaaaaaaaATTACATGCTGAGTCACGATGAGCGTGTACTG3'	151	AF067082	Mustorp, Engdahl-Axelsson, Svensson, and Holck (2008)
Almond	Pru du1	F: 5'TTTGGTTGAAGGAGATGCTC3' R: 5'biotin-TAGTTGCTGGTCTCTTTATG3' P: 5'ALD-aaaaaaaaGTTGGTGGCATCTGCTGATGGA3'	108	EU424251	This study
Oat	Avenin	F: 5'CGGCGATGTGCGATGTATACG3' R: 5'biotin-AGCCCTTGTAGTGTCTTAGAAGC3' P: 5'ALD-aaaaaaaaCCACCGCACTGCCCTGTGCGC3'	84	DQ370180	This study
Sesame	2S albumin mRNA	F: 5'CCAGAGGGCTAGGGACCTTC3' R: 5'biotin-CTCGAATTGGCAITGCTG3' P: 5'ALD-aaaaaaaaTCGAGGTGCAACATGCGACC3'	62	FJ222625	Ma, Cao, Gao, and Ba (2009)

^a Forward PCR primer.

^b Reverse PCR primer.

^c Probe.

^d ALD, aldehyde modification.

The optimal reaction A conditions in the experiments were performed in a DNA thermal cycler in a 25 μ l mixture containing 1 U of HotMaster Taq DNA polymerase (Qiagen, Germany), 0.1 mmol/l dNTPs, 5 μ l of the template DNA, 160 nmol/l of each oligonucleotide primer of celery, almond, oat, and sesame in 1 $\times$ PCR buffer with 2 mmol/l MgCl₂. 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 3% (w/v) agarose gels, stained with EB and observed using UV spectrometry.

PCR reaction parameters and result observation of the optimal reaction B were consistent with that of reaction A except the final concentration of each primer. In reaction B, the final primer concentrations of mustard and hazelnut were 320 nmol/l and 640 nmol/l, whereas those for walnut and lupine were 160 nmol/l, respectively.

2.5. Preparation of the optical thin-film biochips

The biochips were purchased from America Biostar and cut into individual element sizes of 7 mm $\times$ 7 mm by laser. The biochips were prepared according to the research of the pioneers (Bai et al., 2007; Zhong et al., 2003).

2.6. Standard assay protocol of biochips

In order to improve the sensitivity and the clarity of the chips, the method described by Bai et al. (2007) was followed with some modifications. Five different concentrations of aldehyde-labeled probes (1 μ mol/l, 5 μ mol/l, 10 μ mol/l, 20 μ mol/l, and 40 μ mol/l) were used for optimization in this study according to the color reaction of the chips. Each spot comprised 50 nl of 10 μ mol/l probe solution dissolved in 0.1 mol/l sodium phosphate buffer, pH 7.8. Capture probes were assembled on the chips in the order (Fig. 2A). M represented the positive control biotin-dA20 (5'-ALD-AAAAAAAAAAAAAAAAAAAAA-3'-Biotin) that always showed signals if the chip detection system worked, and determined the position of capture probes from eight food allergens on the surface of the chips. In addition, the wash method of the chips was changed to two steps, 200 μ l of ddH₂O following 200 μ l of 0.1 $\times$ 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).

2.7. Absolute detection limit and practical detection limit

Sesame extensively used in food industry and Chinese cuisine is becoming the ninth most common allergen after dairy, eggs, fish, shellfish, peanuts, tree nuts, wheat and soy, and riches in both protein and fat that could affect the DNA extraction and PCR amplification. In order to obtain the truly detection limit, sesame, one of the most difficult materials was chosen for determining the sensitivity of the biochips for detecting food allergens. Sesame 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 sesame genomic DNA per μ l were prepared with ddH₂O. Five microliters of diluted sample was used for PCR, so that the amount of sesame genomic DNA in the final 25 μ l was 50, 5, 0.5, 0.05, 0.005, and 0.0005 ng, respectively. A series of mixture with different percentages of sesame with common wheat powder, that is, 10, 1, 0.1, 0.01, and 0.001% (w/w), was used for determining the relative LOD and prepared as follows. A mixture with 10% (w/w) sesame content was made by mixing 1 g of sesame powder with 9 g of wheat powder, respectively; then, mixtures with 1% (w/w) sesame content were made by mixing 1 g of mixture with 10% (w/wt) sesame content with 9 g of wheat powder, respectively. In a similar manner, mixtures with 0.1, 0.01, and 0.001% (w/w) sesame

content were prepared. DNA extraction was performed according to the CTAB protocol, and detected by the thin-film biochips assay as described above.

