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Multiplex DNA Detection of Food Allergens on a Digital Versatile Disk

Luis A. Tortajada-Genaro,[†] Sara Santiago-Felipe,[†] Sergi Morais,[†] José Antonio Gabaldón,[§] Rosa Puchades,[†] and Ángel Maquieira*[,†]

[†]Centro de Reconocimiento Molecular y Desarrollo Tecnológico (IDM), Departamento de Química, Universitat Politècnica de València, Camino de Vera s/n, E46022 Valencia, Spain

[§]Departamento de Tecnología de Alimentos, Universidad Católica San Antonio de Murcia, Murcia, Spain

ABSTRACT: The development of a DNA microarray method on a digital versatile disk (DVD) is described for the simultaneous detection of traces of hazelnut (*Corylus avellana* L.), peanut (*Arachis hypogaea*), and soybean (*Glycine max*) in foods. After DNA extraction, multiplex PCR was set up using 5′-labeled specific primers for Cor a 1, Ar h 2, and Le genes, respectively. Digoxin-labeled PCR products were detected by hybridization with 5′-biotinylated probes immobilized on a streptavidin-modified DVD surface. The reaction product attenuates the signal intensity of the laser that reached the DVD drive used as detector, correlating well with the amount of amplified sequence. Analytical performances showed a detection limit of 1 μg/g and good assay reproducibility (RSD 8%), suitable for the simultaneous detection of the three targeted allergens. The developed methodology was tested with several commercially available foodstuffs, demonstrating its applicability. The results were in good agreement, in terms of sensitivity and reproducibility, with those obtained with ELISA, PCR-gel agarose electrophoresis, and RT-PCR.

KEYWORDS: *allergen, food, hazelnut, peanut, soybean, DNA microarraying, DVD*

INTRODUCTION

Individuals with food allergies, 2−4% of the total world population, can react adversely to the ingestion of very low amounts of allergens. Food labeling plays a crucial role in the prevention of the reactions by providing the consumer access to the information of health risks.[1] In this way, regulations of several countries oblige producers to indicate allergic food included in priority lists (e.g., EU Directive 2007/68/EC). The major allergenic ingredients come from milk, eggs, fish, peanuts, tree nuts, soybean, and cereals. However, most reported food-allergic events do not come from packaged foods because they have the major ingredient controlled. The problem is the inadvertent presence of food allergens, frequently present at low levels. Then, the challenge is the detection of traces of such allergens.

Detection methods of allergens are used for confirmatory or screening analysis. Mass spectrometry is a confirmatory tool for the unambiguous identification and/or characterization of food allergens.[1−4] Application of proteomics strategies allows the analysis of intact proteins or the use of accurate peptide mass for high-throughput analysis. Specialized personnel, efficiency of protein separation, and biomarkers requirements, together with the high cost of instrumentation, limit the routine application of most of these methods. Screening methods can be easily integrated as part of Hazard Analysis and Critical Control Point programs of industries or regulation agencies. They are classified according to the target molecule: specific protein or coding DNA.[5] Both have advantages and disadvantages. Immunoassays are still preferred methods because they directly detect the compound responsible for triggering the allergic events and they are simple and time-efficient.[5] Also, some industrial derivatives are extrudates with high content in potentially allergenic protein and extremely low DNA content, for example, soy derivatives. Therefore, enzyme-linked immunosorbent assays (ELISAs) or dipsticks are widely used in the food industry.[6] However, methods based on polymerase chain reaction (PCR) techniques provide interesting benefits. The concentration of DNA is nearly independent of the variety and growth conditions. The DNA molecule is relatively stable, allowing its extraction from all kinds of tissues, and the stability of reagents is also higher than for proteins. Although the minimal eliciting doses, the lowest concentration to provoke allergic reaction, are still unknown, the detection limit of PCR-based methods is lower. Intensive processed and heat-treated foods are characterized by a higher degradation of proteins compared to DNA.[7] Also, DNA is efficiently extracted from food matrices and is less affected by extraction conditions as compared to proteins. Then, in recent years, the number of proposed DNA methods for food allergen detection has been growing.

End-point PCR or real-time-PCR methods have been reported for the detection of hazelnut,[8−10] peanut,[11−13] soybean,[14,15] sesame,[16] celery,[16] mustard,[16] lupine,[17] pistachio,[18] almond,[19] or other nuts.[20] However, food safety agencies and the food-processing industry are subjected to increasing scrutiny of their allergen control programs. Then, the multiplex methods offer the possibility to detect several allergens in one run with advantages such as saving time, reducing reagent costs, and lowering the probability of cross-

