

A multiplex, bead-based array for profiling plant-derived components in complex food matrixes

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Abstract Authentication of processed food ingredients is becoming an important issue for customers, and some DNA-based analytical methods have been developed, especially for animal products. As food products typically contain several different ingredients, a current challenge is to increase the multiplexing capacity of DNA-based methods, to develop “all-in-one” assays. Oligonucleotide-coupled, bead-based suspension arrays are sensitive and reproducible multiplex analytical tools. We applied the Multi-Analyte Profile (xMAP™) technology to develop an assay able to concurrently detect five different plant components in mixed flours and in processed feed and food. Capture probes were targeted to species-specific DNA polymorphisms present within the first intron of plant β -tubulin genes, which can be amplified by the tubulin-based polymorphism-amplification method (TBP-PCR). The workflow is very simple and straightforward, consisting of a PCR amplification step with universal primers, followed by the direct hybridization assay. Results are highly reproducible. For each single plant species, the absolute detection limit was as low as one target DNA copy. In complex mixtures of flours derived from seeds or from commercial dry “pasta,” relative limits of detection ranged, in weight, from 2 % for soybean to less than 0.5 % for wheat. The specificity of the capture probes and the high sensitivity of the method allowed the successful determination of the analytical composition of three feeds as well as eleven

food samples, such as snacks, biscuits, and pasta. The multiplexing ability of the assay (up to 100 different analytes) provides scalability and flexibility, in response to specific needs.

Keywords xMAP™ technology · Food traceability · Intron · TBP

Introduction

Food traceability is a major concern in a globalized market where the final products are assembled from raw materials coming from all over the world. Moreover, increasing attention is paid by the consumer to food quality and composition because of health concerns or to ethic and religious beliefs that strongly influence its choice. Food authenticity and composition thus need to be assessed through specific and sensitive analytical methods to be able to detect unintended contamination as well as fraudulent replacement of one raw material with another. The composition of complex food matrixes can be difficult to assess with standard chemical methods, mainly based on the detection of species-specific proteins or metabolites. DNA-based methods, mainly relying on PCR amplification, are becoming an important alternative because of several advantages like the high chemical and thermal stability of DNA, its ubiquitous presence among different tissues and cells [1] and its independency from environmental conditions. In some cases, such as GMO detection, DNA analysis is the only way to distinguish wild type from transgenics [2]. Furthermore, DNA polymorphism may allow deepening the analysis so to distinguish closely related species and even varieties. This is important for plant material where premium products from elite genotypes are protected by protected designation of origin (PDO) or protected geographical indication (PGI) denominations [3, 4]

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as well as for highly valuable products, like coffee species [5]. Analytical methods for the identification of specific allergens of animal or plant origin based on DNA detection are commercially available. They are mostly based on end-point (qualitative) or real-time (quantitative) PCR, which makes use of primers targeting species-specific genes. Different primers can be eventually combined in multiple assays [6, 7]. Assays based on real-time PCR can reach high sensitivity (1–100 ppm) but their multiplexing ability is limited to 5–6 by the number of fluorophores read by the instrument. The possibility of targeting plant DNA barcoding regions, like the *rbcl*, *trnL* or *matk* genes for species identification, has also been investigated [8]. These target sequences have the advantage of being amplifiable with universal primer pairs but the major drawbacks are limited resolution and need for sequencing [9]. Since sequencing approaches can't be applied to mixed samples, hybridization-based methods have been proposed. Multiplex detection methods, based on the microarray technology, allow the simultaneous identification of a high number of components in a single run with advantages such as time saving and a low probability of cross-contamination [4, 10–12], but are quite expensive, scarcely flexible and have a low throughput: one array is needed for each sample. The relative sensitivity reported for mixed samples spans from 0.1 to 5 %. Hybridization on a solid matrix also suffers from a high intra-spot variability and limited reproducibility which can be overcome only by complex statistical analysis and a high number of repetitions, conditions that are unacceptable for a routine diagnostic assay.