2.8. Analysis of commercial samples

Five food samples of different product groups were obtained from local markets in Beijing, China, including sesame biscuits (product components included sesame, gluten, celery, and lupine), Crispy Oatmeal Cookies (product components included gluten and nuts), Chocolate Omelet (product components included gluten, celery, and nuts), Hazelnut Wafers (product components included sesame, celery, lupine, and nuts), and bread sticks (product components included sesame, celery, lupine, and nuts). DNA extraction of these five samples were performed according to the CTAB protocol (Scaravelli et al., 2008), amplified by two tetraplex PCR systems (reaction A and reaction B) and analyzed by the biochips assay as above-mentioned.

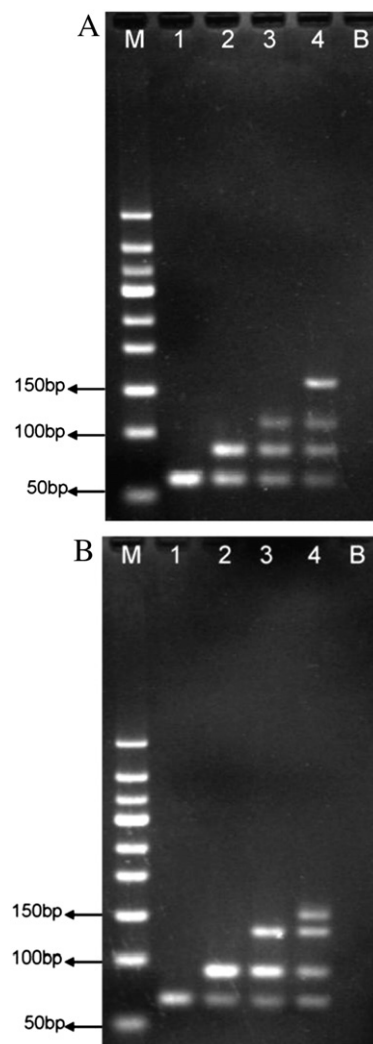


Fig. 1. Multiplex PCR for the simultaneous detection of different allergens. (A) Reaction A for the simultaneous detection of sesame (62 bp), oat (84 bp), almond (108 bp) and celery (151 bp). Lanes: M, 50 bp DNA Ladder marker; 1, sesame; 2, sesame and oat; 3, sesame, oat and almond; 4, sesame, oat, almond and celery; B, blank control (ddH₂O). (B) Reaction B for the simultaneous detection of hazelnut (67 bp), walnut (91 bp), lupine (129 bp) and mustard (147 bp). Lanes: M, 50 bp DNA Ladder marker; 1, hazelnut; 2, hazelnut and walnut; 3, hazelnut, walnut and lupine; 4, hazelnut, walnut, lupine and mustard; B, blank control (ddH₂O).

3. Results and discussion

3.1. Amplification of eight target fragments by two tetraplex PCR

On the basis of the single-factor and orthogonal experiments, different concentration of primers from reaction A and reaction B were optimized, and the best efficiency and specificity of multiplex PCR amplification system were obtained, and laid the foundations for the optical thin-film biochip technology. 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 (Fig. 1). The results from reaction A and reaction B yielded specific amplification of eight targets, celery, almond, oat, sesame, white mustard, lupine, walnut and hazelnut

with comparable band intensities using the PCR cycling parameters and an annealing temperature of 58 °C. The amplicon size of celery, almond, oat and sesame were 151, 108, 84, and 62 bp respectively, while that of mustard, lupine, walnut and hazelnut were 147, 129, 91 and 67 bp, respectively.

