



Microbead-based simultaneous fluorometric detection of three nut allergens

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Abstract

A microbead-based method is developed for the detection of three nut allergens that can occur in food. The bead array constitutes of a suspension of spectrally distinct fluorescent polystyrene microbeads and has been applied for the simultaneous detection of three main and serious allergens (hazelnut, peanut and walnut). The method is based on the simultaneous identification of specific DNA sequences after PCR. The PCR products are biotinylated and hybridized to nut-specific oligonucleotide probes attached to the microbeads. The hybrids are finally detected by a streptavidin-phycoerythrin conjugate and flow cytometric analysis. As low as 0.01% content of the three nuts in processed food (cookies) can be detected by this method.

Keywords Microspheres · Microbeads · Flow cytometry · Fluorescence · DNA · PCR

Introduction

Allergen detection in food is of immense importance, because their consumption, even in trace amounts, can cause severe allergic reactions. For these reasons, national legislation obliges food industries to label their products properly in case of the presence of an allergenic ingredient [1]. However, in many cases, food or raw materials may be contaminated by allergenic substances during their processing, production and storage, leading to serious health risks. Thus, extremely sensitive analytical methods have to be developed and applied to each food manufacturing plant to protect consumer suffering from allergies. Multiplex detection systems are also essential. In addition, multi-analyte systems that enable the simultaneous detection of both allergenic markers: proteins and the related DNA sequences are the most covetable. Several DNA- and protein-based analytical methods are available for the analysis of allergens, having though some difficulties in detectability, inter-laboratory reproducibility and specificity [2].

Nuts are defined as one of the eight most allergenic foods responsible for ~90% of human food allergies. Furthermore,

nuts have been included in the list of the fourteen groups of serious allergens that according to the European Union have to be accurately declared on the label apart from other ingredients [3, 4]. However, threshold values for allergens' labeling have not yet been appointed by the authorities. The analytical methods applied for allergen detection in food are based on the direct detection of the specific allergenic ingredient or the identification of a marker that indicates the presence of this ingredient. The markers usually employed include characteristic proteins or specific DNA sequences of the allergens. The use of both markers reveals some advantages and disadvantages. The choice of the proper marker depends on the analyzed food product. The major disadvantage of protein-based methods is that food processing and climate variations affect proteins. On the other hand, DNA targets are preferred when quantification of the analyte is required, because of their stability, especially during thermal processing, and phenotype differences. Also, the amount of the isolated DNA can be amplified by PCR, increasing the detectability of the method [5]. Emerging analytical methods are focusing on sensing systems that can combine the detection of both proteins and nucleic acids to eliminate the disadvantages of the foresaid techniques. Microbead-based technology is an elegant and versatile alternative for quantitative multianalyte assays. They offer low workload, low sample volume, high-throughput and multiplex ability [6]. A microbead-based array has been developed for the simultaneous detection of 14 different allergenic proteins, offering a detection limit <5 ng/mL [7].

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DNA methods are based on genes that encode an allergenic protein or any other specific DNA sequence as a marker. The different PCR approaches include end-point PCR, quantitative real time PCR and high-resolution melting curve analysis (HRM). Multi-analyte systems are preferred in food allergen detection. Advantages of multi-analyte techniques include short assay times and lower volumes of sample and reagents required for the analysis. The detection of several allergenic nuts includes the simultaneous amplification of species-specific DNA sequences by multiplex PCR. A duplex PCR was developed firstly based on Cy5 labeled primers and a PNA array for the simultaneous detection of peanut and hazelnut. The detection limit was 1 nM [8]. Multiplex quantitative real-time PCR was then introduced for the simultaneous detection of different allergens, e.g. peanut, hazelnut, celery, soy, egg, milk, almond and sesame. Detection systems were based on Taqman probes, SYBR GreenER and subsequent HRM analysis providing detection limits ranging from 0.01–0.1% [9–11].

Other multianalyte techniques involve simultaneous detection of 8 or 10 allergens by ligation-dependent probe amplification (LPA), using labeled probes or fragment length analysis and capillary electrophoresis (CE). The methods offer a detection limit of 0.01–0.1% [12, 13]. Capillary electrophoresis was also used for the simultaneous detection (10plex PCR) of 10 food allergens with a detection limit of 0.005% [14]. Two tetraplex PCR systems were also set for the detection of 8 allergens exploiting silicon-based optical thin-film biochip technology for the visible detection of PCR products within 30 min. The detection limit was 0.001% for single diluted genomic DNA [15, 16]. Another approach was the simultaneous detection of three allergens on a digital versatile disk (DVD) using digoxigenin-labeled PCR products. The detection was accomplished by antidigoxigenin-HRP conjugate combined with HRP chromogenic substrate. Up to 10 targets can be simultaneously detected by this method, with a detection limit of 0.0001% of diluted isolated DNA [17]. An overview of DNA-based methods for the determination of nut allergen in food samples is presented in Table 1.