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Table 1. Primer, Probe, and Control Sequences

target[a]		sequence 5′−3′[b]	T_m (°C)
hazelnut	primer F	Dig-ACTACATAAAGCAAAAGGTTGAAG	53.5
	primer R	TCGTAATTGATTTTCTCCAGTTTG	55.2
	probe	BtnTg-TTTTTCGGACAAAGCATCGCCTTCAATCA	67.1
peanut	primer F	Dig-CTAGTAGCCCTCGCCCTTTT	59.9
	primer R	GGCATCTTCTGTCTCCTTGG	59.8
	probe	BtnTg-TTTTTAGTTCCCACTGCTGCCTC	62.6
soybean	primer F	Dig-TCCACCCCCATCCACATTT	59.2
	primer R	GGCATAGAAGGTGAAGTTGAAGGA	58.8
	probe	BtnTg-TTTTTTTTTTCGAAGCTGGCAACGCTACCGGTT	74.1
C+ immob	probe	Dig-TTTTTTTTTTTTTTTTGTCATGGGCCTCGTGTCGGAAAACC-BtnTg	81.0
C+ hybrid	target	Dig-AGGGTCGTACACCGGCTGTAATCAAA	72.2
	probe	BtnTg-TTTTTTTTTTTTTGATTACAGCCGGTGTACGACCCT	75.9
C− hybrid	probe	BtnTg-ACCGTCGCGCACTATCTGATTTCAAA	73.3

[a]C+ immob, immobilization positive control; C+ hybrid, hybridization positive control; C− hybrid, hybridization negative control. [b]Dig, digoxigenin labeled; BtnTg, biotinTEG-labeled.

contamination. Although an enzyme immunoassay has been described for detecting several allergen proteins, more advances have been achieved detecting targeted oligonucleotide sequences. Recently, simultaneous detection of relevant allergens has been described by multiplex real-time PCR methods.[21−23] The limitation of this technique is the reduced number of fluorophores for determining a few genes simultaneously. Ligation-dependent probe amplification (LPA) has been proposed for the simultaneous determination of 10 allergens.[24] The color channel limitations of real-time PCR are overcome, because the capillary electrophoretical separation after the common PCR amplification allows the use of the same fluorescent label for all allergens. However, the cost of instruments used in real-time PCR or LPA methods limits the application in several food industries or the close monitoring during manufacturing or distribution activities. In this way, microarrays allow an alternative analysis of multiple sequence targets in a single assay by PNA or DNA chips.[25,26]

An innovative approach is using a compact disk-based microarray system.[27,28] The advantages are multiple. First, compact disks (CDs) and digital versatile disks (DVDs) are the most cost-effective platforms with the ability both to conduct assays and to read, and even to record data, from the same disk using standard drives. Second, commercial disks have shown good properties for probe immobilization (proteins and nucleic acids) and performing bioanalytical assays (e.g., chemically resistant, nonspecific interactions). Third, the surface of a single standard disk (94 cm^2) can hold thousands of spots (e.g., analytes, replicates, calibration standards, controls).

The aim of this study was to develop a simple, cost-effective, reliable, and highly sensitive methodology for allergen detection. DVD technology (disks and drives) was selected as the analytical platform, including appropriate controls to avoid false positives and guarantee high-quality results. Moreover, a new immobilization−detection system is proposed for DNA hybridization on disks. Previous studies used chemical or UV activation for the attachment of amino-modified probes in glass or polycarbonate disks; meanwhile, the detection of model sequences was usually performed by fluorescent scanners and CCD cameras or measuring errors by a CD driver.[27,29,30] In this study, several novelties have been incorporated. First, 5′-biotinylated probes and streptavidin were directly printed in a microarray format on the polycarbonate surface of a DVD. Second, targets were PCR products from food samples labeled during amplification. Third, multiplex detection was performed using a common developer for all targets based on peroxidase-labeled digoxigenin antibody. Fourth, the results are shown as an easily interpretable microarray image. In this paper, as a proof of concept, we present the PCR amplification combined with DVD microarraying that allows the simultaneous detection of three relevant allergens (hazelnut, peanut, and soybean) included in the priority list of the European Union (EU), the United States, and other countries. These seeds are considered to be the most difficult allergens to be analyzed, especially by protein-based techniques, due to extraction issues.[3]

MATERIALS AND METHODS

Chemicals and Food Products. Cetyltrimethylammonium bromide (CTAB), tris(hydroxymethyl)aminomethane (Tris), ethylendiaminetetraacetic acid (EDTA), β-mercaptoethanol, and 3,3′,5,5′-tetramethylbenzidine (TMB) were obtained from Sigma-Aldrich (Madrid, Spain). Horseradish peroxidase-labeled digoxigenin antibody (antiDIG-HRP) was from Abcam (Cambridge, U.K.). Tris−borate−EDTA buffer (TBE), RNase, proteinase K, agarose, electrophoresis loading buffer, and 50 bp and 1 kb ladders were purchased from Fisher Scientific (Madrid, Spain). Isopropanol, sodium chloride, chloroform, isoamyl alcohol, and ethanol were from Scharlau (Barcelona, Spain), and SYBR-Safe was from Invitrogen (Barcelona, Spain). Ultrapure water was obtained from a Milli-Q water purification system (Millipore). The oligonucleotides were from Roche Diagnostics (Barcelona, Spain) (Table 1). Several types of foods from different commercial brands were bought in local stores. Seeds, cookies, cereals, chocolates, pastas, soups, and snacks were stored at room temperature in the dark. Dairy products and jams were stored at −4 °C until use. Ice cream and frozen ready meals were stored at −20 °C.

