



Microchip capillary electrophoresis (Lab-on-chip[®]) improves detection of celery (*Apium graveolens* L.) and sesame (*Sesamum indicum* L.) in foods

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ABSTRACT

PCR is an usual analytical method applied to the detection of food allergens but sensitivity is a crucial problem in conventional post-PCR detection phase. The multiplex PCR approach often leads to adjunctive loss of sensitivity. The main goal of our study was to improve sensitivity in order to simultaneously detect sesame and celery in foods by mean of an end-point PCR protocol, by replacing conventional agarose gel electrophoresis with a Lab-on-chip[®] platform (microchip-based capillary electrophoresis). The Lab-on-chip[®]-based detection allowed to obtain the highest sensitivity in singleplex end-point PCR, for celery and sesame-specific primer pairs, using wheat flour as diluting agent. Moreover, in order to simulate a real system, home-made meat balls and commercial soup were artificially spiked with different percentages of sesame/celery (5% cooked meat balls, w/w and 0.1% soups, w/w), and then analyzed. Limits of detection highlighted in this study using Lab-on-chip[®] capillary electrophoresis were significantly lower if compared to those obtained with classical agarose gel electrophoresis.

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1. Introduction

Food allergies represent an important health problem in industrialized countries. People who suffer from these pathologies can undergo several problems, such as digestive disorders, respiratory and circulatory symptoms, skin reactions and sometimes anaphylactic shock (Ortolani, Ispano, Scibilia, & Pastorello, 2001; Sampson, 1999; Wüthrich, 2000). The only effective treatment in food allergy is the avoidance of the offending food. However, because of the possible mislabelling or due to the cross-contamination (along all the production, the transformation and the storage process of the product) sensitized subjects can be exposed to allergenic proteins by eating foods that are supposed to be allergen-free (Poms, Klein, & Anklam, 2003). The European Commission published in November 2007 the Directive 2007/68/EC, amending Annex IIIa to Directive 2000/13/EC of the European Parliament and of the Council, with regard to certain food ingredients. This intended to ensure that all consumers were informed on the complete content of food-stuffs, and to enable consumers with allergies to identify any potential allergenic ingredients that may be present. Foods – or food ingredients – listed are to a large extent in accordance with the list of common allergenic foods adopted by the Codex Alimentarius Commission and the US Food and Drug Administration (FDA

2001). The present work is focused on the analytical determination of celery and sesame, nowadays recognized as “emerging allergens”.

Celery (*Apium graveolens*), which belongs to the *Apiaceae* family, is a frequent cause of pollen-related food allergy, particularly in European countries. The major allergen from celery, named Api g 1, is a 16 kDa homologous to the major birch-pollen allergen Bet v 1, which usually acts as the primary sensitizing agent (Breiteneder et al., 1995). Sesame (*Sesamum indicum*), which belongs to the *Pedaliaceae* family, is a plant coming from the tropical Africa where is widely cultivated for its seeds and oil (Kagi & Wüthrich, 1991), and used in several food preparations. In the last 20 years there has been a great increase of the consumption of sesame seeds in Europe and, in parallel, of the number of sensitized subjects (Wolff et al., 2003). Sesame seeds contain at least 10 allergenic proteins; among them the most important allergen is a 2S albumin (Pastorello et al., 2001).

Nowadays, several techniques are available for the detection of potential allergens in food products based on two principal approaches: (i) the direct search of the protein (allergen or other marker proteins) and (ii) the indirect method based on DNA detection (Arlorio, Cereti, Coisson, Travaglia, & Martelli, 2007; D'Andrea et al., 2009; Dovičovičová, Olexova, Pangallo, Siekel, & Kuchta, 2003; Koppelman et al., 2007; Poms et al., 2003). Methods operating on the DNA level are based on the amplification of a specific genomic DNA fragment by the polymerase chain reaction (PCR). DNA-based methods offer many advantages over protein-based

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methodologies, primarily that the target DNA is efficiently extracted under strong denaturing conditions and is less affected than the protein-based methods by the type of food matrix. Another advantage is its stability to different geographical and seasonal aspects, which may affect protein composition (Poms et al., 2003).

Currently several real-time PCR-based protocols have been developed for detecting both celery (Hupfer, Waiblinger, & Busch, 2007; Mustorp, Engdahl-Axelsson, Svensson, & Holck, 2008) and sesame (Brzezinski, 2007; Hupfer, Demmel, & Busch, 2009; Mustorp et al., 2008; Schoringhumer & Cichna-Markl, 2007) in foodstuffs. In 2009 a duplex real-time PCR protocol for the simultaneous detection of hazelnut and sesame in foodstuffs has been suggested (Schoringhumer, Redl, & Cichna-Markl, 2009).

Real-time PCR technique ensures a more accurate and sensitive measurement, but it requires more expensive laboratory equipments. For these reasons end-point PCR is still considered in many laboratories an “entry-level” low-expensive technique, largely used in screening analysis.