In the present work, we developed a microbead-based array for the simultaneous identification of three severe allergens (hazelnut, peanut and walnut) in raw and processed food. The method involves the simultaneous fluorometric detection of three nut-specific DNA sequences on the surface of fluorescently encoded microbeads. As low as 1.4 pM of PCR product and 0.01% proportion of the three nuts present in processed food (cookies) can be detected by this method.

Experimental

Reagents and apparatus

Agarose was from Applichem (Darmstadt, Germany). Deoxynucleoside triphosphates (dNTPs) and DNA marker

(ΦX174 DNA-HaeIII Digest) were purchased from HT Biotechnology (Cambridge, UK, www.chemicalscout.com). Platinum *Taq* DNA polymerase was obtained from Invitrogen (Waltham, MA, www.thermofisher.com/gr/en/home/brands/invitrogen.html), while Fast Start Master Mix for multiplex PCR was from Roche (Basel, Switzerland, www.roche.com). Trizma base and 2-(N-morpholino) ethanesulfonic acid (MES) was purchased from Sigma (St. Louis, MO, www.sigmaldrich.com). 1-Ethyl-3,3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC) was from Thermo Fisher Scientific (Waltham, MA, www.thermofisher.com/gr/en/home.html). Tetramethylammonium chloride (TMAC) was obtained from Fluka Chemie (Buchs, Switzerland, www.sigmaldrich.com). Fluorescent microbeads (# 33, 80 and 85), 5.6 μm in diameter, were purchased from Luminex (Austin, TX, www.luminexcorp.com). Streptavidin-R-phycoerythrin conjugate (SA-PE) was purchased from Molecular Probes (Eugene, OR, www.thermofisher.com/gr/en/home/brands/molecular-probes.html). Bovine Serum Albumin (BSA) was obtained from Serva (Heidelberg Germany, www.serva.de). Nucleospin Food Genomic DNA Isolation Kit from Macherey-Nagel (Düren, Germany, <http://www.mn-net.com/>) was used for DNA extraction. Ethidium Bromide for gel staining was from Research Organic Inc. (Sigma-Aldrich Corporation). All common reagents were from Sigma. Raw nuts and raw materials for the preparation of cookies were purchased from local markets. Oligonucleotides (primers and probes) are listed in Table 2 (Köppel et al., 2012) and were synthesized by MWG-Biotech/Eurofins Genomics (Ebersberg, Germany, www.eurofinsgenomics.eu). Hybridization solution consisted of 50 mM Tris-HCl pH 8.0, 4 mM EDTA pH 8.0, 1 g·L⁻¹ SDS, and 3 M TMAC. TE buffer consisted of 10 mM Tris-HCl and 1 mM EDTA, pH 8.0. Phosphate-buffered saline (PBS) consisted of 0.14 M NaCl, 2.7 mM KCl, 10 mM sodium phosphate and 1.7 mM potassium phosphate, pH 7.4.

A thermal cycler obtained from Takara (Shiga, Japan, www.clontech.com/) was used for PCR, while Luminex 100 IS flow cytometer (Luminex, Austin, TX, www.luminexcorp.com) was used for the discrimination of the microbeads and fluorescence measurements. An ultrasonic cleaner from Branson Ultrasonics (Danbury, CT) was used for suspension of the microspheres. The Nanophotometer from Implen GmbH (München, Germany, www.implen.de) was used for quantification of isolated genomic DNA. DNA electrophoresis system was obtained by Biorad (CA, USA, <http://www.bio-rad.com>). A digital camera, Konica Minolta DYNAX 5D (Konica Minolta Holdings, Tokyo, Japan, www.konicaminolta.com) and the Gel Analyzer software from Kodak (New York, USA, www.kodak.com/corp/default.htm) were used for PCR products quantification.

