



Multiplex ligation-dependent probe amplification (MLPA) for simultaneous detection of DNA from sunflower, poppy, flaxseed, sesame and soy allergenic ingredients in commercial food products

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ABSTRACT

This work describes a multiplex ligation-dependent probe amplification (MLPA) technique for the simultaneous detection of five food allergens: sunflower, poppy, flaxseed, sesame and soy. Species-specific MLPA half-probes were designed to detect the DNA from the targeted species. Ligated probes were amplified by polymerase chain reaction (PCR) and amplicons were detected using capillary electrophoresis. The specificity of the MLPA system was assessed against DNA from more than 50 plant and animal species. The limit of detection (LOD) of the assay was determined to be 10 mg kg⁻¹ in a model cookie experimentally spiked with different concentrations of the target species. The applicability of the MLPA was demonstrated through the analysis of 56 different commercial products (breads, pastries, cereals, chocolates, drinks, etc.), evidencing the presence of one or more undeclared allergenic ingredients in some of the tested samples. Real-time PCR assays were also performed to confirm and complement MLPA results. The MLPA technique has proved as a qualified screening tool for determining the presence of low amounts of sunflower, poppy, flaxseed, sesame and soy in processed foods.

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Increased attention to food safety has stimulated the interest of consumers and authorities towards authenticity and traceability of food products and their ingredients. In recent years, food allergies have constituted a major health concern in industrialized countries, and it is estimated that may affect up to 3% of the adult population and 6–8% of the children in Europe (Rona et al., 2007). The prevalence of allergies to certain plant species such as sunflower (*Helianthus annuus*), poppy (*Papaver rhoeas*), flaxseed (*Linum usitatissimum*), sesame (*Sesamum indicum*) and soy (*Glycine max*) has shown a positive tendency, associated with an increasing consumption of the seeds and oil of these species in many foods (bakery products, fast foods, vegetarian and ethnic dishes, etc.). Sunflower seeds provide an excellent source of health benefiting nutrients, but anaphylactic reactions ranging from mild to severe

symptoms have been described after their ingestion by sensitized individuals (Axelsson, Ihre, & Zetterstrom, 1994; Hernández, Esteve, & Pla, 2005). Similarly, poppy and flaxseed are highly nutritious and relevant sources of minerals, vitamins and antioxidants. However, allergic type 1 sensitivity to these seeds has also been reported (Gall, 2000; Keskin & Sekerel, 2006; Leon, Rodriguez, & Cuevas, 2002; Panasoff, 2008). Sesame allergy is also a rising problem as new products and recipes are being introduced from around the world on a regular basis. In the UK, a questionnaire-based survey suggested that sesame was responsible for severe reactions (Derby, Gowland, & Hourihane, 2005). Although systemic reactions occur, a single case of death owing to an allergic reaction to sesame has been reported (Pumphrey & Gowland, 2007). A number of soybean products and ingredients is extensively used by a large population worldwide, not only due to its nutrition and health benefits, but also to the introduction of new food trends such as vegetarianism and organic consumption. Nevertheless, soy is considered a major food allergen and the number of patients with allergy to soybeans has greatly increased in the last years (Bauer,

Abbreviations: MLPA, multiplex ligation-dependent probe amplification; PAC, positive amplification control.

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Kirschbaum, Panter, Kenk, & Bergemann, 2011; Tamarcaz, Hauser, & Eigenmann, 2001).

Since sunflower, poppy, flaxseed, sesame and soy are often contained in a wide variety of commercial food products, it is essential for those consumers which are at risk from suffering an allergic reaction to be able to obtain information about these allergens used as ingredients and/or as potential contaminants. In the absence of effective means for combating the allergic responses in sensitized consumers, avoiding the allergen containing food seems the only option. However, this may turn difficult in commercial processed products containing non declared allergens either added deliberately or unintentionally if foods are cross-contaminated during processing storage or shipping (Poms, Anklam, & Kuhn, 2004). In order to safeguard allergic consumers, the declaration of allergenic ingredients used in the preparation of a food results an imperative issue in many countries. According to European Union legislation, 14 allergenic foods and food ingredients, including sesame and soy (and other products thereof), have to be compulsorily given in the food ingredients label (Regulation 1169/2011/EC). Although sunflower, poppy and flaxseed have not been yet included in the list of allergenic ingredients that must be labeled, allergen policies are always in a dynamic state, meaning that new allergens appear and recognized allergens may gain or lose importance depending on consumer habits and new technologies. In this scenario, attention should be placed towards the future inclusion of these and other emerging plant species in the positive list of food ingredients or substances with known allergenic potential (EFSA, 2014). Considering these aspects, the development of suitable analytical methods able to monitor and identify the presence of allergens such as sunflower, poppy, flaxseed, sesame and soy in foods is of prime importance to prevent food-safety risks in sensitive individuals. The detection is, however, not an easy task since minute amounts of one or more allergens are usually present in complex and often highly processed food. Therefore, reliable methods with high specificity and sensitivity, and preferably with multiplexing capacity, are required (Baumgartner, Krska, & Welzig, 2007).