End-point multiplex polymerase chain reaction (m-PCR) provides the simultaneous amplification of multiple regions of DNA templates by adding several primer pairs to the same reaction mixture (Chamberlain, Gibbs, Rainer, Nguyen, & Casey, 1988). Extensive diagnostic and scientific investigations are often restricted by limited availability of material. Therefore, methods like multiplex PCR strategies are needed to save as much sample as possible. Furthermore, this method is less-time consuming and cheaper than singleplex end-point PCR but, unfortunately, the set up of such procedures poses several difficulties. Multiplex PCR assays must be run under the same PCR conditions, thus, primers used should have similar melting temperatures; moreover, complementarity between primer sequences should be avoided to prevent the formation of primer dimers and hairpins. Additionally, an extensive optimization is normally required to obtain a good balance between amplicons from the various loci. However, through stringent initial primer selection, m-PCR minimizes time and costs consumption (Wittwer, Herrmann, & Elenitoba-Johnson, 2001).

Sensitivity is a crucial issue of all end-point PCR protocols, when they are analyzed through conventional agarose electrophoresis. The multiplexed approach complicates this aspect, generally causing an adjunctive loss of sensitivity. The microchip-based capillary electrophoresis technology represents a recent advance for the analysis of complex DNA banding patterns, largely applied on the detection of PCR-RFLP products or microsatellites DNA fingerprints, where gel electrophoresis step is replaced by the automated Lab-on-chip[®] electrophoretic system (Fajardo et al., 2009; Panaro et al., 2000). This approach leads to the separation of nucleic acid amplicons by capillary electrophoresis in a microfluidic chip, automating both the detection and the on line assessment of the fingerprints. Several applications on food authentication (Gonzalez, Guillon, Mas, & Poblet, 2006; Hathaway, Brugger, Martynova, Aebi, & Muhlemann, 2007; Hierro, Gonzalez, Mas, & Guillon, 2006) and GMO detection (Birch, Archard, Parkes, & McDowell, 2001; Burns, Shanahan, Valdivia, & Harris, 2003) are reported in the literature; here for the first time Lab-on-chip[®] technique was applied to food allergens detection. The first objective of this study was to optimize and improve an end-point PCR protocol – both singleplex and multiplex, with a positive internal control of amplification, as previously described (Arlorio et al., 2003) – for the detection of sesame and celery in foods, by replacing the conventional gel electrophoretic step with a chip-based capillary electrophoretic system. A comparative analysis between two detection approaches, particularly focused on the evaluation of the sensitivity, was performed, considering three series of artificially spiked sam-

ples (wheat flour, minced meat balls and vegetables-based soup), specifically prepared for the study.

2. Materials and methods

2.1. Samples

In this study two samples of *S. indicum* L. (sesame, seeds) and *A. graveolens* L. (celery, leaves) were used as referring standards. Other plant/vegetables largely used as food ingredients (seeds or leaves) were used in order to check for primers specificity: *Brassica oleracea* L. var. *botrytis* (cauliflower), *B. oleracea* L. var. *Italica* (broccoli), *B. oleracea* L. var. *sabauda* (cabbage), *Allium ampeloprasum* L. (leek), *Brassica rapaceum* Mill. (turnip tops), *Raphanus sativus* L. (radish), *Eruca sativa* Mill. (rocket), *Cuminum cyminum* L. (cumin), *Foeniculum vulgare* Mill. (fennel), *Petroselinum crispum* (parsley), *Daucus carota* L. (carrot), *A. graveolens* var. *rapaceum* L. (celery-turnip), *Allium sativum* L. (garlic), *Ocimum basilicum* L. (basil), *Solanum tuberosum* L. (potato), *Mentha piperita* L. (mint), *Salvia officinalis* L. (sage), *Triticum aestivum* L. (wheat), *Bos taurus* L. (beef). All samples were purchased from commercial stores in Italy, accurately washed and cleaned before the genomic DNA extraction.

2.2. Preparation of foods (wheat flour, meat balls, vegetable soup) spiked with celery and sesame

The described method has been used both for celery and for sesame, to assess the limit of detection (LOD). Firstly, defatted lyophilized celery and sesame powders were used to create serial dilutions in commercial wheat flour. Five grams of commercial wheat flour were spiked with one gram of celery and/or sesame powder. Subsequently, in order to evaluate the sensitivity of the end-point PCR protocol suggested, different percentages of spiking (10%, 1%, 0.1%, 0.01%, 0.001% and 0.0001%, w/w) of the above described mixture were obtained by serial dilutions.

“Home-made” meat balls (simulating a solid food matrix) and soup (simulating liquid food matrix) were prepared in order to assess the real applicability and the sensitivity of the suggested protocol. Minced meat balls were spiked with celery and/or sesame lyophilized powders in three different concentrations (1%, 5% and 10%). Soups were spiked with 1% and 0.1% of lyophilized celery and/or sesame powders.