Table 1 An overview on multianalyte DNA-based methods for determination of nut allergens in food samples

Sample	Method	Analysis Time	LOD	Multiplicity	Ref.
Raw nuts	Bead array	3.5–4 h	1.4 pM	3	This work
Cookies			0.01%		
Commercial food samples	PNA array	4–4.5 h	1 nM	2	[8]
Raw nuts	Real-time PCR	2–2.5 h	0.01%	4	[9]
Cookies	<i>Taqman probes</i>				
Commercial food samples					
Raw nuts	Real-time PCR	30 min–1 h	0.05%	6	[10]
Cookies	SYBR GreenER				
Commercial food samples	Sequencing				
Raw nuts	Real-time PCR	2–2.5 h	0.1%	6	[11]
	<i>Taqman probes</i>				
	HRM analysis				
Raw nuts	Ligation-depended probe amplification	16–18 h	0.05%	8	[12]
Cookies	Fragment Length analysis				
Commercial food samples	Capillary electrophoresis				
Raw nuts	Ligation-depended amplification	18–20 h	%	10	[13]
	Capillary electrophoresis		of diluted isolated DNA		
Raw nuts	PCR	2–2.5 h	0.005%	10	[14]
Commercial food samples	Capillary electrophoresis				
Raw nuts	Optical thin film biochip	2–3 h	0.001%	4	[15, 16]
Commercial food samples			of diluted isolated DNA		
Raw nuts	Digital versatile disk	4.5–5 h	0.0001%	3	[17]
Commercial food samples			of diluted isolated DNA		

LOD Limit of Detection

Conjugation of oligonucleotide probes to the fluorescent microbeads

The microbeads used in this study are polystyrene, 5.6 μm in diameter, carrying carboxyl groups on their surface. They contain two spectrally distinct fluorescent dyes in various molar ratios. The different proportions of these dyes provide a unique spectral signature. This serves as a barcode for the

microbeads producing up to 100 distinguishable sets. Three different sets of these microbeads (#33, #80 and #85) were covalently coupled to 5'-NH₂-oligonucleotide probes specific to three allergens (hazelnut, peanut and walnut). The coupling protocol was as follows: an aliquot containing 1.25×10^5 microspheres was centrifuged at 12900 g for 2 min and washed twice with 25 μL of 0.1 M MES buffer, pH 4.5. The microspheres were suspended in 25 μL of the same buffer and sonicated for 10 min to keep the microspheres in suspension. Oligonucleotides (25 pmol) specific to each allergen that carry an -NH₂ group at one end were then conjugated to the different sets of microbeads. A volume of 1.25 μL of 10 g·L⁻¹ EDC, freshly prepared in water, was used for the conjugation. The mixture was left for 30 min at ambient temperature in the dark. The addition of the same amount of EDC was repeated with a fresh solution of EDC. The mixture was incubated for another 30 min as above. The microbeads were pelleted at 12900 g for 2 min and washed once with 50 μL of 0.2 mL·L⁻¹ Tween-20 and once with 50 μL of 1 g·L⁻¹ sodium dodecyl sulfate (SDS). The microbeads were finally resuspended in 125 μL of TE buffer, pH 8.0 and stored at 4 °C until use.

Preparation of home-made cookies

Home-made cookies were prepared in the lab containing all three nuts: hazelnut, peanut and walnut. The mixture of doughs contained 250 g regular wheat flour, 100 g regular

Table 2 The sequences of the primers and the specific probes used for amplification and identification of hazelnut, peanut and walnut

Oligonucleotide name	Sequence 5' → 3'
PCR primers	
Peanut R	Biotin-TCCTCGTCACGTTGGATCTTC
Peanut F	GACAGAAGATGCCAGAGCCA
Hazelnut R	Biotin-CACAAAACGTACAACCTCTGG
Hazelnut F	GGAGGTCCCGGAACCATTA
Walnut R	CTCATGTCTCGACCTAATGCT
Walnut F	Biotin-GCGCAGAGAAAGCAGAG
Oligonucleotide probes	
Hazelnut probe	NH ₂ -TTCGATGAAGGTTAATTCTACCA
Peanut probe	NH ₂ -AGGCCCTGCGAGCAACATCTCATG
Walnut probe	NH ₂ -ATTGTGCCTCTGTTGCTCTCTTC CCG

white sugar, 100 g low-fat bovine milk (1.5% fat), 1 pullet egg and all the three analyzed nuts in different portions: 0, 0.01, 0.02, 0.05, 0.2, 1 and 5%. The cookies were then baked in preheated oven (Gallenkamp oven, Fistreem International Ltd., UK, <http://www.fistreeminternational.com>) at 180 °C for 20 min.