DNA Extraction. Two extraction methods based on CTAB and column purification were tested. For the CTAB extraction, 100 g of sample was ground in a kitchen blender (Thermomix Vorwerk, Madrid, Spain) until homogenization. A 100 mg aliquot was mixed with 700 μL of extraction solution previously preheated at 65 °C (20 mg/mL CTAB, 1.4 M NaCl, 0.1 M Tris, 0.02 M EDTA, pH 8), 1.4 μL of β-mercaptoethanol, 10 μL of RNase (10 mg/mL), and 10 μL of proteinase K (20 mg/mL) and incubated at 65 °C for 10 min. After the addition of 100 μL of chloroform/isoamyl alcohol (24:1, v/v), a further incubation step was carried out at 65 °C for 30 min. After centrifugation for 10 min at 12000 rpm (model 5415D, Eppendorf, Westbury, NY), the supernatant was transferred into a 1.5 mL tube, and 700 μL of chloroform/isoamyl alcohol (24:1, v/v) was added. Then, the mixture was centrifuged for 10 min at 12000 rpm and the

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aqueous phase transferred into a new tube. After the addition of 700 μL of ice-cold isopropanol, the tubes were cooled for 10 min at −20 °C and centrifuged for 10 min at 12000 rpm. The supernatant was removed, and the pellet was mixed with 500 μL of ice-cold ethanol. After a further centrifugation step (10 min at 1200 rpm), the supernatant was discarded and the pellet was dried for 60 min at room temperature. Finally, the dried pellet was dissolved in 100 μL of buffer (0.01 M Tris, 0.001 M EDTA, pH 8). The extracted DNA was stored at −20 °C until analysis. For column extraction, aliquots of 5−20 g of homogenized sample were extracted using a GMO extraction kit from Applied Biosystems (Madrid, Spain), according to the manufacturer's instructions. The extracted DNA was also stored at −20 °C until analysis.

For DNA extracts, the absorbances at 260 nm (A_{260}) and at 280 nm (A_{280}) were measured with a spectrophotometer (Agilent 8453 model, Madrid, Spain). An aliquot of the extract with loading buffer (Fisher Scientific, Madrid, Spain) was dispensed on a 0.7% (w/v) agarose gel. The molecular weight of the extracted DNA was determined by comparison with a 1 kb ladder, and the electrophoresis was carried out with TBE buffer at 120 V and room temperature. Gels were stained for 30 min with TBE containing SYBR-Safe at 0.01% (v/v), and bands were visualized on a UV transilluminator.

PCR Amplification. PCR reactions were carried out in a total volume of 50 μL using a thermocycler TC-400 from Bibby Scientific (Staffordshire, U.K.) and kit PCR Core from Roche. Single reactions were performed in ultrapure water, 1× Tris-KCl buffer (100 mM Tris-HCl, 500 mM KCl, pH 8.3), 2 mM $MgC1_2$, 200 μM dNTPs, 1.25 units of Taq DNA polymerase, 0.5 μM of both primers of only one allergen, and 150 ng of the genomic DNA. The thermal program was as follows: initial denaturation at 95 °C for 5 min followed by 40 cycles of denaturation at 95 °C for 30 s, primer annealing at 60 °C for 30 s, elongation at 72 °C for 30 s, and a final elongation at 72 °C for 5 min. Multiplex reactions were carried out similarly, but 0.5 μM of three primer pairs (hazelnut, peanut, and soybean) was added to the PCR mix. The absence of inhibitors in the DNA extracts was verified by parallel amplification of bacteriophage Lambda (Fisher Scientific, Madrid, Spain). An aliquot of PCR products was mixed with loading buffer, and the solution was loaded on a 3% (w/v) agarose gel. The size of the amplicons was determined by comparison with a 50 pb ladder, and electrophoresis was carried out with 1× TBE buffer at 120 V at room temperature. Gels were stained for 30 min with 1× TBE containing SYBR-Safe at 0.01% (v/v), and the bands were visualized on a UV transilluminator.

Microarraying. Bulk DVD-R disks were purchased from MPO Iberica (Madrid, Spain). The disks were first conditioned by gentle ethanol washing and water rinsing and then dried by centrifugation. Streptavidin (10 mg/L) and biotinylated probes (50 nM), diluted in printing buffer (50 mM carbonate buffer, pH 9.6, and 10% glycerol (v/v)), were transferred to the disk (50 nL) with a noncontact printing device (AD 1500 BioDot, Inc., Irvine, CA) in microarray format, controlling the working temperature and relative humidity at 25 °C and 90%, respectively.

DNA Array Hybridization and Data Analysis. The assays were carried out as follows: 10 μL of PCR product was mixed with 90 μL of 5× hybridization buffer (1× saline sodium citrate, NaCl 150 mM,sodium citrate 15 mM, pH 7) containing 10 nM positive hybridization control. Subsequently, the solution was denatured by heating at 95 °C during 5 min and transferred onto the DVD surface. After 120 min of incubation at 37 °C, 1 mL of antiDIG-HRP solution in PBS-T (1:250) was homogeneously dispensed on the washed disk. After 30 min at room temperature, the disk was washed and the product was developed by dispensing 1 mL of TMB solution. The reaction was stopped by washing the disk with water after 2 min. The results were directly read by the DVD drive,[27] the reaction product optical density being related to the allergen amount.