2.3. Genomic DNA extraction and clean-up

Before the genomic DNA extraction, all samples were frozen with liquid nitrogen and then manually ground using a mortar, avoiding cross-contamination among samples. Subsequently celery was directly lyophilized to obtain a fine powder, to improve both the homogenization step during the preparation of the spiked foods and the extraction. Sesame seeds (a lipid rich matrix) were previously defatted in Soxhlet apparatus, using dichloromethane as solvent. All the sieved powders were stored at –20 °C before the use. DNeasy Plant mini kit (Qiagen, Hilden, Germany) and QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) were used for the extraction of (1) celery, (2) sesame, (3) all plant samples used as negative controls and (4) wheat flour, soups and meat balls spiked with celery and/or sesame. Recovery after extraction and clean-up of the genomic DNA were compared. All extraction methods were used according to the manufacturers' protocols. QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) was finally selected to extract all samples, starting from about 200 mg of each one. Isolated genomic DNA was quantified by fluorometer (Qubit[™] instrument, Invitrogen, Milan, Italy).

Table 1Primers used in this study and their features (melting temperature (T_m), gene position and index, amplicon length).

Primer	5'–3' Sequence	T_m (°C)	Locus	Gene index	Amplicon (bp)
<i>Primers specific for Apium graveolens</i>					
AgMTD f	TGATCCCCCTTCTCTAGCACCT	60	AF067082	3283367	233
AgMTD r	CTGACTCATCACACCGTA	61			
<i>Primers specific for Sesamum indicum</i>					
Si2S f	GTAGCGTTGGCCAGCGCCACG	73.6	AF240005	13183174	339
Si2S r	AACCTGCTGTGATTGGCCCTCTGG	73.4			
<i>Primers for Internal Transcribed Spacer sequences (universal control)</i>					
ITS1	TCCGTAGGTGAACCTGCGG	62	AB298970	134048943	~700
ITS4	TCCTCCGCTTATTGATATGC	57	EF565863	146738022	

2.4. Primers design and features

“Primer Express™” software (Applied Biosystems) was used for the design of specific novel primer pairs. Ribosomal ITS primers were obtained from literature (Table 1).

ITS 1/ITS 4 universal primers used in this study as internal control in multiplex PCR protocols, as well as tests on the amplification suitability of genomic DNA, were previously described (White, Bruns, Lee, & Taylor, 1990). All primers were synthesized and purchased from Primm, Biotech customer service, Milan (Italy), and they were dissolved in ultra-pure water MilliQ (Millipore, Milan) to a final concentration of 100 μ M and stored at -20 °C before use.

2.5. PCR reagents and protocols

The ICycler gradient thermal-cycler (Bio-Rad) was used to define the optimal annealing temperature for each primer pair. Selected annealing temperatures (T_a) are listed in Table 2. All reactions (20 μ L) were performed in 200 μ L micro tubes containing ultra-pure water MilliQ (Millipore), 1X Polymerase reaction buffer (Qiagen), 200 μ M of each dNTP (Promega), optimized quantity of each primer, 1.5 mM of $MgCl_2$, 1 unit of Taq DNA Polymerase (Qiagen) and about 100 ng of genomic DNA. The thermal program was set as follows: initial denaturation: 94 °C, 4 min and 35 cycled repetitions of three steps; denaturation: 94 °C, 30 s, annealing: T_a 60 °C (for each primer set), 30 s and elongation: 72 °C, 40 s. The last step was a final extension at 72 °C for 7 min. This temperature program was used both for single and multiplex PCR assays.

The amplified fragments were analyzed on 2% LE agarose gel (Eppendorf, Milan), at 70 V for about 90 min, stained by Sybr Safe™ (Invitrogen, Karlsruhe, Germany) and visualized on UV light at 300 nm, (FluorS Multimager instrument, Bio-Rad, Milan).

2.6. Lab-on-a-chip® capillary micro-electrophoresis analysis

Agilent Bioanalyzer 2100 (Agilent Technologies Ltd., South Queensferry, UK) was used to separate multiplexed profiles using Agilent DNA 1000 reagents and chips (Agilent Technologies Ltd., South Queensferry, UK). All the Lab-on-chip® analyses were then compared with the classical agarose gel electrophoresis (2% agarose). Bioanalyzer 2100 is a successful microfluidic-based platform

Table 2

Primer concentration and annealing temperatures used in optimized PCR protocols.

PCR assay	Primer pairs used	Primer concentration (nM)
Singleplex PCR	AgMTD f–AgMTD r	500
	Si2S f–Si2S r	500
Duplex PCR	AgMTD f–r/ITS 1–4	500/250
	Si2S f–r/ITS 1–4	500/250
Multiplex PCR	Si2S f–r/AgMTD f–r/ITS 1–4	150/500/250

commercially available, based on the capillary micro-electrophoresis on a chip. Reagents and DNA 1000 LabChips were prepared following the manufacturers' instructions. Aliquots (9 μ L) of gel matrix (used to fill chip capillaries) were prepared as required. All reagents were stored at 4 °C, and allowed to reach room temperature for 1 h before use. PCR products (1 μ L) were loaded on the chip following manufacturers' instructions, then analyzed through Agilent Bioanalyzer 2100 (2100 Expert software). Outputs are provided within 30 min, delivering automated, high quality digital data.