DNA extraction from food samples

DNA was extracted from cookies and raw nuts, after peeling, using the Nucleospin Food Genomic DNA Isolation Kit from Macherey-Nagel according to the manufacturer's instructions. Before extraction, the nuts and the cookies were homogenized using a regular mini chopper (Singer, USA). 200 mg of each sample were used for DNA isolation. The concentration and the purity of the isolated DNA were determined by UV absorption at 260/280 nm using a Nanophotometer (Implen).

DNA amplification

Single PCR

The isolated DNA from raw nuts was subjected to separate PCR reactions for the amplification of DNA sequences specific to hazelnut, peanut and walnut, respectively. The mixture of each PCR reaction, final volume of 50 μL , consisted of 50 mM KCl, 20 mM Tris-HCl pH 8.4, 1.5 mM MgCl_2 , 200 μM each of dNTPs, 0.5 μM each of the primers (specific to hazelnut, peanut or walnut), 1.5 U of Platinum *Taq* DNA polymerase and 5 μL of the isolated genomic DNA ($\sim 70\text{--}100\text{ ng}\cdot\mu\text{L}^{-1}$). The cycling conditions were: 95 °C for 5 min, followed by 45 cycles at 95 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s. A final extension step at 72 °C for 10 min was also included. Negative samples, that contained H_2O instead of DNA, were also included in the reaction. The PCR products were biotinylated due to the biotinylated primer (Table 1) used during the amplification reaction. The PCR products were then confirmed by 2% agarose gel electrophoresis and ethidium bromide staining, while quantitated by densitometric analysis using appropriate DNA marker.

Triplex PCR

The isolated DNA from home-made cookies, that contained the three nuts, was subjected to triplex PCR reaction for the simultaneous amplification of DNA sequences specific to hazelnut, peanut and walnut. The mixture of the reaction had a final volume of 25 μL and consisted of 12.5 μL of Fast Start Master Mix, 0.6 μM each of the primers specific to hazelnut and peanut and 0.4 μM each of the primers specific to walnut and 100 ng of isolated genomic DNA from cookies. The thermal conditions of the reaction were: 95 °C for 15 min, followed by 45 cycles at 95 °C for 30 s, 62 °C for 30 s and 72 °C for

30 s. A final extension step at 72 °C for 10 min was also included at the end of the reaction. A negative sample, that contained H_2O instead of DNA, was also included in the analysis.

Microbead-based array

The PCR products were analyzed by a microbead-based array. In more detail, the PCR products were subjected to a multiplex hybridization assay on the surface of spectrally distinct fluorescent microbeads. The assay was performed in a total volume of 55 μL . The mixture contained 1000 microspheres of each of the three sets of microbeads, conjugated to the specific probes to hazelnut, peanut and walnut, respectively, in 47 μL of the hybridization solution. The mixture was then sonicated for 10 min and preheated at 37 °C. The biotinylated PCR products were heat-denatured for 5 min at 98 °C and placed on ice for 2 min. Immediately, a 5- μL aliquot was added to the microbead-mixture and incubated for 1 h at 37 °C, while it was occasionally hand-stirred. The microspheres were then centrifuged at 12900 g for 2 min and washed once with 50 μL of the hybridization solution. The microspheres were resuspended in 55 μL of the hybridization solution and 12 μL of streptavidin-phycoerythrin (SA-PE) conjugate ($10\text{ mg}\cdot\text{L}^{-1}$) were added. The solution was incubated for 20 min at room temperature, in the dark. The microbeads, without washing, were then analyzed using the Luminex IS 100 flow cytometer.

Results and discussion

A new and sensitive microbead-based DNA array was constructed for food allergen determination. This method is based on multiplex PCR followed by multiplex hybridization assay on fluorescent microbeads for simultaneous detection of 3 main allergens in food (hazelnut, peanut and walnut). The method includes a) DNA isolation from raw nuts (hazelnut, peanut and walnut) and home-made cookies containing the three analyzed nuts, b) triplex PCR for the simultaneous amplification of specific DNA sequences for hazelnut, peanut and walnut and c) multiplex hybridization assay on the surface of different sets of fluorescent microbeads and d) detection and identification of the amplified DNA sequences by flow cytometric analysis. The analysis, after genomic DNA isolation, is completed within 3.5–4 h. The microbeads used in this study have a diameter of 5.6 μm , are polystyrene and carry carboxyl groups on their surface. They also contain two spectrally distinct fluorescent dyes in different molar ratios producing up to 100 different sets. Thus, up to 100 different analytes can be detected simultaneously by this method. Each set of microbeads was covalently linked to 5'- NH_2 -oligonucleotide probe specific to hazelnut, peanut or walnut through carbodiimide chemistry. The PCR products were