Reference Methods. Identification of hazelnut and peanut was performed using ELISA kits from R-Biopharm AG (Darmstadt, Germany) according to the manufacturer's instructions. Absorbance was read with a Spectra MAX340pc microplate reader from Molecular Devices (Toronto, Canada) at 450 nm. The presence of soybean was detected by RT-PCR method with forward primer, 5′-TCCACCCC-CATCCACATTT-3″ reverse primer, 5′-GGCATAGAAGGT-GAAGTTGAAGGA-3′; and probe, FAM-AACCGGTAGCGTTGC-CAGCTTCG-TAMRA. The reaction conditions were as follows: 10 min at 95 °C for enzyme activation, 50 cycles of denaturation at 95 °C (15 s), and annealing at 60 °C (1 min). Readings were taken using a real-time sequence detection system 5700 from Applied Biosystems. The threshold was set at 10 times the standard deviation of the mean baseline emission calculated between the 3rd and 15th cycles. Gel electrophoresis was also used for the detection of three allergens. An aliquot of end-point-PCR products (8 μL) was mixed with 2 μL of 5× glycerol loading dye, and the solution was loaded on a 3% (w/v) agarose gel. DNA standard of known size (50 bp DNA) was used for the determination of the DNA fragment size. Electrophoresis was run in 1× TBE buffer solution at 120 V and room temperature. PCR product bands were stained with SYBR-Safe at 0.01% (v/v), and bands were visualized on a UV transilluminator.

RESULTS

DNA Extraction. Analyte extraction is considered to be the most critical step in food allergen analysis, especially in processed foods. The extraction of genomic DNA from complex matrices, such as fat-rich and polyphenol-rich foods, is especially difficult because these compounds can potentially be coextracted and inhibit the PCR. Another problem is achieving a representative fraction from a food sample containing the trace allergen. A high-quality template DNA is an important requirement to facilitate the detection of the target sequences.[31] For that, two genomic DNA extraction methods based on different principles (CTAB-based and column purification) were compared in food samples with different processing techniques and compositions. The DNA concentration was calculated according to the equation c (ng/μL) = A_{260} × 50 × dilution factor. The ratio A_{260}/A_{280} was used to evaluate the quality of the isolated DNA. The results of the extraction study are shown in Table 2. The amounts of DNA extracted were similar using the column method or the CTAB method. In terms of quality, the ratio of absorbance A_{260}/A_{280} was evaluated, being higher than 1.5 for all samples (raw or processed) using both methods. The integrity of the DNA extracts was assessed through agarose gel electrophoresis. Well-defined bands were observed with good accordance between the two extraction methods. PCR sensitivity is strongly dependent on the amount and quality of the DNA template, but inhibition was not observed in any extract, allowing the correct amplification and detection of allergen in spiked samples, as explained in the following sections. Because a

Table 2. Yield and Purity of DNA Extracted Using the CTAB and Column Methods

	μg DNA/g of food		A_{260}/A_{280}	
	CTAB	column	CTAB	column
raw hazelnut	32.2 ± 1.6	25.5 ± 1.0	1.50 ± 0.03	1.93 ± 0.19
soy cookies	34.5 ± 0.5	18 ± 2	1.56 ± 0.04	1.71 ± 0.10
dried soup	36.9 ± 1.6	19.7 ± 0.9	1.51 ± 0.09	1.67 ± 0.08
soybean seeds	35.9 ± 1.1	61.4 ± 1.3	1.55 ± 0.08	1.74 ± 0.07
soybean flour	38.6 ± 1.5	59.0 ± 1.9	1.50 ± 0.11	1.69 ± 0.12
cocoa powder	40.4 ± 1.4	74.0 ± 1.9	1.63 ± 0.07	1.55 ± 0.06
cow's milk	21.1 ± 3.0	17.5 ± 1.6	1.66 ± 0.18	1.64 ± 0.15
peanut butter	29.5 ± 1.3	15.4 ± 1.1	1.56 ± 0.05	1.56 ± 0.04
chocolate spread	38.7 ± 1.7	47.8 ± 1.9	1.49 ± 0.07	1.59 ± 0.05
soy yogurt	37.5 ± 1.0	21.9 ± 0.6	1.65 ± 0.10	1.70 ± 0.07

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good extraction yield, a high purity, and a good amplification of the target sequences were obtained, both extraction protocols can be used for DNA-based allergen detection.