Conversely, arrays performed in a liquid format, in which the capture probe is coupled to suspended microspheres, may represent a valid alternative to conventional microarrays for diagnostic applications. In particular, the xMAP™ technology developed by Luminex utilizes a set of 100 commercially available color-coded fluorescent beads that are very rapidly identified by a specialized flow analyzer. Each set of beads can be covalently coupled to a different capture molecule, like an antibody or an oligonucleotide, and is then hybridized to the analytical sample. The fluorescence associated to a fixed number of beads for each sample is individually measured by a flow analyzer: in this way, each sample is split in thousands of replicated data points, providing a very high accuracy of measurement. Widely applied in the biomedical field, particularly as an alternative to ELISA immunoassay [13], xMAP™ technology is now increasingly used to develop multiplex assays for microbial identification and genotyping, gene expression profiling, as well as for the screening of genetic diseases [14]. Some applications of this technology have only very recently appeared in the field of food chemistry. Hildebrandt [15] developed a multiplex assay for the identification of eight edible fish species, while Li et al. [16] set up a sevenplex assay for oil characterization. Advantages of this technology include excellent precision

and sensitivity, handiness, very high throughput, high flexibility in array preparation and rapid data acquisition. Reading a 96-well plate for a 100-plex assay takes about 40 min. Moreover, the whole process can be automated, thus reducing the cost per analysis [17]. In brief, specific PCR target products, tagged with a biotinylated primer, are mixed with beads which have been coupled to complementary sequence-specific capture probes. Each bead-set (probe) hybridizes to its complementary PCR product, when present. Biotinylated amplicons are subsequently labeled with streptavidin-R-phycoerythrin. A red laser identifies the spectral region of the color-coded beads and a green laser registers the amount of labeled target captured by each probe [14].

In the current study, we present a direct DNA-based application of the xMAP™ technology that makes use of the tubulin-based polymorphism (TBP) method, to generate species-specific targets. The TBP method is a special multiplex PCR amplification reaction able to provide a fast fingerprinting of any plant genome by exploiting the intron length polymorphism occurring among the members of the plant β -tubulin gene family [18, 19]. In fact, in land plants, two introns lie at fixed positions within β -tubulin genes and are flanked by highly conserved exon region. This gives the opportunity to amplify any plant β -tubulin intron by a unique, universal primer set, annealing to the conserved exon regions. According to the variable size of the β -tubulin gene family, the number of targeted loci varies from 8 to as many as 20 per haploid genome, in different plant species. The assay we propose, based on the design of species-specific capture probes targeting β -tubulin introns, allows the simultaneous detection of five different plant species in complex feeds or commercial foods.

Materials and methods

Plant and food samples

Seed and feed samples were provided by the Regional Association of Lombardy Farmers (ARAL).

Food samples were from a local supermarket.

DNA extraction and TBP-PCR amplification

Samples were pulverized by grinding in stainless steel jars (Retsch GmbH, Germany) with one sphere (10 mm diameter) using the vibration mill TissueLyser (QIAGEN, USA) at 30 Hz for 1 min. Genomic DNA was then extracted using the GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, USA) for seeds and the NucleoSpin® Food kit (Macherey-Nagel, GmbH, Germany) for feed and food samples, following the manufacturer's instructions.

Concentration of the eluted DNA was determined spectrophotometrically by the A260 absorption (NanoDrop spectrophotometer, Thermo Scientific). DNA quality was also checked on agarose gel.

PCR amplification with the TBP method (TBP-PCR) of genomic DNA was performed according to the original method [19] in 30 μ l final volume with the exception that, for xMAP™ assays, the universal reverse primer (Rex) was 5'-biotinylated (Integrated DNA Technologies). No further purification was needed before carrying out the xMAP™ analysis.

The method that leads from TBP to the design of plant intron-specific diagnostic probes is under European pending patent protection PCT/IB2011/000146.

Sequence alignments for probe design were performed using the VectorNti software (Invitrogen, CA, USA).