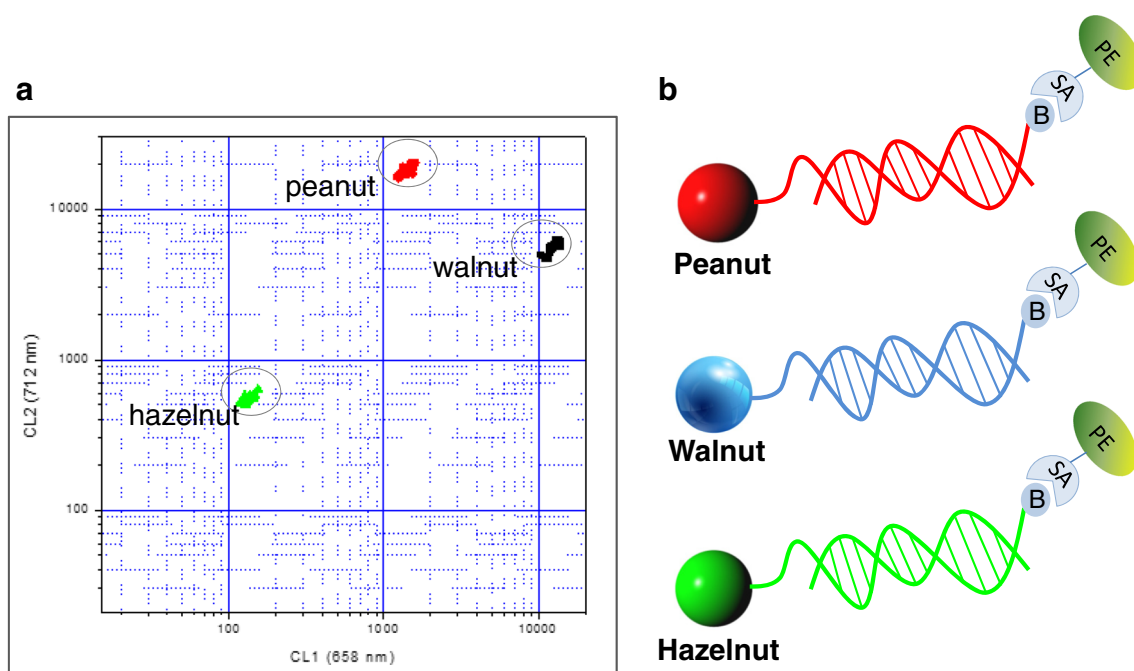


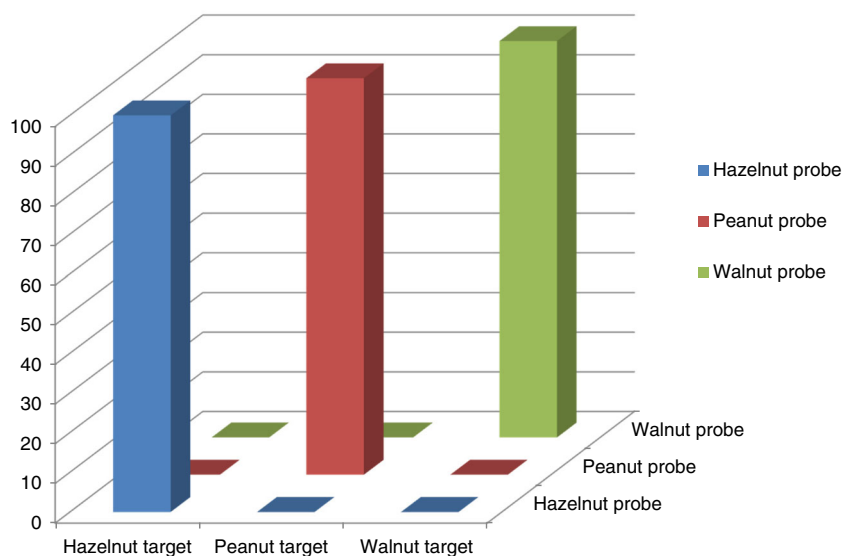
Fig. 1 **a** Classification of the three different sets of microbeads based on the ratio of the emitted fluorescence at 658 and 712 nm. **b** Schematic illustration of the microbead array. Biotinylated PCR products are

hybridized to specific probes attached onto the surface of the microbeads and detected by streptavidin-phycoerythrin. CL: channel, B: Biotin, SA: streptavidin, PE: phycoerythrin