3. Results

The extraction of genomic DNA was performed using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), a commercial pre-cast extraction kit, specific for complex matrices, which allows to reduce the presence of PCR inhibitors (polyphenols, lipids). The integrity of genomic DNA was then assessed using agarose gel electrophoresis (0.8%). As described in Section 2, specific primer pairs used in this work were designed on the target sequences selected using “Entrez” Database integrated system (National Centre for Biotechnology Information; database “Nucleotide”), performing a multiple alignment with the free on line software MultAlin (Cor-

Table 3

Specificity of each primer set in duplex PCR (with ITS in-tube positive control) evaluated using as negative controls different plant species genetically related to celery or sesame or other common food ingredients.

Species	Family	End-point PCR		
		ITS (internal control)	Celery (AgMTD f-r)	Sesame (Si2S f-r)
Celery	Apiaceae	+	+	–
Sesame	Pedaliaceae	+	–	+
Broccoli	Brassicaceae	+	–	–
Red crauto	Brassicaceae	+	–	–
Cauliflower	Brassicaceae	+	–	–
Cabbage	Brassicaceae	+	–	–
Turnip tops	Brassicaceae	+	–	–
Radish	Brassicaceae	+	–	–
Rocket	Brassicaceae	+	–	–
Cumin	Apiaceae	+	–	–
Fennel	Apiaceae	+	–	–
Celery-turnip	Apiaceae	+	–	–
Parsley	Apiaceae	+	–	–
Carrot	Apiaceae	+	–	–
Leek	Alliaceae	+	–	–
Garlic	Alliaceae	+	–	–
Basil	Lamiaceae	+	–	–
Mint	Lamiaceae	+	–	–
Sage	Lamiaceae	+	–	–
Wheat	Poaceae	+	–	–
Potato	Solanaceae	+	–	–
Beef	Bovidae	–	–	–

pet, 1988). 2S albumin genomic sequence (g.i.: 13183174) and the mannitol dehydrogenase gene (g.i.: 3283367) were selected as targets for sesame and celery, respectively. In multiplex PCR approach amplicons must not overlap each other, in order to ensure specificity and to allow the interpretation of the fingerprint both in agarose gel and in chip-assisted device. Primers designed on celery and sesame genomic DNA lead to a single PCR band of 233 and 339 bp, respectively. Most important parameters referred to all primer pairs used in this work are reported in Tables 1 and 2.

Firstly, we checked out primer pairs specificity using a singleplex end-point PCR; all of them resulted specific for their own matrix. Each single primer pair was also used in combination with Internal Transcribed Spacers (ITS) universal primer pair as internal positive control, setting up a duplex PCR protocol. The internal control allows to prevent the presence of false negatives, confirming the good quality of the genomic DNA extracted either from polyphenols-rich plant samples or from "model foods", such as meat balls and soups. In this case we assessed the specificity of each primer set using different negative controls (plant species genetically related to sesame or celery, as well as other common food ingredients), as showed in Table 3. Beef samples (used for the preparation of meat balls) resulted negative when tested with ITS primers. Once efficiency and specificity of each primer pair were assessed using conventional agarose gel electrophore-

sis as detection system, we kept on checking the limit of detection (LOD), in order to evaluate the sensitivity of our protocols for celery and sesame. We firstly set-up a sensitivity test analyzing total genomic DNA extracted from wheat flour spiked with different known amounts of celery and sesame (previously prepared as defatted powder). The detection of all PCR products was carried out using the Agilent Bioanalyzer 2100 system, which allows the most sensitive and objective detection of amplicons. A direct comparison between conventional agarose gel electrophoresis and Lab-on-chip detection was then performed (Figs. 1 and 2).

Concerning electropherograms, sesame specific amplicon (Si2S f-r) was detected down to 1 ppm, while celery's (Ag MTD f-r) specific product was detected down to 100 ppm.

Bioanalyzer electropherogram profile is probably the clearest and the most sensitive way to detect PCR amplicons. Even if it does not provide quantitative information, a functional sensitive detection is clearly achievable, as previously confirmed by works focused on the detection of GMO's (Birch et al., 2001; Burns et al., 2003). Lab-on a chip technology also allows to confirm the presence of a signal undetectable using the conventional agarose gel staining, as pictured in Figs. 1 and 2.