Capture probe coupling

HPLC-purified oligonucleotide was synthesized with a 5'-end amino C12 modification (Integrated DNA Technologies) were coupled to MagPlex™ magnetic beads (Luminex Corporation TX, USA) following the established protocol for carbodiimide coupling. For each probe, 1.25×10^6 beads were centrifuged at $8,000 \times g$, 4 min, the supernatant was removed, and the beads were re-suspended in 50 μ l of 0.1 M 2-(N-morpholino) ethanesulfonic acid, pH 4.5 (MES-Sigma). The amino-modified probe (2 μ l, 100 μ M) was added to the beads and mixed by vortexing. To each coupling reaction, 2.5 μ l of a fresh 10 mg mL⁻¹ 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride solution (EDC-PIERCE) was added and the mix was incubated for 30 min at room temperature in the dark. After addition of fresh EDC solution, incubation was repeated. After the second round of EDC, the beads were centrifuged at $8,000 \times g$ for 4 min, washed once with 1 ml of 0.02 g l⁻¹ Tween 20 and once with 1 ml of 0.1 g l⁻¹ sodium dodecyl sulfate. The beads were resuspended in 300 μ l of TE 0.1 % BSA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0, 1 g l⁻¹ BSA, Sigma) and stored in the dark at 4 °C until used. The coupling was confirmed with biotinylated oligonucleotides, reverse complementary of the probes.

Hybridization assay

Following PCR amplification, 5 μ l (unless otherwise stated) of the reaction mix was added to 33 μ l of 1.5× TMAC hybridization solution (4.5 M tetramethylammonium chloride, 0.15 % sarkosyl solution, 75 mM Tris-HCl, and 6 mM EDTA, pH 8.0) with a mixture of coupled beads (approximately 2,500 beads) and additional 12 μ l of TE (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). The hybridization was performed by incubating tubes at 95 °C for 5 min followed by 15 min at the established hybridization temperature, on a thermal cycler. The beads were then washed twice with 100 μ l of 1× TMAC

detection buffer (3 M tetramethylammonium chloride, 0.1 % sarkosyl solution, and 50 mM Tris-HCl, pH 8.0 and 4 mM EDTA, pH 8.0) with the Biorad Pro II wash station and resuspended in 75 μ l of 1× streptavidin-R-phycoerythrin (Biorad, CA) diluted in 1× TMAC detection buffer. A second incubation was performed at the same temperature of the hybridization step for 5 min, followed by a second round of wash. The beads were resuspended in 125 μ l of 1× TMAC detection buffer and further incubated at the hybridization temperature for 5 min. Fifty microliters of beads were analyzed on the Bio-Plex 200 platform (Biorad, CA) at the hybridization temperature, using a high photomultiplier tube setting. Each sample was tested in duplicate.

Data analysis and cutoff definition

The Median Fluorescence Intensity (MFI) was calculated by the software of the instrument after counting at least 50 beads for each bead set in a sample, as recommended by the user manual. For background calculation, negative controls containing all the hybridization components except target DNA, were used in each experiment. Background MFI values were subtracted from each MFI value and duplicate data were mediated to determine the net MFI±SD. Unless otherwise stated, a cutoff value was defined, for each bead set, as the mean of the net MFI values of two negative PCR controls+3 SD, accordingly to common procedures adopted for diagnostic assays, i.e., ELISA [20].