biotinylated at the one end and had size of 85, 86 and 88 bp for hazelnut, peanut and walnut, respectively. In general, DNA as a target is more stable than proteins during thermal processing. However, because a small degree of DNA degradation may occur during food processing, the PCR primers are designed so that short DNA fragments can be amplified even if the DNA is partially degraded. The biotinylated PCR products, specific to the three nuts, were heat-denatured and let to hybridize to their complementary probes on the microbead surface. The hybrids were then detected by streptavidin-

phycoerythrin conjugate and flow cytometric analysis (Fig. 1). The microbeads are analyzed in a row, one by one, passing in front of two laser beams. A laser beam at 635 nm is used for the classification of microbeads into different distinct sets. The classification is based on the ratio of the emitted fluorescence of the two dyes of each microsphere at 658 and 712 nm (Fig. 1). A second laser beam (532 nm) is used for the excitation of phycoerythrin. This molecule gives an emission peak at 578 nm and the intensity of the emitted fluorescence is proportional to the concentration of the DNA sequence

Fig. 2 Specificity of the array. Each PCR product of three specific DNA sequences for peanut, hazelnut and walnut is analyzed separately with the microbead array



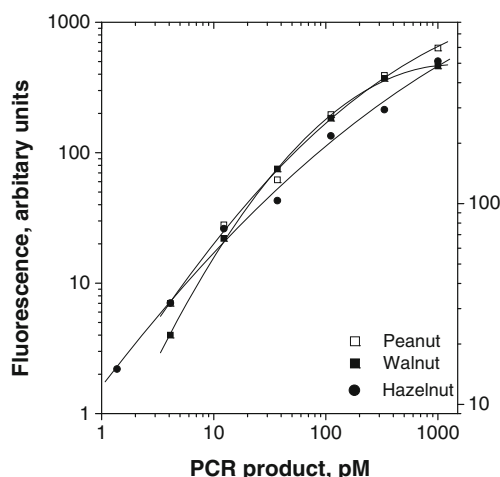


Fig. 3 Calibration graphs of the three allergens: peanut, hazelnut and walnut. A mixture of the three DNA sequences specific to the three nuts are analyzed simultaneously in different concentrations (1.4–1000 pM) with the microbead array. The emitted fluorescence is plotted versus the concentration of the PCR product

hybridized on the surface of the bead. At least 50 beads of each set were analyzed during each run. The median value of the measured fluorescence is then plotted against the concentration of amplified DNA sequence for calibration curve construction. The method was applied successfully for the simultaneous detection of 3 allergens (nuts) in raw and processed food (cookies).

The specificity of the hybridization assay on the bead array was firstly studied. For this aim, each PCR product at the high concentration of 1000 pM was subjected separately to hybridization assay with a mixture containing 1000 beads of each of the three different sets. Each set was coupled to a specific probe to hazelnut, peanut or walnut. The optimum number of beads (1000 beads) used in the assay was also determined. After analysis of the beads by the flow cytometer, the normalized intensity of the measured fluorescence for all three DNA targets is calculated and presented in Fig. 2. We observed that

Table 3 Reproducibility of the method

Proportion of nuts in cookies	% Coefficients of Variation (CVs)		
	Microbeads/ Hazelnut probe	Microbeads/ Peanut probe	Microbeads/ Walnut probe
0.02%	7.8	10.0	0.9
5%	5.4	5.2	3.5

all three oligonucleotide probes were strictly specific to their corresponding DNA targets.

The method was then optimized for the detection of each DNA sequence specific to hazelnut, peanut and walnut. DNA was isolated from the three raw nuts. Each DNA was subjected separately to single PCR to amplify a nut-specific DNA sequence. After amplification, the three different PCR products were mixed at different concentrations (1.4–1000 pM) and subjected to multiplex hybridization assay, after heat-denaturation. The hybridization reaction consisted of a mixture of the three different sets of fluorescent microbeads coupled to the specific probes. The microbeads, after incubation, were analyzed by flow cytometry. Streptavidin-phycoerythrin was used for the detection of the formed DNA hybrids. The calibration curves for the three nuts are presented in Fig. 3. As low as 1.4 pM of hazelnut-specific PCR product and 4.1 pM of PCR products specific to peanut and walnut were detected by the method.