Optimization of PCR. According to PCR product specificity, minimal self-annealing, and similar melting temperatures, the target sequences were selected from the allergen genes (GenBank database). The selected genes were Cor a 1 (accession no.Z72440) of hazelnut (*Corylus avellana* L.), Ara h 2 (accession no. L77197.1) of peanut (*Arachis hypogaea*), and Le (accession no. K00821) of soybean (*Glycine max*). The design of a novel peanut-specific primer pair was performed on the Ara h 2 coding region sequence using the software program Primer3Plus. The primer sequences of soybean and hazelnut were selected from the literature,[21,32] ensuring the absence of cross-dimers. All primers and probes were successfully checked for relevant homologies by BLASTNr search. Before the setup of triplex assay, the efficiency of each PCR system was tested. For that, an experimental design was carried out to optimize the PCR conditions, studying the response from dilution series and mixtures of allergens in blank food samples. A temperature gradient was performed from 56 to 65 °C to determine a common annealing temperature; it was optimal at 60 °C. The concentrations of primers tested were from 0.3 to 0.7 μM; it was optimal at 0.5 μM. Each PCR reaction generated the predicted product (109 bp for hazelnut, 82 bp for peanut, and 81 bp for soybean). The optimization of multiplex PCR was performed with mixtures of DNA extracted from the allergens and blank food samples. Under the working conditions (0.5 μM for each primer), any preferential amplification was detected, even for mixtures in which an allergen was present in a minor amount and other allergens were in excess.

DNA Array Hybridization and Detection. Multiplexed PCR products were thermally denatured and dispensed on the DVD modified surface. For that, the array layout on the DVD surface consisted of nine blocks of nine dots each: three blocks corresponding to single-target systems (specific allergen); two blocks corresponding to multitarget systems (three allergens); two positive controls (immobilization and hybridization); and two negative controls (immobilization and hybridization). A total of 10 arrays were printed on the polycarbonate surface of the DVD-R, allowing the simultaneous analysis of 10 samples (Figure 1). In this configuration, spots are 500 μm in diameter with a track pitch (center-to-center distance) of 1.5 mm, achieving an array density of 1.0 spot/mm^2. The storage stability of the modified DVD was tested at 4 °C until 7 weeks. The same analytical performances were observed.

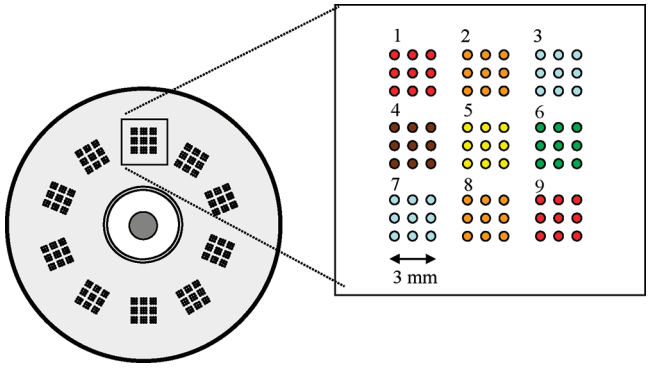

Figure 1. Scheme of DVD arrays. The blocks correspond to hybridization positive control (1), allergen probe mix (2), hybridization negative control (3), hazelnut probe (4), peanut probe (5), soybean probe (6), immobilization negative control (7), allergen probe mix (8), and immobilization positive control (9).

The hybridization assays were carried out at different incubation conditions (25−55 °C, 30−180 min) and concentrations of hybridization buffer (1−20×) to achieve the maximum yield. The best results on the basis on relative signal intensity were obtained using hybridization buffer 5× at 37 °C during 120 min. Next, the digoxigenin-labeled hybridization product was developed by the HRP−antidigoxigenin antibody−TMB system (Figure 2). The resulting solid changed the intensity of reflected light at 650 nm, allowing the quantification by the DVD drive. To verify that the assay steps are well-developed, several assay controls were implemented. The immobilization controls correspond to a double-labeled probe (positive) and a nonprobe block (negative). The hybridization controls correspond to a specific probe to digoxigenin-labeled oligonucleotide added together with PCR products (positive) and a noncomplementary oligonucleotide (negative). Non-cross-hybridization or nonspecific responses were observed.

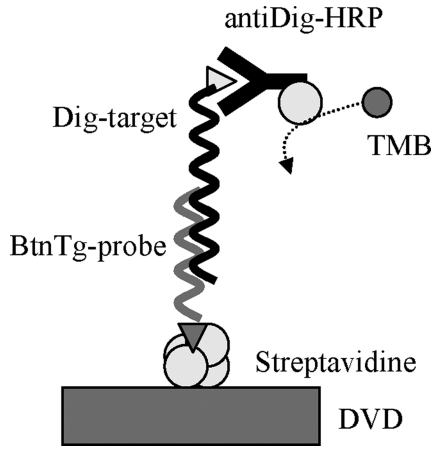

Figure 2. Scheme of the assay format developed on DVD surface.

Although specificity was successfully checked by BLAST search, DNA from several plant species and other food ingredients was isolated and tested in the selected multiplex PCR system. Almond, barley, bean, Brazil nut, cashew, chestnut, chickpea, corn, egg, kidney bean, lentil, lupine, oat, olive oil, pea, pecan, pistachio, powdered milk, rice, rye, sesame, sunflower seed, tomato, walnut, and wheat were tested. No cross-reactivity was observed, negative responses, confirming the high specificity of the developed assay.