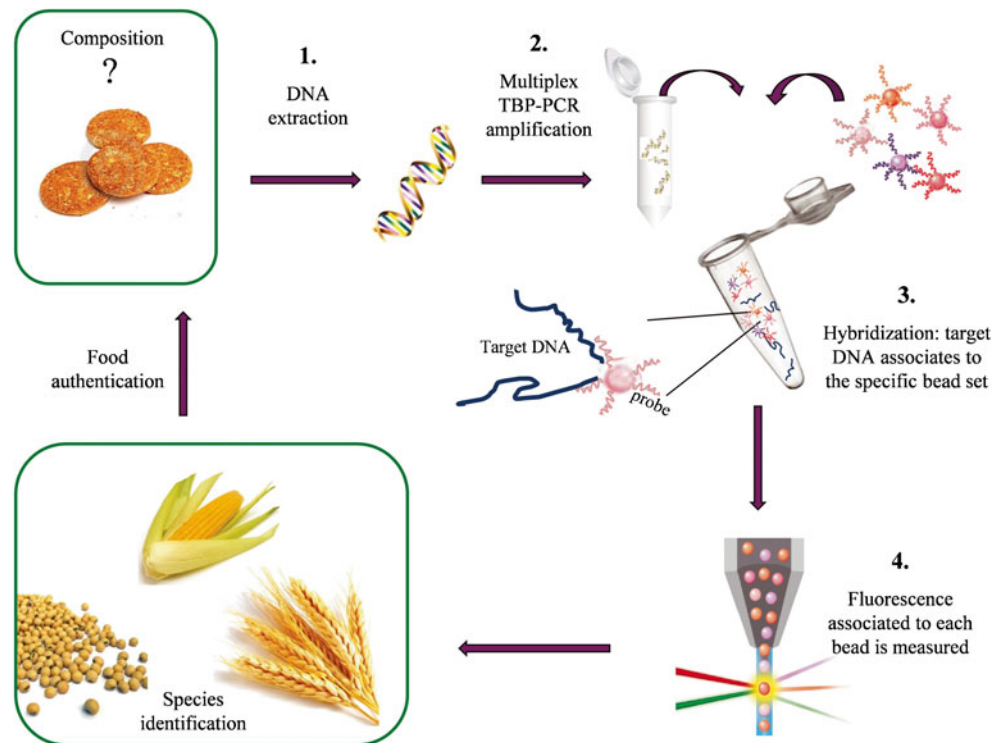
Results

Assay and probe design

The workflow of the proposed assay for food authentication consists of four steps: DNA extraction from the matrix, multiplex TBP-PCR amplification, liquid array hybridization, and fluorescence detection (Fig. 1). The rate-limiting step is DNA extraction, that can be speeded by means of an automated extraction system. A plate containing 48 duplicate DNA and control samples can be processed, from amplification to hybridization and reading, in about 4 h. *Triticum aestivum*, *Hordeum vulgare*, *Glycine max*, *Arachis hypogea*, and *Zea mays* are the five plant species that have been selected to test the feasibility of the multiplex assay. They are all widely used in the feed and food industry. β -tubulin introns of each of these plant species were first isolated, cloned and sequenced (Ponzoni et al., unpublished data) to design specific oligonucleotide capture probes that could selectively match with their complementary sequence if present among those amplified by the multiplex TBP-PCR reaction.

An example of The TBP fingerprinting profile obtained from each of the five plant species after agarose gel electrophoresis of

Fig. 1 Schematic representation of the four-step assay for the food composition analysis



the amplification products are shown in Fig. 2. Each band corresponds to the first intron of a β -tubulin gene. The amplification profile of a mixed DNA sample derived from the five plant species is also shown. The correspondence of each of the amplification products of the mix with one or more bands of the single plant species as well as the absence of any unrelated band was demonstrated elsewhere by electrophoresis on acrylamide sequencing gel followed by silver staining or by capillary electrophoresis [21, 22].

Despite their faster evolution rate with respect to coding sequences, genomic introns can still show a certain degree of sequence homology between orthologous genes of closely related species. For this reason barley, wheat, and maize capture probe sequences were designed on a multiple intron alignment of putatively orthologous genes, to identify the most polymorphic region. Conversely, the wheat probe was designed on an intron sequence shared between soft and durum wheat, to recognize both (Electronic supplementary material, Fig. S1). To verify their uniqueness, all probe sequences were also tested for sequence similarity against the whole NCBI genomic database (<http://blast.ncbi.nlm.nih.gov>) using the Megablast algorithm. Capture probe sequences and target features are listed in Table 1.

Capture probe specificity

After validation against its own complementary oligonucleotide (Electronic supplementary material, Table S1), each set of oligonucleotide-coupled beads was first hybridized at 42 °C

to 5 μ l of the TBP-PCR amplification reaction obtained from 20 ng of template DNA of the corresponding target species. To determine probe specificity, the products of each amplification were hybridized to the multiplexed set of beads at the increasing temperatures of 42, 45, 48, 52, and 56 °C. Figure 3, which reports the MFI values after background subtraction, summarizes the results of a representative experiment. Maximum fluorescence intensity signals, ranging from 5,000 MFI for wheat (Fig. 3b) to about 10,000 for soybean (Fig. 3d),