The method was then applied for the simultaneous detection of the three nuts present in low amounts in processed food. Home-made cookies were prepared in the lab containing different proportions (0–5% w/w) of the three nuts. DNA was then isolated. A “negative” dough that contained no nuts (0%) was also prepared and included into the study. The isolated DNA was subjected to triplex PCR and analyzed with the microbead array. The results are shown in Fig. 4. As low as

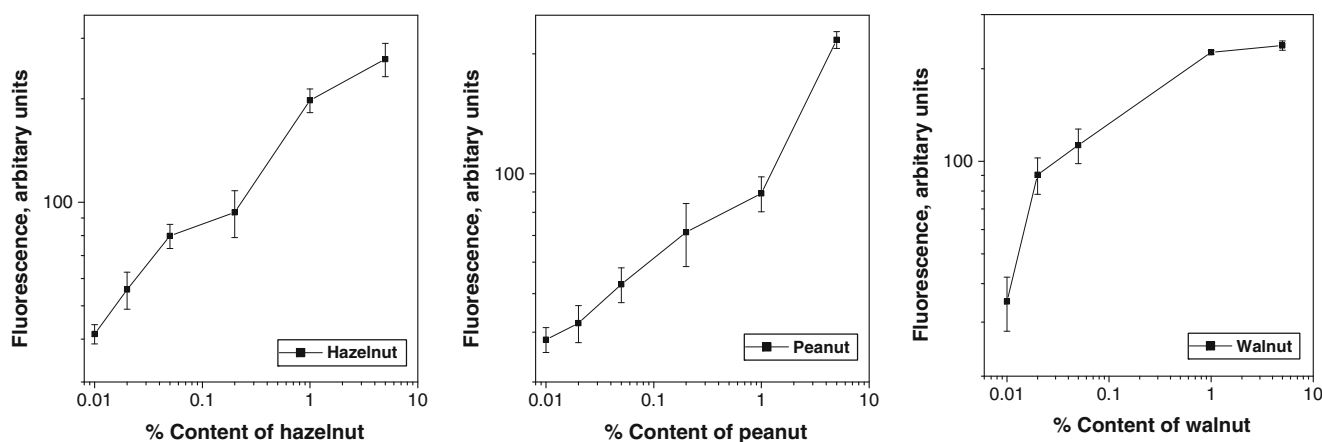


Fig. 4 Simultaneous detection of the three allergens (nuts) in home-made cookies prepared with different nuts' proportions (0–5% w/w) based on the combination of multiplex PCR and microbead-based array. The

emitted fluorescence is plotted versus the %content of nuts in cookies. Error bars are also provided in the calibration graphs ($n = 3$)

Table 4 Statistical data: the number of analysed beads within a run, the mean fluorescence value and the intra-assay reproducibility (%CV) of the multiplex hybridization assay

Proportion of nuts in cookies	Microspheres/hazelnut probe			Microspheres/peanut probe			Microspheres/walnut probe		
	N	Mean	%CV	N	Mean	%CV	N	Mean	%CV
0.02%	83	60.5 ± 6.8	11.3	54	20.8 ± 2.0	9.8	52	76.95 ± 5.4	7.0
	101	51.5 ± 5.6	10.9	53	25.4 ± 3.8	15.2	58	108.2 ± 10.8	10.6
	65	43.0 ± 3.3	7.7	52	22.2 ± 4.3	19.4	57	95.7 ± 10.6	11.1
5%	95	225.8 ± 19.8	8.8	46	259.2 ± 31.6	12.2	58	169.9 ± 17.6	10.4
	128	229.2 ± 18.8	8.2	55	249.0 ± 31.9	12.8	52	159.5 ± 19.9	12.5
	136	254.9 ± 19.4	7.6	53	222.1 ± 33.8	15.3	59	188.0 ± 23.7	12.6

N: number of microspheres measured at each run

0.01% proportion of each of the three nuts in cookies was detected by the method.

Finally, the reproducibility of the multi-analyte microbead-based array was assessed by analyzing, in triplicates, the PCR products from the multiplex amplification reaction corresponding to a low and a high content (0.02% and 5%) of the three nuts in cookies. The calculated % Coefficients of Variation (CVs) are presented in Table 3. All CVs were ≤10% indicating the excellent reproducibility of the bead array. A minimum of 50 microbeads of each set was analyzed during each run. A statistical analysis of a flow cytometric run is presented in Table 4.