DNA-based methods have been increasingly used as valuable alternatives to immunoassays for allergen detection, taking advantage of the greater thermal stability of DNA molecules compared to proteins. In the last years, real-time PCR technology has proved to be a very effective tool for the detection of food allergens (De la Cruz et al., 2013; López-Calleja et al., 2013; Pafundo, Gulli, & Marmiroli, 2009). By combining several real time PCR amplifications in one assay (multiplex real-time PCR), simultaneous detection of two or more targets can be achieved (López-Calleja, De la Cruz, González, García, & Martín, 2015b, 2016, 2015a; Hashimoto, Makabe, Hasegawa, Sajiki, & Miyamoto, 2007; Hernández et al., 2005; Pafundo, Gulli, & Marmiroli, 2010). However, multiplex real time PCR may lead to different amplification efficiencies for each target and the assays are laborious to validate. Furthermore, the multiplex PCR format is not particularly flexible, as it is based on single primer sets which cannot be removed or added easily without influencing the whole assay. In this way, the assay known as Multiplex Ligation dependent Probe Amplification (MLPA), firstly described in 2002 by Schouten, McElgunn, Waaijer, Zwijnenburg, Diepvens and Pals to detect copy number variations in the human genome, is a high throughput and easy-to-learn technique for detection of multiple DNA sequences providing a flexible system that can be extended by adding further probes for a higher level of multiplexing. While MLPA technique has been mainly used for molecular diagnosis of human diseases (Gille et al., 2002), its application has been recently extended to the analysis of foods, including relative quantitation of GMO (Ehlert, Moreano, Busch, & Engel, 2008; Moreano, Ehlert, Busch, & Engel, 2006) or

simultaneous detection of DNA from allergenic food ingredients (Ehlert, Demmel, Hupfer, Busch, & Engel, 2009; Mustorp, Dromtorp, & Holck, 2011; Unterberger et al., 2014).

Considering all the previous aspects, this work describes the development and evaluation of a MLPA screening system for the simultaneous detection of DNA from sesame, flaxseed, poppy, sunflower and soy in a variety of marketed foods. These five plant species were chosen because they are known to elicit life threatening anaphylactic reactions and since an increasing number of retail foodstuffs may contain any of these seeds alone or in combination.

1. Materials and methods

1.1. Sample selection

Sunflower, poppy, flaxseed, sesame and soy, together with various commercial food products were purchased from different local stores and several delicatessen markets. Moreover, a wide range of plant (flesh and kernels) and animal (flesh) species was also included in the assays for specificity control purposes (Table 1).

1.2. Preparation of reference spiked cookies

In order to check the performance and sensitivity of the assay to detect the five targets simultaneously, and due to the lack of appropriate reference materials, model cookie samples were experimentally elaborated with different concentrations of the target species (0.1–10,000 mg kg⁻¹). First, a 100 g mixture containing 100,000 mg kg⁻¹ (10%) of each sunflower, poppy, flaxseed, sesame and soy (referred as “10% cookie sample”), was prepared as follows: 20 g of each target were ground with a Thermomixer (Vorwerk, Wuppertal, Germany). Additionally, a dough was prepared containing equal parts of wheat flour, sugar and butter. The whole target seed mixture (100 g) and 100 g of dough were mixed together using the Thermomix at maximum speed (9000 rpm) to favor a complete homogenization. This 10% cookie sample and sufficient amount of dough were then baked separately at 180 °C for 20 min and ground afterwards, before performing the rest of the model cookie spiking levels. For this purpose, 20 g of the corresponding cookie concentration was successively spiked in 180 g of dough and ground afterwards to ensure a thorough homogenization of each sample until the lowest spiking concentration of 0.1 mg kg⁻¹.

1.3. DNA extraction

Genomic DNA was digested and extracted using the Wizard DNA Clean-up System kit (Promega, Madison, WI, USA) as described by López-Calleja et al. (2007). The DNA was eluted in 50 µL of sterile deionized water. DNA concentration and quality was determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Montchanin, DE) and stored at –20 °C. Three DNA replicates were extracted from each sample and a negative control sample, which strictly contained the reagents used for the DNA extraction procedure, was included in every DNA extraction.

1.4. Oligonucleotide half-probes and primers

Alignment and computer analysis of *internal transcribed spacer* (ITS) sequences available in the NCBI (National Center for Biotechnology Information) database (<http://www.ncbi.nlm.nih.gov>) allowed the design of 5 sets of MLPA probes specific for sunflower, poppy, flaxseed, sesame and soy, according to the general suggestions of MRC-Holland (Amsterdam, The Netherlands) (<http://www>.

Table 1
Specificity of the Allergen-MLPA system.