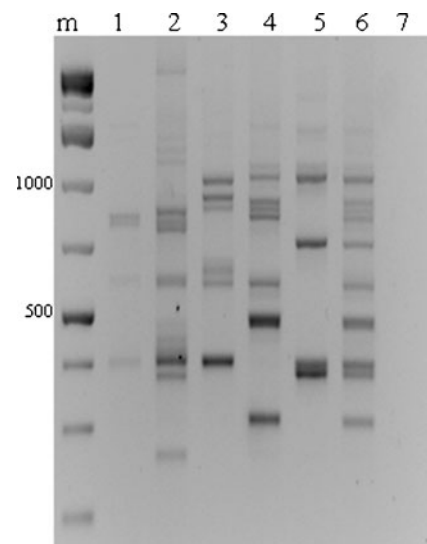


Fig. 2 TBP-PCR fingerprinting profiles of the five selected plant species. *m* molecular marker, *1* barley, *2* wheat, *3* maize, *4* soybean, *5* peanut, *6* mix of the five species, *7* no DNA

Table 1 Capture probe and target features

Target species	GenBank accession no. ^a	Capture probe sequence (5'–3')	Probe length (bp)	<i>T_m</i> (°C)	%GC	Target amplicon (bp)	Beads region
<i>Hordeum vulgare</i>	AM502849.1	GAAGAGGGCCTGCCACTTGTAC	22	56.6	59	753	8
<i>Triticum aestivum</i>	U76745.1	ATATGAAATGTATGCCATAATAGT	24	44.8	25	758	59
<i>Zea mays</i>	EU960070.1	TGATACTTATTGGTCCTTGCCG	22	45.2	39	899	68
<i>Glycine max</i>	XM_003546228.1	AGCTGTCATTCTCAACTAAAGT	23	46.6	34.8	488	70
<i>Arachis hypogea</i>	–	ACTCTGATATGCAACTGTGGTA	22	46.6	40.9	1,028	86

^a The GeneBank accession numbers refer to the β -tubulin cDNA sequences

were reached between 45 and 52 °C. Starting from 48 °C, hybridization of each TBP-PCR product to its complementary capture probe was specific. An unpredictable cross reaction was observed between the soybean capture probe and some intron sequences of wheat (Fig. 3d). This nonspecific signal disappeared only at 56 °C (91 MFI), a temperature at which MFI values for the wheat and the barley probes were halved with respect to 52 °C (2,560 vs. 5,973 MFI for barley and 2,677 vs. 5,249 MFI for wheat). To this specific regard, we believe that a compromise between specificity and sensitivity of the assay was best reached at a hybridization temperature of 52 °C. Specificity was also tested against other closely related plant species or species which may be present in feed or food samples such as: durum wheat (*Triticum durum*), emmer (*Triticum dicoccum*), oat (*Avena sativa*), rice (*Oryza sativa*), bean (*Phaseolus vulgaris*), flax (*Linum usitatissimum*), rapeseed (*Brassica napus*), sunflower (*Helianthus annuus*), and buckwheat (*Fagopyrum esculentum*). As expected, the wheat probe was not able to discriminate among the two species of the genus *Triticum*. No other cross-reaction was recorded (data not shown).

Limit of detection

To determine the minimal DNA amount detectable, the multiplex TBP-PCR amplification was performed on tenfold serial dilutions of starting gDNA, from 10 ng to 10 pg, for each of the analyzed species. Experiments were performed hybridizing, at 52 °C, the fiveplex capture probe set with 5 μ l of each PCR-amplified target sample. These data are reported in Table 2.

In these conditions, the absolute limit of detection (LOD) was estimated to be lower than the highest DNA dilution tested, 10 pg, for barley, maize and peanut, and between 10 and 100 pg for wheat, while soybean was detectable down to a limit of 1 pg (data not shown). The observed difference in the detection limit is in agreement with the genome size of selected plant species, considering that similar amounts of gDNA contain different numbers of target sequences. The minimum number of target copies detected in the multiplex assay was around ten and even lower in the case of barley,

where 10 pg of DNA correspond to approximately 5.3 target copies. For all five species, signal saturation was reached between 1 and 10 ng of template gDNA, likely due to PCR saturation, as confirmed by agarose gel analysis (Electronic supplementary material, Fig. S2). In fact, bead signal saturation with complementary oligonucleotides was reached at MFI values of more than 20,000 (Electronic supplementary material, Table S1).