Conclusions

This work describes a microbead-based DNA array for the simultaneous detection and identification of three main food allergens (hazelnut, peanut and walnut) in raw nuts and processed food (home-made cookies). The array was constructed by different sets of fluorescently encoded microbeads that were distinguished by flow cytometric analysis. The method was based on a) triplex PCR for the simultaneous amplification of DNA sequences specific to hazelnut, peanut and walnut and b) a simultaneous identification of the three nuts by a multiplex hybridization assay on spectrally distinct microbeads. The detection limit of the method was 1.4 pM of PCR product corresponding to hazelnut and 4.1 pM of peanut- and walnut-specific PCR product with good reproducibility (≤10%). Regarding food analysis, as little as 0.01% of the three nuts in home-made cookies can be determined. This is better than protein- and DNA-based methods that have been previously reported.

Conclusively, microbead-supported technology is a useful analytical tool offering high multiplexing ability, high-throughput (sample) analysis, great detectability, sensitivity, reproducibility and robustness. As there are

available 100 spectrally different sets of microbeads, the technology can provide simultaneous detection, identification and quantification of up to 100 different parameters/analytes in a single tube [7, 18]. Finally, this method can combine simultaneous protein and DNA analysis, developing an innovative and versatile multiparametric assay for a thorough study of food allergens.

Compliance with ethical standards The author(s) declare that they have no competing interests.

References

- Alves RC, Barroso MF, González-García MB et al (2016) New trends in food allergens detection: towards biosensing strategies. *Crit Rev Food Sci Nutr* 56:2304–2319
- Prado M, Ortea I, Vial S et al (2016) Advanced DNA- and protein-based methods for the detection and investigation of food allergens. *Crit Rev Food Sci Nutr* 56:2511–2542
- Costa J, Mafra I, Carrapatoso I, Oliveira MBPP (2016) Hazelnut allergens: molecular characterization, detection, and clinical relevance. *Crit Rev Food Sci Nutr* 56:2579–2605
- Directive 2000/13/EC regarding certain food ingredients. 68/EC of 27 November amending Annex IIIa. Off. J Eur Union 2007, L3, 11–14
- Poms RE, Klein CL, Ankam E (2004) Methods for allergen analysis in food: a review. *Food Addit Contam* 21(1):1–31
- Rödiger S, Liebsch C, Schmidt C et al (2014) Nucleic acid detection based on the use of microbeads: a review. *Microchim Acta* 181: 1151–1168
- Cho CY, Nowatzke W, Oliver K, Garber EAE (2015) Multiplex detection of food allergens and gluten. *Anal Bioanal Chem* 407: 4195–4206
- Rossi S, Scaravelli E, Germini A et al (2006) A PNA-array platform for the detection of hidden allergens in foodstuffs. *Eur Food Res Technol* 223:1–6
- Köppel R, Dvorak V, Zimmerli F et al (2010) Two tetraplex real-time PCR for the detection and quantification of DNA from eight allergens in food. *Eur Food Res Technol* 230:367–374
- Pafundo S, Gulli M, Marmioli N (2010) Multiplex real-time PCR using SYBR GreenER for the detection of DNA allergens in food. *Anal Bioanal Chem* 396:1831–1839
- Köppel R, van Velsen-Zimmerli F, Bucher T (2012) Two quantitative hexaplex real-time PCR systems for the detection and

- quantification of DNA from twelve allergens in food. *Eur Food Res Technol* 235:843–852
12. Engel K-H, Demmel A, Ehlert A, Hupfer C, Busch U (2009) Simultaneous detection of DNA from ten food allergens by ligation-dependent probe amplification. *Food Addit Contam* 26(4):409–418
 13. Mustorp SL, Dromtorp SM, Holck AL (2011) Multiplex, quantitative, ligation-dependent probe amplification for determination of allergens in food. *J Agric Food Chem* 59:5231–5239
 14. Cheng F, Wu J, Zhang J et al (2016) Development and inter-laboratory transfer of a decaplex polymerase chain reaction assay combined with capillary electrophoresis for the simultaneous detection of ten food allergens. *Food Chem* 199:799–808
 15. Wang W, Han J, Wu Y et al (2011) Simultaneous detection of eight food allergens using optical thin-film biosensor chips. *J Agric Food Chem* 59:6889–6894
 16. Wang W, Li Y, Zhao F et al (2011) Optical thin-film biochips for multiplex detection of eight allergens in food. *Food Res Int* 44:3229–3234
 17. Tortajada-Genaro LA, Santiago-Felipe S, Morais S et al (2012) Multiplex DNA detection of food allergens on a digital versatile disk. *J Agric Food Chem* 60:36–43
 18. Hildebrandt S (2010) Multiplexed identification of different fish species by detection of parvalbumin, a common fish allergen gene: a DNA application of multi-analyte profiling (xMAP) technology. *Anal Bioanal Chem* 397:1787–1796