Species	Allergen-MLPA					
	Sesame	Soybean	Sunflower	Poppy	Flaxseed	PAC (18S)
Sesame (<i>Sesamum indicum</i>)	+	–	–	–	–	+
Soybean (<i>Glycine max</i>)	–	+	–	–	–	+
Sunflower (<i>Helianthus annuus</i>)	–	–	+	–	–	+
Poppy (<i>Papaver rhoeas</i>)	–	–	–	+	–	+
Flaxseed (<i>Linum usitatissimum</i>)	–	–	–	–	+	+
Walnut (<i>Juglans regia</i>)	–	–	–	–	–	+
Macadamia (<i>Macadamia intergrifolia</i>)	–	–	–	–	–	+
Cashew nut (<i>Anacardium occidentale</i>)	–	–	–	–	–	+
Almond (<i>Prunus dulcis</i>)	–	–	–	–	–	+
Hazelnut (<i>Corylus avellana</i>)	–	–	–	–	–	+
Peanut (<i>Arachis hypogaea</i>)	–	–	–	–	–	+
Pistachio (<i>Pistacia vera</i>)	–	–	–	–	–	+
Brazil nut (<i>Bertholletia excelsa</i>)	–	–	–	–	–	+
Pecan (<i>Carya illinoensis</i>)	–	–	–	–	–	+
Green bean (<i>Phaseolus vulgaris</i>)	–	–	–	–	–	+
Green pea (<i>Pisum sativum</i>)	–	–	–	–	–	+
Chickpea (<i>Cicer arietinum</i>)	–	–	–	–	–	+
Lentil (<i>Lens culinaris</i>)	–	–	–	–	–	+
Tiger Nut (<i>Cyperus esculentum</i>)	–	–	–	–	–	+
Lupine (<i>Lupinus albus</i>)	–	–	–	–	–	+
Acorn (<i>Quercus ilex</i>)	–	–	–	–	–	+
Chestnut (<i>Aesculus hippocastanum</i>)	–	–	–	–	–	+
Pine nut (<i>Pinus pinea</i>)	–	–	–	–	–	+
Barley (<i>Hordeum vulgare</i>)	–	–	–	–	–	+
Oat (<i>Avena sativa</i>)	–	–	–	–	–	+
Rye (<i>Secale cereal</i>)	–	–	–	–	–	+
Rice (<i>Oryza sativa</i>)	–	–	–	–	–	+
Maize (<i>Zea mays</i>)	–	–	–	–	–	+
Wheat (<i>Triticum aestivum</i>)	–	–	–	–	–	+
Cocoa (<i>Theobroma cacao</i>)	–	–	–	–	–	+
Grape (<i>Vitis vinifera</i>)	–	–	–	–	–	+
Pear (<i>Pyrus rosaceae</i>)	–	–	–	–	–	+
Apple (<i>Malus domestica</i>)	–	–	–	–	–	+
Apricot (<i>Prunus armeniaca</i>)	–	–	–	–	–	+
Cherry (<i>Prunus avium</i>)	–	–	–	–	–	+
Sour cherry (<i>Prunus cerasus</i>)	–	–	–	–	–	+
Peach (<i>Prunus persica</i>)	–	–	–	–	–	+
Plum (<i>Prunus domestica</i>)	–	–	–	–	–	+
Banana (<i>Musa cavendishii</i>)	–	–	–	–	–	+
Kiwifruit (<i>Actinidia deliciosa</i>)	–	–	–	–	–	+
Watermelon (<i>Citrullus lanatus</i>)	–	–	–	–	–	+
Orange (<i>Citrus Sinensis</i>)	–	–	–	–	–	+
Potato (<i>Solanum tuberosum</i>)	–	–	–	–	–	+
Garlic (<i>Allium sativum</i>)	–	–	–	–	–	+
Onion (<i>Allium cepa</i>)	–	–	–	–	–	+
Eggplant (<i>Solanum melongena</i>)	–	–	–	–	–	+
Zucchini (<i>Cucurbita pepo</i>)	–	–	–	–	–	+
Tomatoes (<i>Solanum lycopersicum</i>)	–	–	–	–	–	+
Asparagus (<i>Asparagus officinalis</i>)	–	–	–	–	–	+
Carrot (<i>Daucus sativus</i>)	–	–	–	–	–	+
Olive (<i>Olea europaea</i>)	–	–	–	–	–	+
Cattle (<i>Bos taurus</i>)	–	–	–	–	–	+
Sheep (<i>Ovis aries</i>)	–	–	–	–	–	+
Goat (<i>Capra hircus</i>)	–	–	–	–	–	+
Swine (<i>Sus scrofa domestica</i>)	–	–	–	–	–	+

PAC: Positive amplification control for eukaryotics on the 18S rRNA gene.

mrc-holland.com/WebForms/WebFormMain.aspx). To avoid cross homologies, the specificities of the probe sequences were evaluated by BLAST search within NCBI GenBank by Beacon Designer software. An additional probe set was designed based on conserved nuclear 18S rRNA gene sequences from various plant and animal species and was used as a positive amplification control (PAC) of the MLPA reactions. The synthesis of the probes was performed by IDT (Integrated DNA Technologies, Iowa, USA) (<https://eu.idtdna.com/site>). Primers for the PCR were included in the MLPA reagents kit (MRC-Holland). Table 2 shows the sequences of the 6 MLPA probe sets and primers, together with the GenBank accession numbers of the selected targets and amplicons generated in the MLPA

reactions.

1.5. Multiplex ligation-dependent probe amplification

MLPA reactions were performed in a Progene Thermal Cycler (Techne Ltd., Cambridge, UK), using the SALSA MLPA EK1 reagent kit (MRC-Holland) according to the manufacturer's instructions. All components, except DNA mix, probe mix, water and TE buffer are included in the SALSA MLPA EK1 reagent kit.

Routinely, 5 µL of sample DNA (20–100 ng) was denaturated at 95 °C for 5 min. A non-template control was used along the experiment to discard possible contaminations. After the samples

Table 2
Half-probes and primers used in the MLPA method.

Target/GenBank accession nr	Half-probe name	LPO (5' → 3')	RPO (5' → 3')	Target sequences	Amplicon size (bp) calculated/Observed
Sesame/JN115050	SesLPO/SesRPO	GGGTTCCTAAGGGTTCGATCAGCACG TGGTGTGTAACGCT	CAACTCGCTGCTGCTGCTCTCTAGATTGGATCTTCTCTGGCAC	ITS	85 86
Flaxseed/FJ169526	FlaxLPO/FlaxRPO	GGGTTCCTAAGGGTTCGAGGGGCTTTTGG CTTAAGTGTCAAGTCTC	TGCCAACAATAACAAACCCCGGCA ATCTAGATTGG ATCTTCTGGCAC ACCGCTGAATTAAGCATATCAATAAGCGTCT	ITS	94 93
Poppy/X83836	PopLPO/PopRPO	GGGTTCCTAAGGGTTCGACACTCTCGACCCC AGGTCAAGCGGACT	AGATTGGATCTTCTGGCAC GTGGCTTCTTGTAAACTTAAACGACTCTCGTCTA	ITS	101 99
Sunflower/KF767534	SunLPO/SunRPO	GGGTTCCTAAGGGTTCGATTTGCGGCCCG TTCGGGTGTCGGCGTCTTC	GATTGCACTTCTGGCAC ATTCACTACCTCGGAGGGGATGACACGGCTC	ITS	108 107
Soy/FJ609734	SoyLPO/SoyRPO	GGGTTCCTAAGGGTTCGATTTGCGATGCTCACAATCAGA TTGACCCGCGAAGCTGTT	TAGATTGGATCTTCTGGCAC TAAGTTGTTGCACTTAAAGCTCTTATTTGGACTTTGGGATGGTTC	ITS	120 119
18S	18SLPO/18SRPO	GGGTTCCTAAGGGTTCGAGGTCGCCGA GCCGCGTAATTCAGCTCCAATAGCGTATATT	TAGATTGGATCTTCTGGCAC	18S rRNA	128 127
Primer R -unlabeled Primer F - labeled	5'- GTGCCAGCAAGATCCAATCTAGA - 3' FAM - 5'-GGGTTCCTAAGGGTTCGGA - 3'				