To verify how much the presence of multiple components influence the detection limit of the assay, we tested serial dilutions of blends, made by mixing gDNAs of the five plant species (barley, wheat, maize, soybean, and peanut (BWMSP)), on a mass base. The total amount of DNA was kept the same as that used for individual gDNA samples (tenfold serial dilutions from 10 ng), so that the DNA mixtures contained 1/5 of the DNA amount for each species. The products of two parallel TBP-PCR reactions were pooled in a single analytical sample, to reduce sampling problems, due to the presence of very low target copy numbers at the highest dilution rates (close to or less than one copy).

Three independent sets of PCR reactions were performed on serial dilutions of the same gDNA mix and subjected in duplicate to the xMAP™ assay (Table 3). Despite the competition of many targets for the same primers in the multiplex TBP-PCR system, the sensitivity of the assay was comparable to that obtained in reactions run with gDNA of single species: each of the five target plant species was identified in a complex sample, even at high dilution rates, although with some reduction in the fluorescent signal.

The detection limits were more finely defined here, with lower DNA concentrations tested. Down to 2 pg amount for peanut, barley, maize, and soybean and 20 pg for wheat were detected in the mix. For each probe, the limit of detection was actually dictated by the target copy number in the template. In fact, in the case of barley, maize, and peanut, where 2 pg of DNA were expected to contain a number of target copies close to one, the assay resulted positive in two out of three cases.

When template DNA concentrations are low but above the LOD, the fluorescent signal can be increased by hybridizing a higher volume of the TBP-PCR sample. Dose–response curves with different DNA amounts, corresponding to 1, 2,

Fig. 3 Probe specificity at different temperatures in the multiplex assay. Assays were conducted on a mixed DNA sample of the five plant species. Net MFI values after background subtraction were reported for each bead set

5, and 10 μ l of the TBP-PCR reaction, show a steadily increase of the MFI values for all the capture probes (Electronic supplementary material, Fig. S3).

The precision of the method was evaluated by analyzing data of four hybridization replicates obtained from the same TBP-PCR reaction run with 20 ng gDNA for each of the five different plant species. The intra-assay coefficient of variation (CV) was equal to 2 % with a maximum fluctuation of 7 % (not shown).

Inter-assay variability was also calculated, from the mean MFI values of the three analytical experimental repetitions done on the mixed DNA samples shown in Table 3 (BWMSP mixed samples, 20 ng). In this case, the variability of the MFI values was mainly due to variations among PCR amplifications, but CV values of replicates remained low, with maximum values around 10 %, thus confirming a high level of repeatability of the method. Of course, this does not hold true for PCR samples obtained from the highest gDNA dilutions (10 pg), close to the detection limit, where a greater variability, likely due to sampling, was observed (Table 3).

A split assay format

As anticipated in the results of Fig. 3d, the soybean capture probe gave a weak cross-reaction against wheat (500–800 net MFI in saturating PCR conditions accounting for approx 1/10 of the specific signal), which could lead to a small overestimation of wheat and even to false positives in complex sample analyses. An increase in the hybridization temperature to 56 °C was shown to reduce this cross reactivity, but it also reduced the intensity of the signal (Fig. 3). To maximize both sensitivity and specificity, we checked the possibility to split the assay in a fourplex format (run at 52 °C) plus a singleplex hybridization of the soybean-specific beads performed at higher hybridization temperature. As the soybean probe was the one showing the highest signal and having the highest sensitivity in the fiveplex assay, no relevant reduction in sensitivity was expected.

The soybean capture probe was thus tested in singleplex at two hybridization temperatures, 56 and 58 °C, on TBP-PCR amplifications obtained from mixed genomic samples of two different dilutions of the four test species, added or not with soybean DNA (Electronic supplementary material, Table S2). Results were then compared with those obtained at 52 °C in the fiveplex format assay. While the intensity of the specific soybean signal was not influenced, the nonspecific hybridization signal due to cross-reactivity with wheat was reduced from 775 to 681 MFI at 56 °C and to 202 at 58 °C. Similarly, the nonspecific signal was reduced when testing the soybean probe