3.2. Simultaneous detection of eight allergens with biochips

The biochips spotted by robotic pipetting (50 nl per spot) were used to detect eight food allergens (Fig. 2A). The PCR-amplified products were hybridized to the chips. In this system, all other genes were negative controls for the detected gene. Results showed that specific signals were detected from eight food allergens (Fig. 2B). For example, ddH₂O instead of PCR-amplified products was used for hybridization in a

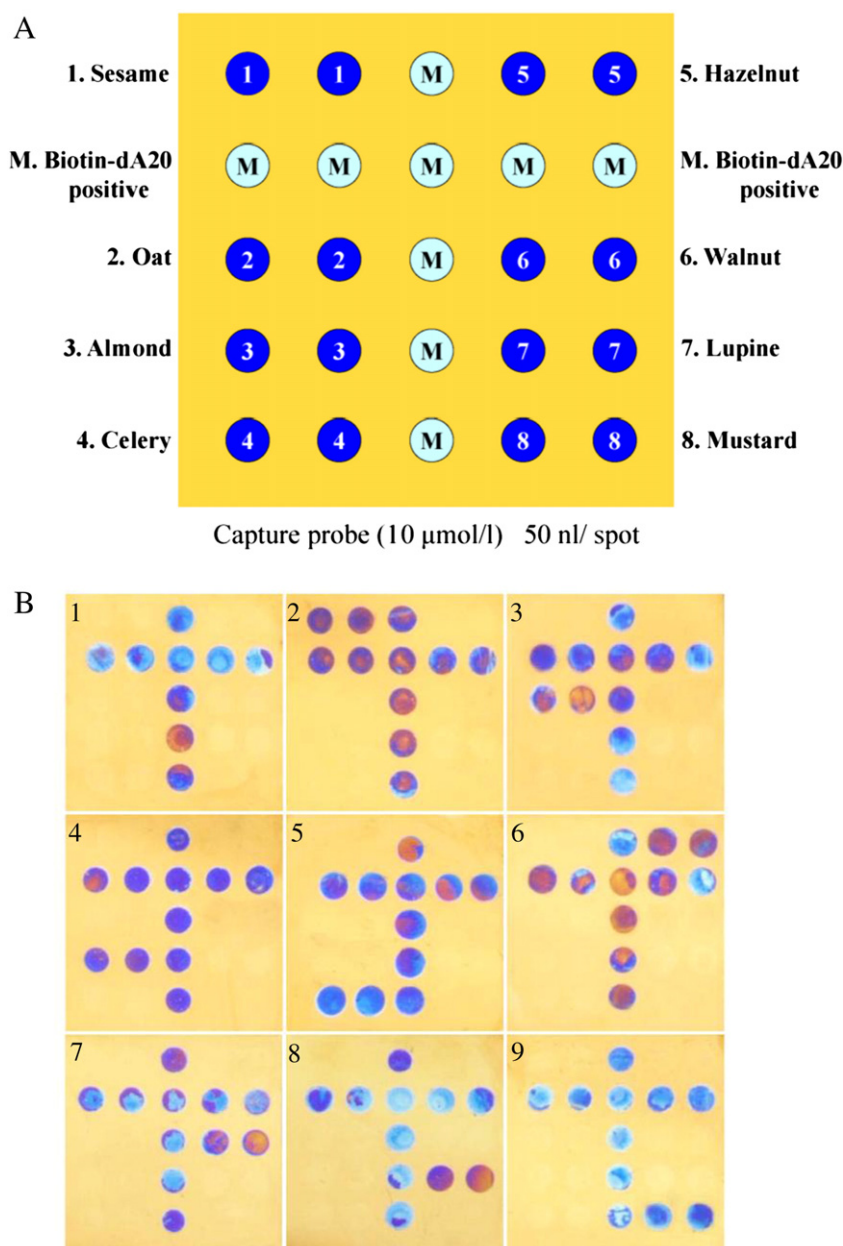


Fig. 2. Specificity of food allergen detection on thin-film biochips with capture probes spotted by a computer-controlled dispenser. Capture probes were arranged in the order shown in (A). M biotin-dA20 (positive control and marker); 1, 2S albumin mRNA gene (sesame); 2, Avenin gene (oat); 3, Pru du1 gene (almond); 4, Mtd gene (celery); 5, Oleosin gene (hazelnut); 6, Jug r 2 gene (walnut); 7, ITS (18S–26S) gene (lupine); 8, Sin a I gene (mustard). (B) Detection results of the food allergen-specific genes on thin-film biochips: 1, blank control (ddH₂O); 2, sesame; 3, oat; 4, almond; 5, celery; 6, hazelnut; 7, walnut; 8, lupine; and 9, mustard.

100 μ l reaction, and the assay showed that only positive signals were observed. The Avenin gene targets amplified from oat were used for hybridization to the biochip, and the assay resulted in one set of colored dots. The other seven gene targets also showed their own specific sets of colored dots. Results showed that specific signals were detected from eight food allergens, and no false-positive signals were observed among these tests (Fig. 2B, 2 and 4–9). Our study indicated that this chip could be proposed as a specific and ready-to-use analytical technique to detect the presence of these eight food allergens, and may be improved to meet the needs of analysis of commercialized food allergens.

3.3. Sensitivity and limitations of biochips