Common half-probe sequences (primer binding sites) are marked in bold capitals.

were cooled to 25 °C, 1.5 µl SALSA MLPA buffer and 1.5 µl probe mix were added and incubated at 60 °C for 16 h, to achieve hybridization. 5 fmol of each half-probe was used.

The ligation was performed by adding 3 µl ligase buffer A, 3 µl ligase buffer B, 25 H₂O and 1 µl Ligase-65 before incubating the mixture for 15 min at 54 °C and then for 5 min at 98 °C to inactivate the enzyme. The ligated probes are templates for the universal SALSA PCR primers mix. For subsequent PCR amplifications, 10 µl of polymerase mix (7.5 µl H₂O, 2 µl PCR Primer Mix and 0.5 µl SALSA polymerase) were added. The PCR amplification was performed under the following conditions: 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min, followed by 1 cycle of extension at 72 °C for 20 min. Finally, samples were cooled down to 4 °C and used for fragment separation by capillary electrophoresis. To assess MLPA reproducibility, two replicates of each DNA extract were analyzed in each experiment.

1.6. Capillary electrophoresis

For each sample, 1 µl of the PCR reaction, 9 µl of HiDi formamide and 0.5 µl of Genescan 500 LIZ size standard were mixed. The samples were denatured for 5 min at 95 °C and then cooled on ice. The plate was run on a 3730 DNA analyzer (Applied Biosystems) and data were collected during fragment separation. The injection time was 5–22 s, and the electrophoretic conditions were as follows: run time 1800 s at 15,000 V; run current, 5 µA; and 60 °C run temperature. GeneMapper 4.0 (Applied Biosystems) was used to extract data on the height, area, and size of fluorescent PCR fragments in the obtained electropherograms.

1.7. Real-time PCR confirmatory assays

Taqman real-time PCR assays previously developed by our group (Table 3) were performed to check the MLPA results. Duplex PCR reactions for sesame/flaxseed and sunflower/poppy, and simplex PCR reactions for soy were run under generic cycling conditions using a LightCycler 2.0 Instrument (Roche Applied Science, Penzberg, Germany).

2. Results and discussion

2.1. Choice of target sequences and design of the MLPA-based detection system

Sunflower, poppy, flaxseed, sesame and soy MLPA probes were designed upon published sequence data from the nuclear *ITS1* region of ribosomal DNA (rDNA). Plants possess three nuclear ribosomal genes (18S-5.8S-26S) which are repeated several hundred to a few thousand times in the genome, and are typically arranged within tandem arrays separated by different *internal transcribed spacer (ITS)* regions. It is known that DNA sequences of the rRNA coding genes are well-conserved between plants. However, the *ITS* sequences are species-specific, providing this region with a valuable potential for identification studies of closely related species (Hirao, Watanabe, Temmei, Hiramoto, & Kato, 2009; Pereira, Carneiro, & Amorim, 2008). Moreover, the use of a multi-copy gene favors the recovery of at least a few DNA copies when the targets are subjected to intense thermal treatments, such as those applied to commercially processed foods (López-Calleja, De la Cruz, González, García, & Martín, 2016). Considering these premises, specific ligation probes were designed by choosing specific *ITS1* regions on the vicinity or overlapping with previously described PCR primer or probe binding sites showing species-specific differences (López-Calleja

Table 3

Description of the primers and probes used in the Taqman real time PCR confirmatory assay.

Primers	Length (bp)	Sequence (5' → 3')	Description	Target gene/References	Fragment length (bp)
<i>SunflowerITSdir</i>	18	TGGCTTCTTTGTAACCTT	Sunflower-specific forward primer	ITS ^a	66
<i>SunflowerITSinv</i>	15	TTCTTCATCGATGCG	Sunflower-specific reverse primer		
<i>PoppyITSdir</i>	18	GGTCGGTCTAACACAGG	Poppy-specific forward primer	ITS ^a	72
<i>PoppyITSinv</i>	20	TGAAGAGAGACACGAGTGT	Poppy-specific reverse primer		
<i>SesameITSdir</i>	18	CCAGTGGTGGTTGAACGC	Sesame-specific forward primer	ITS ^a	84
<i>SesameITSinv</i>	19	CCTTTGGGTCCAACGTGAT	Sesame-specific reverse primer		
<i>FlaxseedITSdir</i>	18	AACCTGCTCGGCAGAAATG	Flaxseed-specific forward primer	ITS ^b	72
<i>FlaxseedITSinv</i>	20	CCTAGAAGCACACCCAGCTT	Flaxseed-specific reverse primer		
<i>SoyITSdir</i>	18	TCCCGCGGAGTCATCAC	Soy-specific forward primer	ITS ^c	89
<i>SoyITSinv</i>	22	CGTACGTTCTTCATCGATGCG	Soy-specific reverse primer		
<i>18Sdir</i>	16	TGGTGCCAGCAGCCGC	Positive control forward primer	18S rRNA ^{a, b}	77
<i>18Sinv</i>	25	TCCAACACTACGAGCTTTTAACTGCA	Positive control reverse primer		
Probes					
<i>SunflowerITSP</i>	24	FAM-AACGACTCTCGGCAACGGATATCT-BBQ	Sunflower probe	ITS ^a	
<i>PoppyITSP</i>	19	HEX-GTCACGATTCTGTGGTGGTC-BBQ	Poppy probe	ITS ^a	
<i>SesameITSP</i>	18	FAM-TCAACTCGCGTGTGTGCG-BBQ	Sesame probe	ITS ^b	
<i>FlaxseedITSP</i>	19	HEX-AACTGTCGTTAAATGCGGG-BBQ	Flaxseed probe	ITS ^b	
<i>SoyITSP</i>	22	FAM-TGACTCTCGGCAACGGATATCTCGGCT-BBQ	Soy probe	ITS ^c	
<i>18SP</i>	22	FAM-CGCTATTGGAGCTGGAATTACC-BBQ	Universal probe	18S rRNA ^{a, b}	