The detection limit (LOD) was determined in two ways, by analyzing serially diluted DNA extracted from allergenic foods and the whole food product spiked with certain amounts of allergens. In both cases, allergen-free food samples were selected by obtaining blank responses with the DVD-based method or reference methods (ELISA or RT-PCR). They included cookies, jams, sponge cake, powdered milk, and frozen ready meals. First, mixtures of DNA extracted from three allergens were diluted with allergen-free food extracts, keeping a total DNA concentration constant (30 ng/μL). Figure 3 shows the images after scanning for a 10-fold serial dilution. Although the aim of this research was not the quantification of allergen, a correlation between the DNA amount of food allergens and the optical intensity measured by DVD detector was found.

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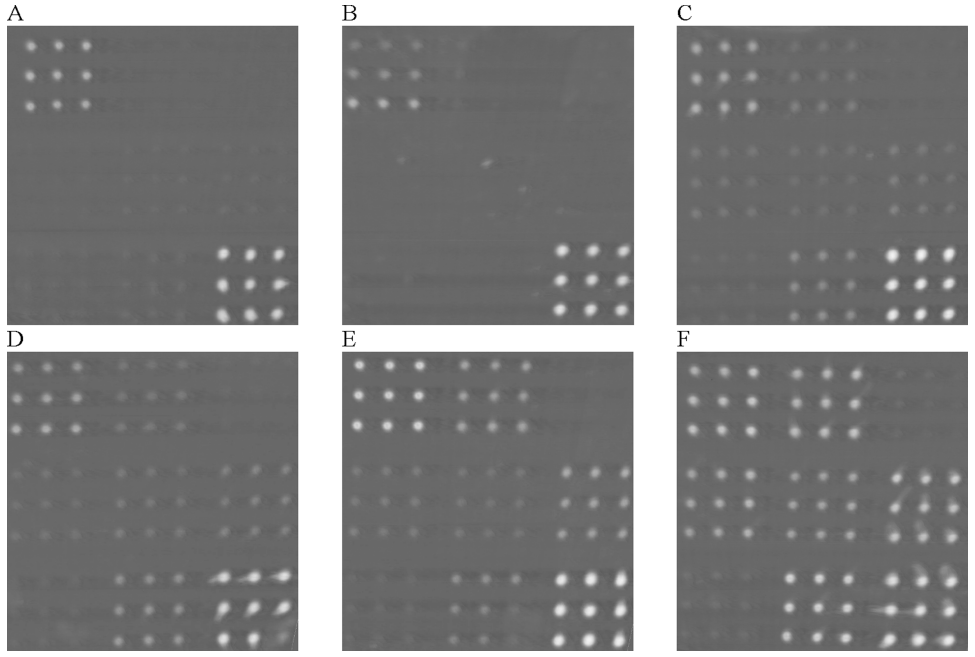

Figure 3. Optical density images from different assays. Panels A–F correspond to 0, 0.1, 1, 10, 100, and 1000 μg/g of allergen mixture (hazelnut, peanut, and soybean), respectively.

Table 3. Comparison of Screening Results of Allergens in Commercial Foodstuffs Analyzed by DVD and Reference Methods (Number of Replicates = 3)

		DVD-based method[a]			ELISA method		RT-PCR method
category	food	hazelnut	peanut	soybean	hazelnut	peanut	soybean
jam	quince paste 1	+	−	−	+	−	−
	quince paste 2	−	−	−	−	−	−
	quince paste 3	−	+	−	−	+	−
	quince paste 4	−	−	−	−	−	−
	quince paste 4	−	−	−	−	−	−
frozen ready meal	meatballs	−	−	+	−	−	+
	scalloped ham and cheese	−	−	+	−	−	+
	chicken nuggets	−	−	+	−	−	+
	vegetable rice	−	−	+	−	−	+
	cauliflower	−	−	−	−	−	−
	scrambled eggs with shrimp	−	−	−	−	−	−

[a]Detection: +, presence; −, absence.

Nevertheless, the sensitivity of the screening assay was related to the signal-to-noise ratio (SNR) calculated from background signal. The 1 μg/g dilution was positive for three allergens, because SNR was >3, but lower concentrations were not detected. Second, commercial food products free from the three allergens were spiked with different combinations of hazelnut, peanut, and soybean until 10% of each one. The spiked samples were extracted, amplified, and analyzed. The measured LODs were 1 μg/g (0.0001%) for the three allergens, demonstrating the absence of inhibition effects during extraction. Also, method sensitivity was independent of food processing, the relative amount of allergen, or the nature of the food in which these allergens are usually contained as ingredients. These values were similar to or better than those calculated by RT-PCR methods or the LPA method for allergen detection (>1 μg/g).[3]

Assay reproducibility was also determined from the optical density of spots. To determine intradisk and disk-to-disk relative standard deviations (RSDs) of positive controls, three DVDs were tested, each with 10 arrays and 9 replicates per array. The intradisk RSD varied from 3.6 to 5.3%, whereas the disk-to-disk RSD ranged from 5.8 to 7.3%, indicating their suitability. Assay reproducibility was also investigated by analyzing replicates of DNA extracts (n = 3), varying from 3.1 to 14.5%. No dependence of allergen nature was statistically confirmed by an ANOVA test. Therefore, the results were suitable for routine qualitative detection of food allergens.