Six concentrations of sesame genomic DNA in serial diluted samples were applied to the confirmation of 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 was consistent with the decrease in sesame genomic DNA concentration, and when sesame genomic DNA content was reduced to 0.5 pg, weak signals could also be observed (Fig. 3A), which means that the absolute LOD of the biochips was 0.5 pg. The practical LOD of the biochips was determined by hybridizing different-percentage sesame mixtures including 10, 1, 0.1, 0.01, and 0.001% (w/w) sesame. As indicated in Fig. 3B, hybridization signals could be observed from blended samples with sesame concentration down to 0.001%, which means

that the practical LOD of this assay was 0.001%. Both of them had a much greater sensitivity than reported conclusions (Brzezinski, 2007; Schoringhumer, Redl, & Cichna-Markl, 2009).

3.4. Detection of eight allergens in commercial samples by biochips

In order to confirm the reliability and accuracy of biochips, five different commercial samples were chosen as detected objects. Chips were spotted by robotic pipetting as indicated in Fig. 2A. The DNA targets amplified by reaction A and reaction B were hybridized to the chips in order to analyze potentially allergenic ingredients in retail samples. The result indicated that positive controls all showed color reactions, which ensured that the biochip detection had proceeded normally and excluded the possibility of false negatives; food allergens in commercial samples declared on the label could be detected by the chips method (Fig. 4). This technique would be an effective monitoring and assessment tool to verify food allergen labeling and track sources of allergen contamination in food.

4. Conclusion

Food allergies have been becoming a concern for government, consumers and food industries. A great number of different assays and strategies are available for detecting allergenic ingredients and contaminations. However, in order to meet the need of the large-scale and simple operation of application, the development of thin-