References

^a López-Calleja et al., 2016.^b López-Calleja et al., 2015b.^c Unpublished results.

et al., 2015b, 2016). A total of five MLPA probe sets specific for each sunflower, poppy, flaxseed, sesame and soy targets were designed according to the general suggestions of MRC-Holland (Schouten et al., 2002). Since PCR inhibition can result in negative test results, the inclusion of an additional set of conserved 18S rRNA probes (positive amplification) is useful to evidence the presence of amplifiable DNA in the samples.

The detection of each target sequence by MLPA requires the design of two adjacent half-probes, a left probe oligonucleotide (LPO) and a right probe oligonucleotide (RPO), containing their respective target-specific hybridization sites together with identical universal primer binding sites (PBS) at their 5'- or 3'- ends. The allergen-MLPA probes are chosen with a suitable number of nucleotides to give amplicons that would be easily separated and differentiated from each other. One of the primers is labeled with a fluorescence dye which is included in the amplification products during the PCR. When the adjacent half-probes bind to the target and are ligated, ligation products of specific size for specific target DNA are generated and can be detected by means of capillary gel electrophoresis. Details of the selected target sequences and MLPA primers and probes are listed in Table 2.

2.2. MLPA functionality and specificity

To check for possible cross homologies, the specificities of sunflower, poppy, flaxseed, sesame and soy MLPA probe sequences were first evaluated in silico by BLAST search within NCBI GenBank by Beacon Designer software. Preliminary experiments using single templates from known allergens in combination with single probe sets gave single peaks with the expected sizes and showed that all probe pairs amplified their specific targets (results not shown). The functional performance of the MLPA system was then examined by testing DNA extracts (adjusted to 20 ng/μL) of the five targets simultaneously with the six probe sets. Species specific peaks close to their calculated sizes (sunflower 108 nt/poppy 101 nt/flaxseed 94 nt/sesame 85 nt/soybean 120 nt/PAC 128 nt) were reproducibly generated, and showed that the five selected allergens could be detected concomitantly when present in a sample (Fig. 1). Differences between expected and observed amplicon size (peaks) are commonly reported (Ehlert et al., 2008) and pose no problem as the

fragment lengths determined for each target are reproducible.

The varied number of plant and animal components that can be present in commercial food products indicates the need to test the cross reactivity of the MLPA system against a wide range of species. Specificity of each MLPA probe set was therefore assessed by analysis of DNA extracted from 46 plant species (target species not included) and 4 animal species which constitute possible food ingredients. No unspecific peaks were obtained with any of the probes, meeting the desired MLPA specificity requirements. The positive amplification control (PAC) evidenced the presence of amplifiable DNA in all the analyzed samples (Table 1).

2.3. MLPA sensitivity

Since suitable reference material containing representatives of the target allergens was not available, the sensitivity of the MLPA method was assessed using a model cookie that was self elaborated by spiking a cookie matrix (dough) with different concentrations of the target species (0.1–10,000 mg kg⁻¹). The spiked cookie was used as reference of the hypothetical presence of any of the target allergens at different concentrations in a food, either due to deliberate incorporation or to unintended contamination. Starting materials and ingredients of the cookie dough were preliminary checked for the absence of any target species. Three independent DNA extractions of each spiking level were analyzed in two replicates per extraction using the MLPA. The limit of detection (LOD), expressed as the lowest spiking concentration for which all the results were consistently positive, was determined to be 10 mg kg⁻¹ (Fig. 2A).

Although the peak intensities varied somewhat depending on the allergen, especially with soy which showed the lowest signal, the overall assay sensitivity remained essentially unchanged when varying the conditions of the MLPA reactions (e.g. probe concentrations). Despite the MLPA technique developed in this work does not allow a quantitative estimate of the allergen concentration in a sample, the results obtained suggest that the assay has the potential to screen for the presence of low amounts of sunflower, poppy, flaxseed, sesame and soy in a complex matrix that resembles a commercial food product.