Determination of Allergen Presence in Food Samples. A two-laboratory validation study was performed by analyzing 10 blind samples (unknown allergen content for participants). The methods were based on DVD technology (three allergens, simultaneously) and ELISA (hazelnut and peanut, individually) or RT-PCR (soybean). Detection limits of both ELISA methods were 0.00025%, and that of the RT-PCR method was 0.0005%. Table 3 shows the excellent agreement between the proposed triplex assay and the three reference

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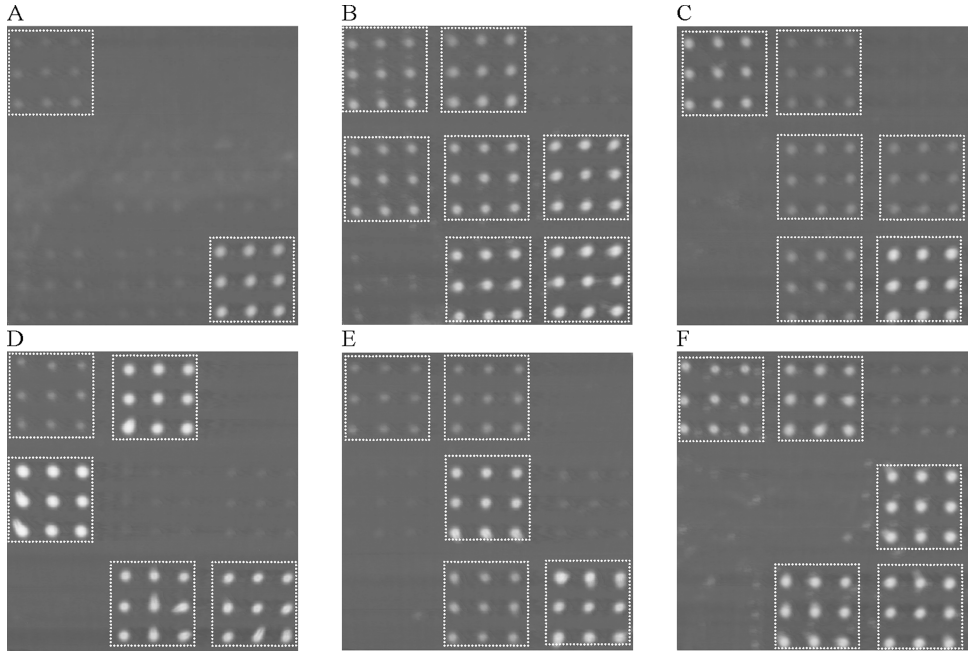

Figure 4. Optical density images from food samples: noodles (A); chocolate wafers (B); flat breadsticks (C); chocolate bar (D); peanut butter (E); ice cream (F).

methods developed in an external laboratory. No allergen was detected in three food samples; meanwhile, one allergen was detected in the positive food samples. It is worth mentioning that the multiplexed analysis by DVD technology is able to analyze simultaneously several allergens, a goal that is very difficult to reach with screening protein-based methods.

Twenty-five food samples were analyzed for determining the presence of DNA from allergens using the triplex PCR method and DVD technology. Foodstuff was selected including several categories of them and a large variety of food-processing methodologies. Figure 4 shows examples of results from food samples. Besides positive controls, when only one allergen was present in food sample, the signal was observed in both multitarget block and specific block. In the case of the presence of some allergens, the associated blocks were detected. For all cases, specific hybridizations were obtained, because each target sequence hybridized to their corresponding specific capture probe. The presence of allergens was assessed according to the declaration on the label (Table 4). Allergen DNA was not found in sponge cake, corn flakes, cocoa powder, strawberry yogurt, cow's milk, and noodles. Hazelnut was detected in one cookie sample, cereal bars, and several chocolate products. Peanut was detected in several cookies, one cereal product, flat breadsticks, and peanut butter. Twelve samples were positive to soybean, including cookies, chocolate products, ice cream, soups, and snacks. To check the performances of our technique, some protein derivatives of soybean were included. In the case of cookies containing traces of soybean lecithin, it was not detected by any DNA-based technique. Cookies containing protein extrude (<1%), an industrial derivative with extremely low DNA content, were positive by DVD technology. These results confirm the reliability and sensitivity of the proposed method in the qualitative detection of allergens even in some samples having a low DNA content (impurities of protein isolation process).

Several allergens were detected in seven food samples (37% of positive samples), demonstrating the importance of multiplexing to reduce the efforts of screening methods. The same food samples were also analyzed using an agarose gel electrophoresis technique to establish the presence or absence of specific bands. Good agreement was observed when allergen was found at high concentrations. However, in several cookies, ice cream, dried soups, and flat breadsticks, the presence of at least one allergen was detected only by the DVD technique.