Once the specificity and the sensitivity of single primer pairs were confirmed, the work went on setting up the multiplexed

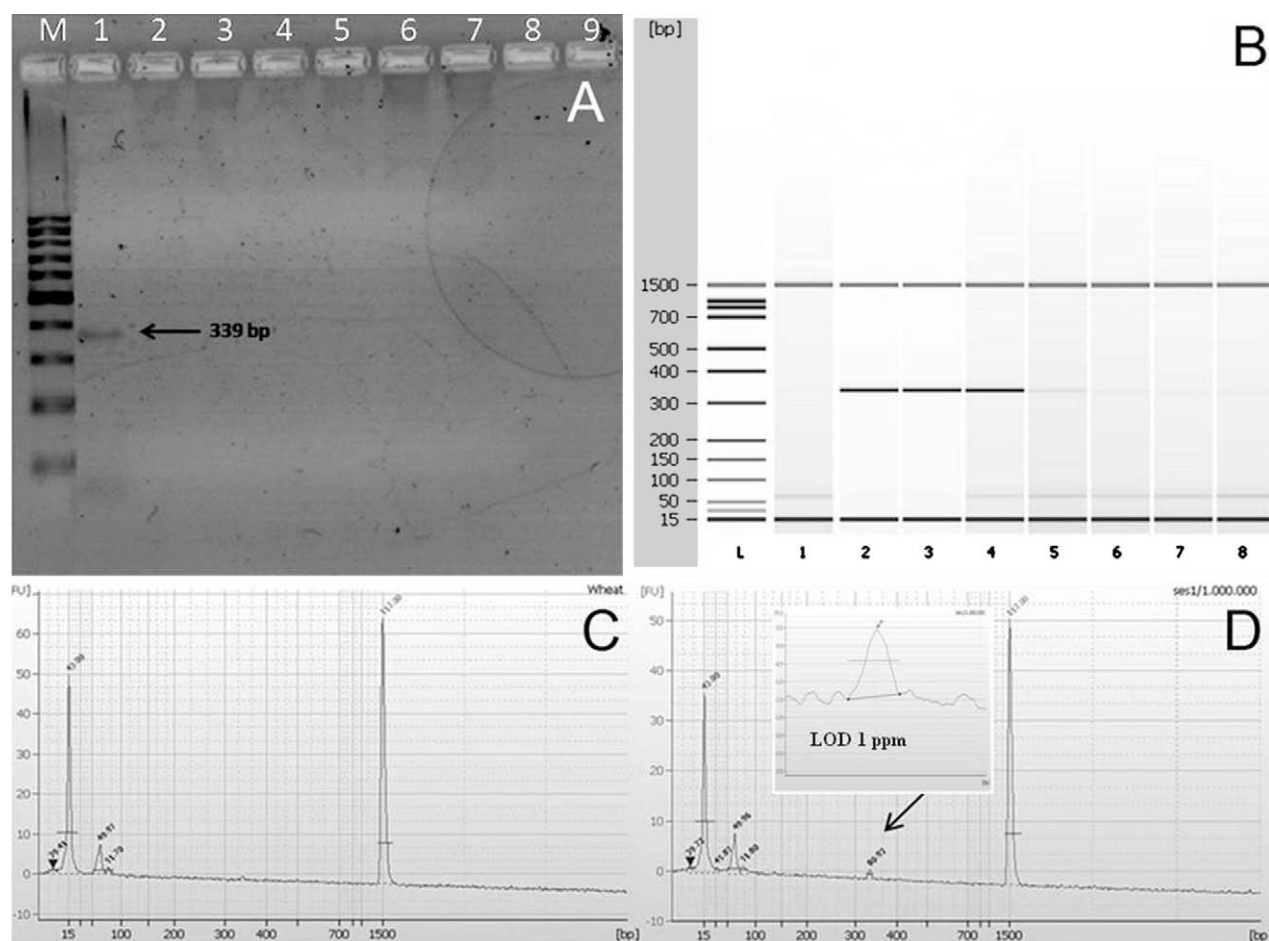


Fig. 1. Sensitivity assessed using conventional agarose gel (2%, **A**) and Agilent 2100 Bioanalyzer DNA 1000 chip (**B**, **C**, **D**). Samples: wheat flour spiked with different known amounts of defatted sesame, amplified with sesame-specific primer pairs Si2S f-r (339 bp). **A**: M: molecular marker (100 bp); 1: sesame; 2: wheat flour spiked with 10% of defatted sesame; 3: wheat flour spiked with 1% of defatted sesame; 4: wheat flour spiked with 0.1% of defatted sesame; 5: wheat flour spiked with 0.01% of defatted sesame; 6: wheat flour spiked with 0.001% of defatted sesame; 7: wheat flour spiked with 0.0001% of defatted sesame; 8: wheat flour; 9: no template control. **B**: Gel virtual image on 2100 Bioanalyzer. L: Ladder (molecular marker); 1: wheat flour; 2: sesame; 3: wheat flour spiked with 10% of defatted sesame; 4: wheat flour spiked with 1% of defatted sesame; 5: wheat flour spiked with 0.1% of defatted sesame; 6: wheat flour spiked with 0.01% of defatted sesame; 7: wheat flour spiked with 0.001% of defatted sesame; 8: wheat flour spiked with 0.0001% of defatted sesame. **C**: Electropherogram performed by the 2100 Bioanalyzer relative to wheat flour amplified (no spiked sample). **D**: Electropherogram performed by the 2100 Bioanalyzer relative to wheat flour spiked with 0.0001% of defatted sesame. Arrow shows the specific amplified DNA.