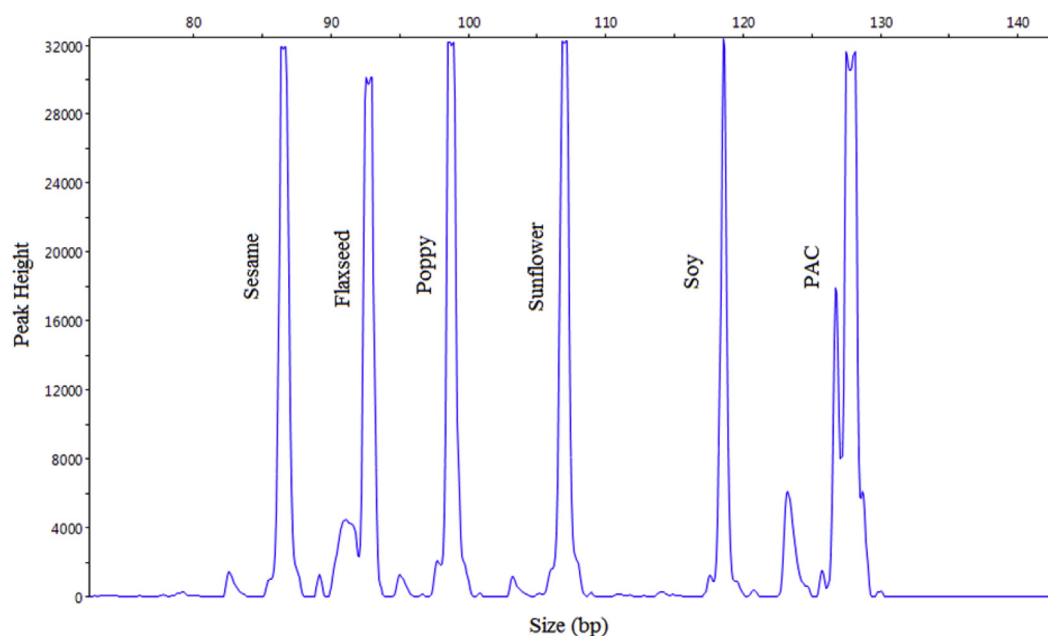


Fig. 1. Capillary electrophoresis of the MLPA system using a mixture of sunflower, poppy, flaxseed, sesame and soy DNAs (20 ng/ul) as template. All five targets plus the positive amplification control (PAC) are detected in one experiment.

2.4. Analysis of commercial food products

To evaluate the applicability of the system for routine testing, the MLPA technique was applied to the analysis of 56 commercial food products (breads, pastries, cereals, chocolates, drinks, etc.) of different brands, ascribed to three groups of label declarations: A) foods that declare to contain sunflower, poppy, flaxseed, sesame and/or soy; B) foods that declare “may contain” traces of sunflower, poppy, flaxseed, sesame and/or soy. These products may or may not state the presence of any of the targets in the labeling; C) foods that do not declare sunflower, poppy, flaxseed, sesame and soy, nor their traces in the labeling. Products are arranged by groups and label statements and results are shown in Table 4. As can be seen, the MLPA technique successfully detected the presence of the targeted species (one or more) in all the products that declared their presence in the labels. The electropherograms of two representatives of these samples are shown in Fig. 2 (products 4 and 9), to illustrate the ability of the assay to detect multiple targets in a complex commercial food matrix. However, 20 of the 56 (36%) food products analyzed (products 7, 10, 11, 12, 15, 16, 17, 23, 26, 29, 30, 32, 34, 36, 39, 40, 42, 43, 45 and 46) showed distinct discordances between the MLPA results and the labeling statements respecting the declared targets, revealing the presence of undeclared sunflower, poppy, flaxseed, sesame, and/or soy in such samples (Table 4). The presence of non declared allergenic ingredients in these commercial foods may be due to cross-contamination, since they were produced by companies that process different seeds and products in their facilities. This may happen when a production line cannot be completely cleaned after producing an allergen containing product, or due to cross-contact at some point of the raw material or ingredient supply chains (Mustorp et al., 2011; Spanjersberg, Knulst, Kruizinga, Van Duijn, & Houben, 2012). Moreover, the omission of an allergen on a food label may result from their use as additives in packaged food, the use of recycled materials, or substitution of a more valued ingredient by other of lower price when it is not possible to recognize the characteristics of the ingredients in processed products. Whichever the case, the degree of undeclared contamination found in the analyzed products may

compromise the health of allergic individuals and, therefore, detection methods should provide both, a selective detection and a sensitive threshold level of the allergen/s in question. In this way, the MLPA technique developed herein proved to be a useful screening tool for the specific and simultaneous detection of low amounts (LOD of 10 mg kg^{-1}) of sunflower, poppy, flaxseed, sesame and soy in a variety of commercial food products.

Because even small amounts of food allergens may cause a serious disease in sensitized individuals, the precautionary warning “may contain” is adopted as a voluntary health-protecting labeling tool among factories with cross-contamination opportunities in their manufacturing processes (Spanjersberg et al., 2012; Van Hengel, 2007). However, in line with other studies (Pegels, González, García, & Martín, 2015), results obtained in the present work suggest that the use of precautionary statements does not always comply with the true allergic ingredients of a product, leading to unnecessary restriction of consumer's choice (products 8, 9, 13, 14, 16, 20, 22, 23, 36 and 44).

To confirm and complement the results obtained by the MLPA technique, the commercial food products shown in Table 4 were further analyzed by two duplex (sesame/soy and sunflower/flaxseed) and a simplex (soy) Taqman real time PCR previously designed by us. The majority of the MLPA and real time PCR results were coincident, indicating an adequate correlation between both techniques. However, in some commercial food products, certain undeclared species did not yield a positive MLPA signal, but were positively detected by the real-time PCR technique (products 5, 6, 11, 17, 28 and 34). Discrepancies in these results may be justified by the lower sensitivity achieved in the MLPA technique developed (10 mg kg^{-1}), compared to that of 0.1 mg kg^{-1} reported for the real-time PCR assays (Table 3), suggesting the presence of minute amounts of the undeclared PCR-positive ingredients in these samples.

The detection of allergens in food can be challenging, as often various targets are present in trace amounts and are masked by the food matrix. Moreover, as the number of food components with allergenic potential increases, multiplex DNA-based methods are preferable for enforcing the legislation in relation to labeling.