DISCUSSION

A multiplexed DNA-based method using compact disk technology has been developed, demonstrating the usefulness for allergen determination. The proposed oligonucleotide microarrays are capable of detecting target genes of regulated allergens, including controls that guarantee the reliability of the analysis. As has been demonstrated, low-cost DVD technology can be used as a simple screening test of their presence/absence in foodstuffs of different nature. The assay specificity was good, showing no cross-reactivity in the experiments, and high reproducibility reinforces the use of this methodology for screening purposes. The multiplexed analysis on DVD is flexible to increase significantly the number of samples, replicates, or allergen targets to be simultaneously detected. A change in array layout is totally compatible with proposed immobilization−detection systems, and it would allow the determination of other priority or emergent allergens at one time. Besides, the universal nature of PCR and DVD technology suits the needs of the food industry, production or distribution areas, having significant concerns about food-allergenic consumer complaints.

The analytical performances of DVD microarray technology were compared to other methods reported for screening determination of food allergens. A limitation of our approach is that allergenic protein is not directly analyzed, because it is based on the detection of a specific gene of the allergen. However, it was demonstrated that DNA from allergens was successfully extracted and detected in several processed foods, which is difficult for some protein-based methods. Also, some

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Table 4. Comparison of Screening Results of Allergens in Commercial Foodstuffs Analyzed by DVD and Electrophoresis Techniques (Number of Replicates = 3)

		declared allergen[a]			detected allergen[b]					
					DVD-based method			electrophoresis		
category	food	hazelnut	peanut	soybean	hazelnut	peanut	soybean	hazelnut	peanut	soybean
seeds	raw hazelnut	+	−	−	+	−	−	+	−	−
	roasted peanut	−	+	−	−	+	−	−	+	−
	soybean flour	−	−	+	−	−	+	−	−	+
cookies	chocolate cookies	±	±	+	−	+	+	−	+	+
	wheat cookies	−	±	±	−	+	+	−	−	−
	muesli cookies	±	±	+	+	+	+	−	−	+
	butter cookies	−	±	±	−	+	+	−	−	−
	sponge cake	−	−	−	−	−	−	−	−	−
	soy cookies	−	−	+	−	−	+	−	−	+
	soy lecithin cookies	−	−	+	−	−	−	−	−	−
	soy protein cookies	−	−	+	−	−	+	−	−	−
cereals	cereals bar	+	±	−	+	+	−	+	+	−
	corn flakes	−	±	−	−	−	−	−	−	−
chocolates	chocolate bar	+	−	−	+	−	−	+	−	−
	chocolate spread	+	−	−	+	−	−	+	−	−
	cocoa powder	−	−	−	−	−	−	−	−	−
	chocolate wafer	±	+	+	+	+	+	+	+	+
dairy products	strawberry yogurt	−	−	−	−	−	−	−	−	−
	soy yogurt	−	−	+	−	−	+	−	−	+
	cow's milk	−	−	−	−	−	−	−	−	−
	ice cream	−	−	±	−	−	+	−	−	−
soups	dried soup	−	−	±	−	−	+	−	−	−
pasta	noodles	−	−	−	−	−	−	−	−	−
snacks	flat breadsticks	−	+	±	−	+	+	−	+	−
butters	peanut butter	−	+	−	−	+	−	−	+	−

[a]Declaration: +, hazelnut, peanut, or soybean listed; −, hazelnut, peanut, or soybean not listed; ±, may contain trace levels. [b]Detection: +, presence; −, absence.

researchers have pointed out that DNA-based methods are more suitable than ELISA when closely related species have to be analyzed.[3] The detection limit, analysis time, reproducibility, and/or multiplexing capacity are the main advantages of the proposed method against gel electrophoresis analysis, ELISA, or single RT-PCR.

Multiplex RT-PCR is a potential competitive technique, because similar analytical performances have been reported achieving a tetraplex quantitative assay.[22] This technique without post-PCR steps provided shorter assay times, minimizing the risk of cross-contamination, although the detection limits are better for DVD technology. However, the main drawback of RT-PCR could be the maximum number of allergens than can be determined, including internal control, to guarantee the correct analysis performance. Fluorescent detection is limited by the availability of different labeling dyes for probes. Multiplexed analysis on DVD is flexible to increase significantly the number of samples, replicates, or allergen targets to be simultaneously detected, due to the spatial separation of probes. Another potential competitive method is LDA, which allows the simultaneous determination of up to 10 allergens with similar selectivity, sensitivity, or reproducibility.[24] The main limitation is the analysis time (the duration of the ligation−hybridization step is 17−18 h), the sequential detection mode, and cost by reaction. Both alternatives (RT-PCR and LDA) are less portable and more expensive than DVD technology. Therefore, the proposed method is especially suitable for screening applications in the point-of-control facilities with a low availability of equipment or resources and maintaining the analytical performances. On the other hand, the compact disk based PCR microarray technology could be quite compatible with isothermal amplification or other simple developing approaches, to improve the competitiveness and simplify the working protocols.

AUTHOR INFORMATION

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