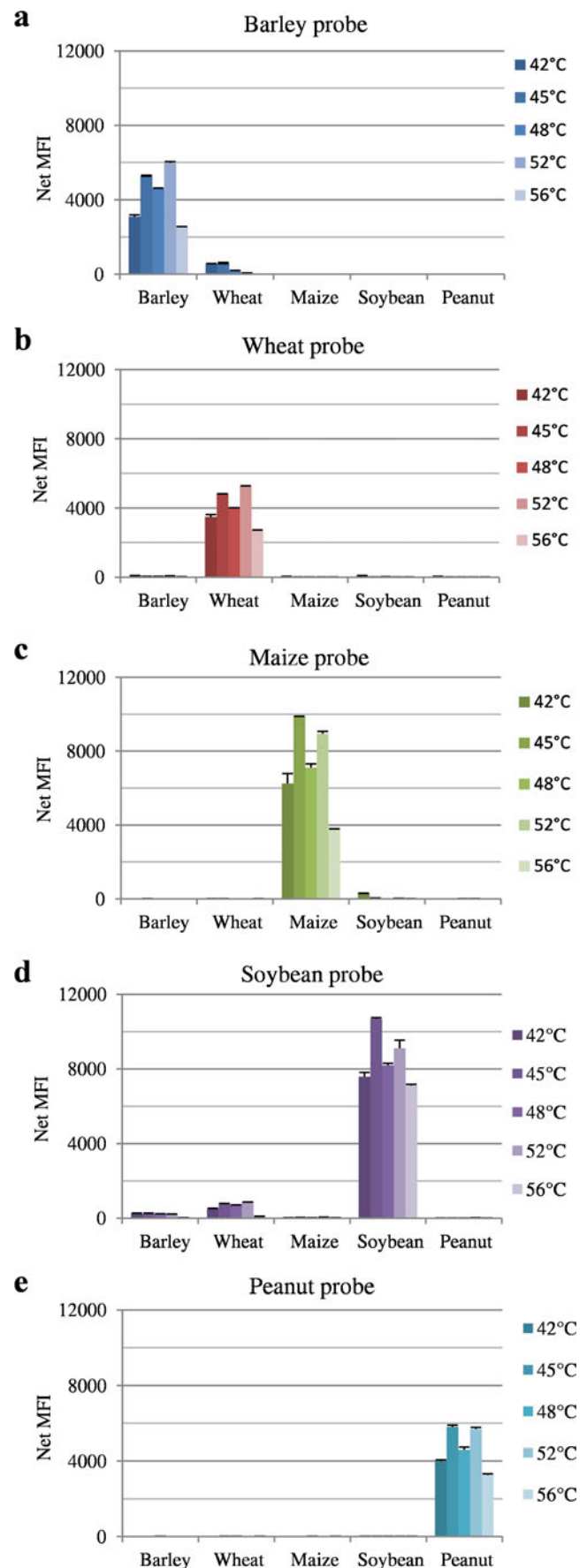


Table 2 Specificity and absolute sensitivity of each plant-specific capture probe

gDNA sample			net MFI				
Species	ng	Target copies ^a	Barley	Wheat	Maize	Soybean	Peanut
Barley	10		5,868	136	61	170	25
	1		5,118	93	88	381	30
	0.1		1,224	117	90	89	40
	0.01	5.3	1,051	32	38	29	-46
Wheat	10		-6	2,440	-2	194	23
	1		8	2,994	4	12	39
	0.1	13	-23	1,381	1	0	2
	0.01		-24	14	-1	2	11
Maize	10		-12	17	8,222	105	-14
	1		-63	-46	8,217	-54	-135
	0.1		-52	-47	7,652	-64	-134
	0.01	7.7	-50	-8	409	-27	-120
Soybean	10		-4	0.8	-14	7,392	-16
	1		9	5	-13	7,267	-9
	0.1		-1	5	-15	6,717	-1
	0.01	17.5	1	-2	-11	6,452	-20
Peanut	10		10	3	-5	5	4,277
	1		-1	1	-22	1	2,827
	0.1		8	-7	-20	-6	2,682
	0.01	6.9	2	3	-9	-12	1,599

^a The expected target copy number was calculated from the estimated *C* value of each plant genome, expressed as DNA pg/haploid genome (<http://data.kew.org/cvalues>)

against pure wheat gDNA samples, with the maximum MFI value that decreased from 675 in the fiveplex at 52 °C, to 208 in the singleplex format at 58 °C. We thus chose this split assay format for subsequent experiments. The MFI value+3 SD of the soybean nonspecific signal in a blank sample containing wheat DNA was assumed as the threshold, to avoid false positive.