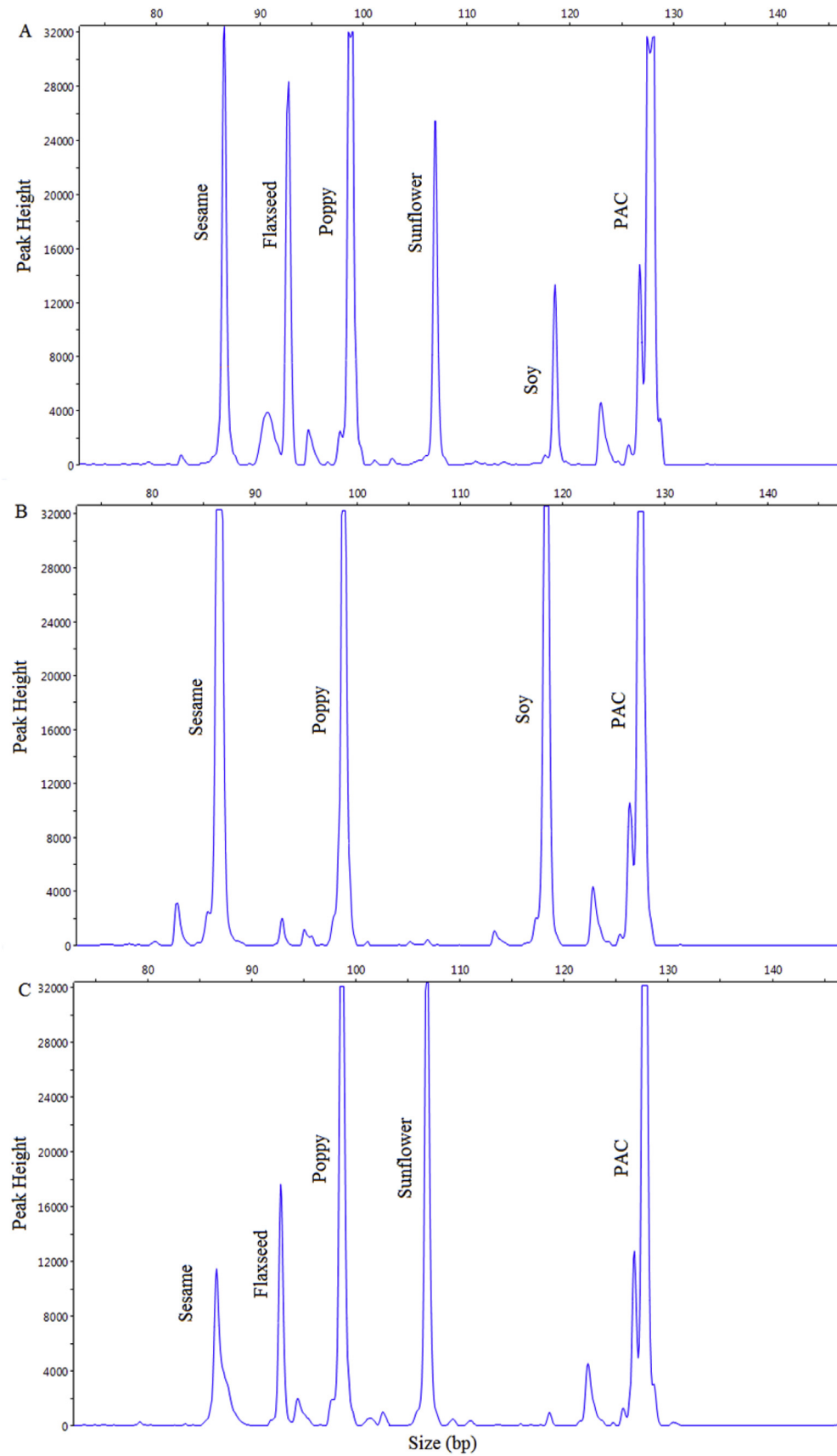


Fig. 2. (A): Capillary electrophoresis after allergen MLPA of a model cookie spiked with sunflower, poppy, flaxseed, sesame and soy at 10 mg kg^{-1} concentration. (B, C): MLPA analysis of commercial food products 4, 8 respectively (Table 3).

Table 4

Results after MLPA and real time PCR analysis of 56 commercial food products.