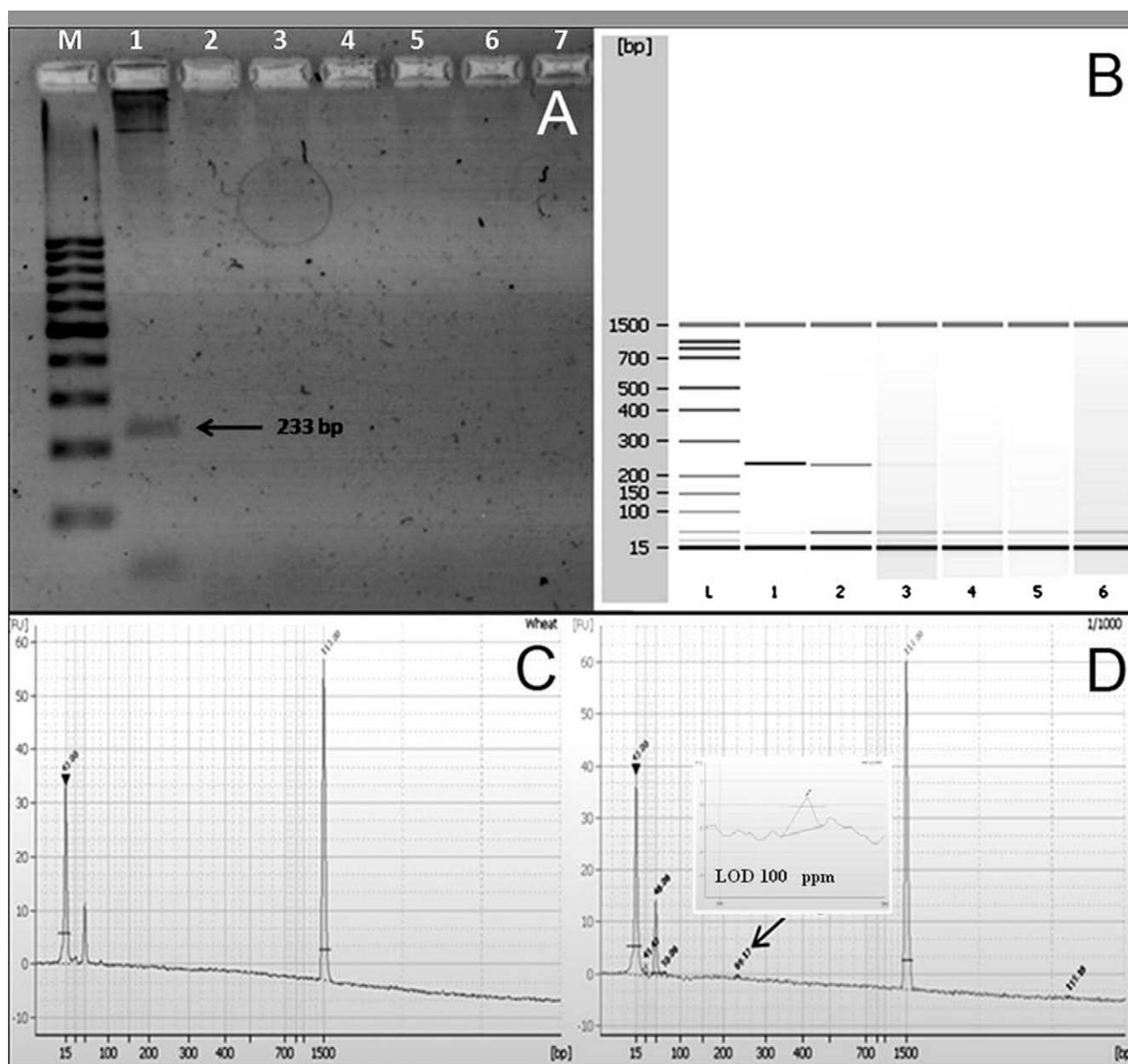


Fig. 2. Sensitivity assessed using conventional agarose gel (2% **A**) and Agilent 2100 Bioanalyzer DNA 1000 chip (**B, C, D**). Samples: wheat flour spiked with different known amount of celery, amplified with celery-specific primer pairs Ag MTD F-r (233 bp). **A:** M: molecular marker (100 bp); 1: celery; 2: wheat flour spiked with 10% of celery; 3: wheat flour spiked with 1% of celery; 4: wheat flour spiked with 0.1% of celery; 5: wheat flour spiked with 0.01% of celery; 6: wheat flour; 7: no template control. **B:** Gel virtual image on 2100 Bioanalyzer. L: ladder (molecular marker); 1: celery; 2: wheat flour spiked with 10% of celery; 3: wheat flour spiked with 1% of celery; 4: wheat flour spiked with 0.1% of celery; 5: wheat flour spiked with 0.01% of celery; 6: wheat flour. **C:** Electropherogram performed by the 2100 Bioanalyzer relative to wheat flour (no spiked sample). **D:** Electropherogram performed by the 2100 Bioanalyzer relative to wheat flour spiked with 0.01% of celery. Arrow shows the amplified specific DNA.