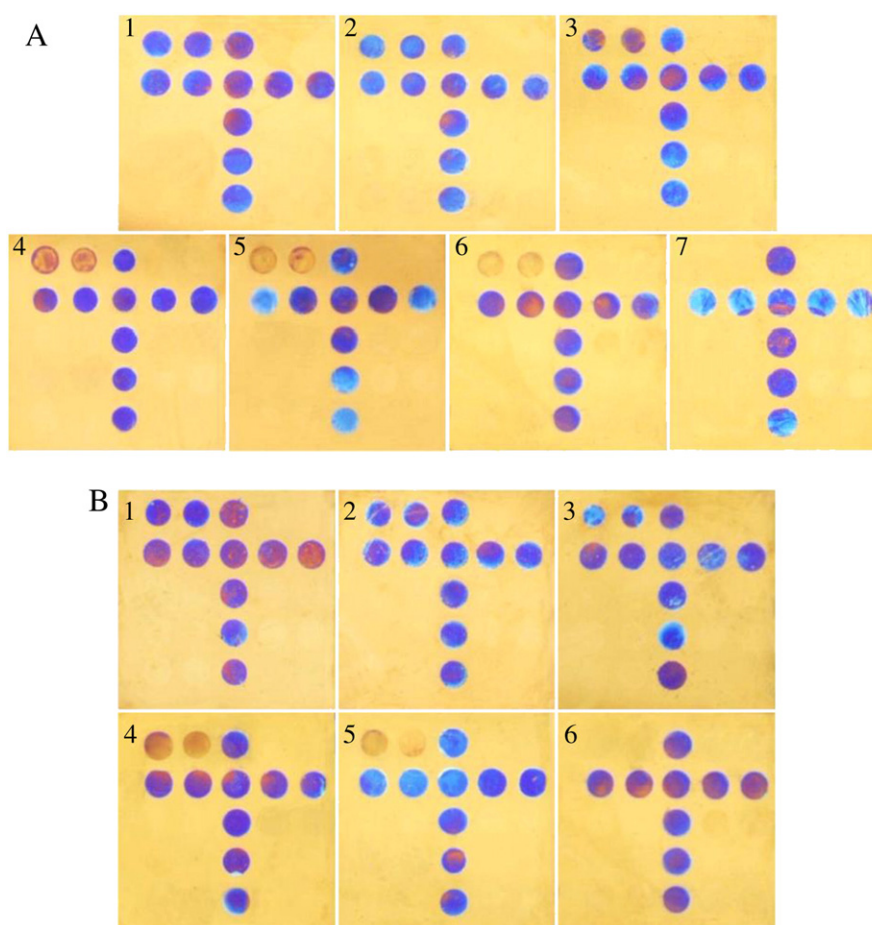


Fig. 3. Absolute sensitivity (A) and Practical detection limit (B) of thin-film biochips method for detecting sesame. The results of A indicated the change of hybridization signals with different sesame genomic DNA levels: 1, 50 ng; 2, 5 ng; 3, 0.5 ng; 4, 0.05 ng; 5, 0.005 ng; 6, 0.0005 ng; 7, blank control (ddH₂O), and the results of B showed the hybridized signals obtained from different concentrations of sesame in wheat powder: 1, 10%; 2, 1%; 3, 0.1%; 4, 0.01%; 5, 0.001%; and 6, blank control (ddH₂O).

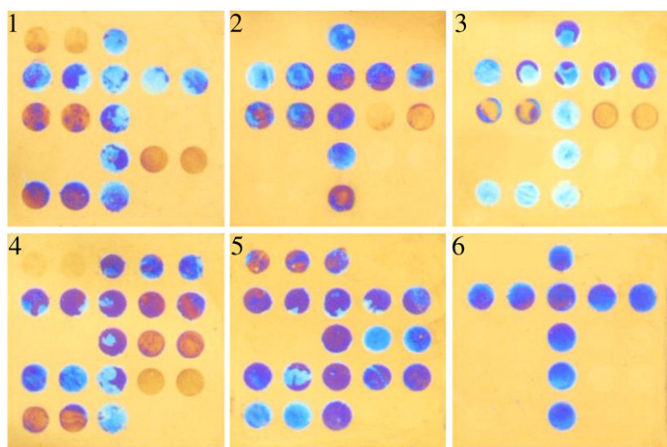


Fig. 4. Detection of allergen components in commercial food products with the biochips method. 1, Sesame biscuits (sesame, oat, celery and lupine showed positive signals); 2, Crispy Oatmeal Cookies (oat and walnut showed positive signals); 3, Chocolate Omelet (oat, walnut and celery showed positive signals); 4, Hazelnut Wafers (sesame, almond, celery, hazelnut, walnut and lupine showed positive signals); 5, Bread sticks (sesame, almond, celery, walnut and lupine showed positive signals); and 6, blank control (ddH_2O).

film biochip assays, based on two tetraplex PCR systems, has been presented. This method meets the needs of fast, high throughput, and available detection and little reagent-consuming. In addition, experimental results can be visible to the naked eye without any expensive and specific instruments. Therefore, this assay as described above for the detection of food allergens on biochips has laid the foundation for future development of chip detection technology. This chip may be used to help the manufacturer and the retailer in food allergen labeling matter.

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