No/product	Target ingredients declared on the label	Precautionary labelling	Detection (MLPA)	Detection (real time PCR)
Bread				
1 Bread multicereal	Sunflower, flaxseed, sesame, soy	—	Sunflower, flaxseed, sesame, soy	Sunflower, flaxseed, sesame, soy
2 Bread roll	Sunflower, flaxseed, sesame	—	Sunflower, flaxseed, sesame	Sunflower, flaxseed, sesame
3 German bread	Sunflower, flaxseed, soy	—	Sunflower, flaxseed, soy	Sunflower flaxseed, soy
4 Rye sticks	Poppy, sesame, soy	—	Poppy, sesame, soy	Poppy, sesame, soy
5 NY style bagel	Sunflower, poppy	—	Sunflower, poppy	Sunflower, poppy, sesame, soy
6 Poppy bagel	Poppy	—	Poppy	Poppy, sesame, soy
7 Bread sticks	Sunflower	—	Sunflower, flaxseed, sesame, soy	Sunflower, flaxseed, sesame, soy
8 Swedish flat bread	Sunflower	May contain traces of sesame and soy	Sunflower	Sunflower
9 Bread 5 seeds and cereal	Sunflower, poppy, flaxseed, sesame	May contain traces of soy	Sunflower, poppy, flaxseed, sesame	Sunflower, poppy, flaxseed, sesame
10 Muesli bread	Soy	May contain traces of sesame	Soy, sunflower, sesame	Soy, sunflower, sesame
11 Pretzels	—	—	Sunflower	Sunflower, soy
Cereals				
12 Cereals with flaxseed	Flaxseed	—	Flaxseed, sunflower, soy	Flaxseed, sunflower, soy
13 Granola Apple and cinnamon	Sunflower, flaxseed	May contain traces of sesame	Sunflower, flaxseed	Sunflower, flaxseed
14 Treacle and pecan	Sunflower	May contain traces of sesame	Sunflower	Sunflower
15 Cruesli	Sunflower	May contain traces of nuts	Sunflower, sesame, soy	Sunflower, sesame, soy
16 Muesli 4 nuts	—	May contain traces of sesame and soy	Soy	Soy
17 Muesli chocolate amaranth	—	—	Sesame	Sesame, soy
Nut bars				
18 Muesli nut bar	Sesame, soy	—	Sesame soy	Sesame, soy
19 Soy nut bar	Soy	—	Soy	Soy
20 Himalayan goji	Sunflower	May contain traces of sesame and soy	Sunflower	Sunflower
21 Chocolate nut bar	Sunflower, soy	May contain traces of nuts and seeds	Sunflower, soy	Sunflower, soy
22 Brazilian nut	Sunflower	May contain traces of sesame and soy	Sunflower	Sunflower
23 Almond nut bar	Sunflower sesame	May contain traces of tree nuts and soy	Sunflower, sesame, flaxseed	Sunflower, sesame, flaxseed
24 Organic food bar Acai	—	—	—	—
Biscuits and cakes				
25 Chocolate and oat biscuit	Soy	—	Soy	Soy
26 Chocolate noisettes biscuit	Sunflower	—	Sunflower, soy	Sunflower, soy
27 Whole wheat biscuit	—	May contain traces of sesame	Sesame	Sesame
28 Spelt and ginger	—	May contain traces of sesame	Sesame	Sunflower, sesame
29 Rice cake	—	—	Sesame	Sesame
30 Chocolate macadamia	—	—	Sesame	Sesame
Chocolates and nougat				
31 Gourmet chocolate	Sunflower, soy	—	Sunflower, soy	Sunflower, soy
32 Peanut butter chocolate	Soy	—	Soy, sesame	Soy, sesame
33 Dark chocolate	Sunflower	May contain traces of other nuts	Sunflower	Sunflower
34 Almond bonbon	Soy	May contain traces of peanuts and nuts/cereals	Soy, sunflower, poppy	Soy, sunflower, poppy, sesame
35 70% cocoa	—	May contain traces of sesame	—	—
36 Peanut bonbon	—	May contain traces of other nuts	Sunflower	Sunflower
37 Walnut and cream nougat	—	—	—	—
38 Diet chocolate	—	—	—	—
39 Orange with dark chocolate	—	—	Sunflower, sesame	Sunflower, sesame
40 Nut chocolate	—	—	Sunflower, sesame	Sunflower, sesame
Beverages and yogurt				
41 Tiger milk	Sunflower	—	Sunflower	Sunflower
42 Vanilla soy	soy	—	soy, sesame	soy, sesame
43 Kamut and goyi milk	Sunflower	—	Sunflower, sesame, soy	Sunflower, sesame, soy
44 Yogurt with walnuts	—	—	—	—

Table 4 (continued)

No/product	Target ingredients declared on the label	Precautionary labelling	Detection (MLPA)	Detection (real time PCR)
45 Macadamia milk	—	May contain traces of soy	Sunflower, soy	Sunflower, soy
46 Muesli yogurt	—	May contain traces of soy	Sesame, soy	Sesame, soy
47 Rice milk	—	May contain traces of sesame	—	—
Spread creams and sauce	—	—	—	—
48 Chocolate cream	Soy	—	Soy	Soy
49 Cashew cream	—	May contain traces of sesame	Sesame	Sesame
50 Peanut cream	—	—	—	—
51 Pecan butter syrup	—	—	—	—
52 Tomato sauce	—	—	—	—
Ice creams	—	—	—	—
53 Praline ice cream	—	—	—	—
54 Chocolate fondant	—	—	—	—
55 Chocolate chip	—	—	—	—
56 Caramel and nuts	—	—	—	—

Recently, MLPA has proved to be a reliable approach for screening multiple food allergens, although these works are still scarce. Among them, Ehlert et al. (2009), and Mustorp et al. (2011) developed multiplex ligation-dependent probe amplification (MLPA) assays for the simultaneous detection of different allergenic plant ingredients in foods. The reported LOD varied from 5 to 1000 mg kg⁻¹ (Ehlert et al., 2009) and from 5 to 400 gene copies (Mustorp et al., 2011), depending on the targeted allergen. Also, Unterberger et al. (2014) described a LPA-based multiplex screening system for the detection of DNA from fish, shellfish and cephalopod species in food with LOD between 20 and 100 mg kg⁻¹.

Compared to other multiplex probe-based approaches such as Taqman real-time PCR, the MLPA technique has a number of advantages: a) the probes are less expensive as merely the 5'-end of the right probe needs a modification (phosphate); b) only minute amounts of probes are used when setting up a probe mix and therefore can be used for large sample number screenings; c) due to small interaction between the ligation MLPA probes, the method shows high reproducibility; d) possibilities for higher levels of multiplexing exist since it is a flexible system that can be complemented with further hybridization probes to broaden the range of targets or, by the contrary, by removing probe pairs when needed.

3. Conclusions

The MLPA system developed in this work has shown to be a specific and sensitive method suitable for the simultaneous detection of low levels of sunflower, poppy, flaxseed, sesame and soy in processed food products. The assay proved to be reliable, fast and easy to perform, and could be used as a complementary screening tool in routine programs to monitor the compliance with labelling rules regarding food allergens.

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