PCR protocols (not-linked PCR). To optimize the preparation of a successful reaction mixture, a great attention was paid to the primer balancing step, in order to obtain a similar amplification yield for each amplicon. The optimized output is showed in Fig. 3: the simultaneous detection of celery and sesame mixture (50/50 genomic DNA) is showed in lane 3. The presence of celery and sesame was confirmed in home-made meat balls, spiked at 5% (lanes 4 and 6) and 10% (lanes 5 and 7). ITS pair gave a very low amplification signal using sesame samples: this was probably due to the competition between ITS and sesame-specific primer pair. This competitive effect was highlighted particularly when the concentration of sesame genomic DNA was below 5%. Nevertheless, this fact does not affect the specificity of the amplification (see Fig. 4).

4. Discussion

The complexity related to a multiplexing PCR assay requires an accurate strategy, firstly based on primer design. In multiplex PCR, in order to clear up the electrophoretic recognition, it is extremely important to obtain amplicons with different lengths. Moreover, an optimized multiplex protocol requires primers working at the same reaction conditions (importantly the same annealing temperature) (Wittwer et al., 2001). All these requirements were considered in this work, particularly concerning the simultaneous use of an internal positive control of amplification, useful to exclude PCR false negative responses (Arlorio et al., 2003). Internal Transcribed Spacer (ITS) regions (ITS 1 and ITS 2) are characterized by high repetition degree (high yield of amplification) and a certain

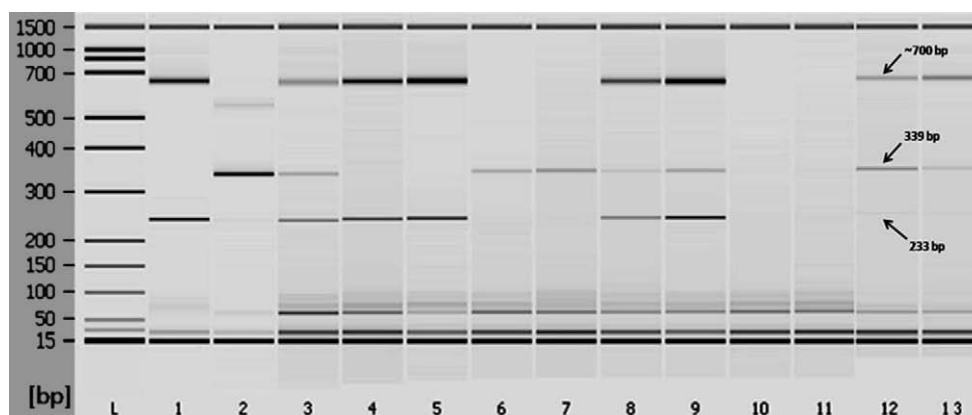


Fig. 3. Gel virtual image on 2100 Bioanalyzer of the triplex-PCR performed using celery-specific primer pair AgMTD f-r (233 bp), sesame-specific primer pair Si2S f-r (339 bp) and ITS primer pair (as internal control, ~700 bp). L: Ladder (molecular marker); 1: celery; 2: sesame; 3: celery-sesame DNA mixture, 1:1; 4: meat ball spiked with celery (5%); 5: meat ball spiked with celery (10%); 6: meat ball spiked with sesame (5%); 7: meat ball spiked with sesame (10%); 8: meat ball spiked with sesame-celery mixture (5%, 1:1); 9: meat ball spiked with sesame-celery mixture (10%, 1:1); 10: meat; 11: no template control; 12: soup spiked with sesame-celery mixture (1%, 1:1); 13: soup spiked with sesame-celery mixture (0.1%, 1:1).

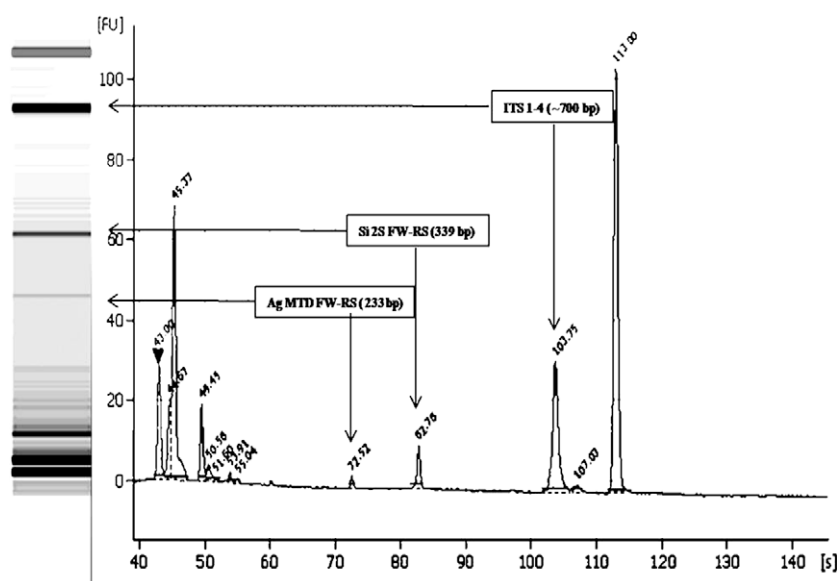


Fig. 4. Electropherogram performed by the 2100 Bioanalyzer relative to detection of triplex-PCR with in-tube positive ITS control (left: electrophoretic profile; right: electropherogram). Sample: soup spiked with celery-sesame mixture (0.1%). Arrows show the specific amplicons and the ITS internal positive control.

sequence polymorphism variability. They usually occur in the non-coding regions flanking the 5.8S rRNA gene, as well as in the intergenic loci between the large and the small highly conserved rRNA subunits, containing the annealing site of ITS related primers (White et al., 1990). Because ITS primers are designed on high conserved regions among plants and fungi, these primers are a useful tool to evaluate the quality of genomic DNA. The great size homology among ITS plant products (around 700 bp) allows to compare different species all together, although it does not exclude the presence of polymorphisms. The amplification of ITS genes has been previously applied to the identification of fungi at inter-specific level, i.e. in the truffle case (Lanfranco, Arlorio, Matteucci, & Bonfante, 1995). The co-amplification of the ITS sequences is a useful tool for excluding the presence of false negative amplification signals, which normally corresponds to poor quality DNA samples, not suitable for amplification (Arlorio et al., 2003; Berg, Tesoriero, & Hailstones, 2006).

The m-PCR was previously employed in food analysis to discriminate for the presence of ruminant, poultry, fish and pork meat in foodstuffs, but the use of the internal positive control was not considered by the Authors (Dalmaso et al., 2004).

Furthermore, Germini and colleagues used the multiplex PCR to simultaneously detect five different maize transgenes in food products which claimed to contain maize and/or soybean as an ingredient (Germini, Salati, Quartaroli, & Marchelli, 2005). The multiplexed approach is also developed in real-time PCR, as recently suggested for the simultaneous detection of allergenic ingredients. In this recent work, two protocols able to simultaneously detect peanut, hazelnut, celery, soy, and almond, sesame, eggs, milk were validated (Köppel et al., 2010).

The use of two different detection methods (conventional agarose gel electrophoresis and Lab-on-chip micro-electrophoresis) enabled us to compare different sensitivity degrees. In this work we intentionally avoided the analysis of commercial foods, because, to our understanding, only the analysis of spiked samples (model system) allows to know exactly the limit of detection (LOD). As a matter of facts, depending on food composition and on thermal processing, extraction of genomic DNA may be incomplete (Scaravelli, Brohè, Marchelli, & van Hengel, 2009), leading to underestimates due to an amplification reduction, with a consequent loss of sensitivity.

Successfully optimized multiplex PCR protocol is a complex process depending on several critical factors, most importantly primer selection step. All primer pairs selected enabled us to amplify all desired loci, without any unspecific product, achieving similar yields among respective amplicons. Additionally, the introduction of an internal control (universal primers ITS) provided for the exclusion of false negative amplification results. Our suggested method measures indirectly the presence of ingredients in traces in a very fast, cheap and simple way. This method can be applied in food industry to easily identify an unexpected ingredient present as a possible cross-contamination.

The employment of Agilent Bioanalyzer instrument allows to obtain highly sensitive results in terms of limits of detection. Comparing our protocol to the previously reported one by Dovičovičová et al. (2003), also performed in end-point PCR on celery, we achieved a reduction of LOD (100 ppm). Regarding sesame, the LOD that we reached (1 ppm) was even lower than those obtained via real-time PCR by Brzezinski (50 ppm) and Hupfer and colleagues (10 ppm), although our spiked samples did not undergo thermal processes before the DNA extraction (Brzezinski, 2007; Hupfer et al., 2007).

Concluding, this work describes two duplex protocols to identify celery and sesame as a single ingredient with an internal standard, as well as a well-working triplex PCR protocol. We suggest this multiplexed protocol followed by detection through Lab-on-chip technique as an efficient way for the simultaneous, time-saving, accurate and sensitive detection of celery and sesame, recently added in the list reported in EU Directive 2007/68/EC. The application of this protocol should be considered as a relatively low-cost improvement of the sensitivity of the end-point PCR platform, avoiding the use of real-time PCR.

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