As our analytical method is based on a multiplex PCR, where target competition for primers can be high, it was important to establish the validity of the assay on mixed flour samples in which the proportion of the different components was unbalanced, on a mass base. We thus tested the sensitivity of the assay adding different, increasing amount of soybean (1, 5, 10, and 25 %) in a background mix made of barley, wheat, and maize flours in a 1:1:1 ratio. Figure 4, reporting net

MFI values obtained by singleplex analysis vs. soybean percentage, clearly shows a linear relationship. This also shows the possibility of building a calibration curve on a specific background to obtain semi-quantitative data.

The threshold value was established as the MFI value of the sample deprived of soybean. Given the established threshold value, the relative LOD for soybean in these flour mixtures was around 1 % (w/w).

Applicability to processed food: Italian pasta

To test the ability of the assay to work on processed food products rather than raw material, we applied it to the analysis of different kind of Italian dry pasta, obtained from a local

Table 3 Sensitivity of the five capture probes in mixed gDNA samples of the five target plant species and repeatability of the assay

Total gDNA (ng)	Net MFI values														
	Barley			Wheat			Maize			Soybean			Peanut		
	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
10	1,620	1,596	1,647	708	731	791	2,781	2,104	2,919	7,138	6,997	7,612	2,683	2,177	2,586
1	1,225	1,464	1,353	456	822	547	1,993	2,075	2,198	6,680	6,833	6,937	2,069	2,132	2,158
0.1	1,062	805	1,205	383	432	512	2,187	2,238	2,508	5,828	6,098	5,991	1,922	1,070	1,952
0.01	709	7	750	0	11	77	586	12	668	4,613	138	4,752	728	13	769

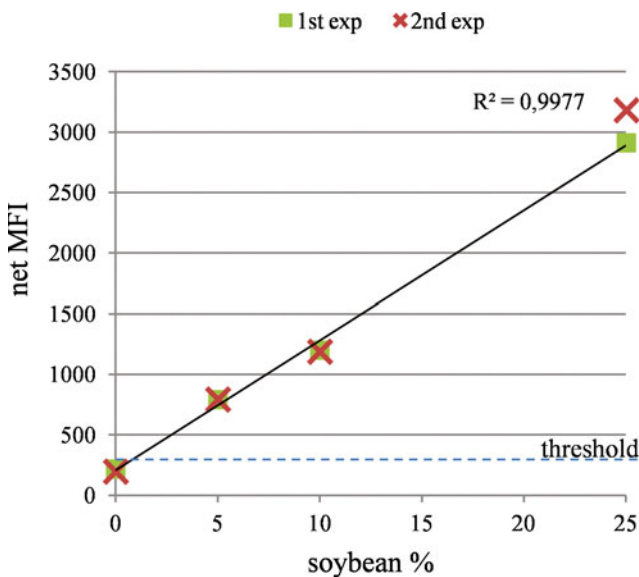


Fig. 4 Limit of detection of soybean in a mixture of barley, wheat, and maize flours. Net MFI values of two datasets were plotted against soybean percentage. The threshold was fixed as the mean value+3 SD of two samples deprived of soybean

supermarket. The assay was used to verify the ability of the method to detect wheat flour contaminations in gluten-free pasta made with maize. Flour (10 g) obtained by finely grinding maize pasta, was spiked with different percentages (0.1–10 %, *w/w*) of wheat flour, similarly obtained by grinding durum wheat pasta. DNA was extracted from 200 mg of each sample and 20 ng of genomic DNA were PCR amplified with the TBP method. Samples were assayed in the fiveplex format and MFI values for wheat and maize are reported in Table 4, after background subtraction. Setting the cutoff as the mean MFI value plus 3 SD of the blank samples (0 % wheat), the limit of wheat detection resulted lower than 0.5 % (*w/w*). Signal intensity was in a linear range up to 10 % of wheat amount, indicating the possibility of a rough quantitative estimation, once a calibration curve is established.

Table 4 Multiplex assay on flour from maize pasta spiked with different percentage of flour from durum wheat pasta

Wheat flour (%)	Maize pasta		Maize pasta+maize gDNA	
	Wheat	Maize	Wheat	Maize
0	72	237	59	6,495
0.1	99	356	58	6,424
0.5	307	182	70	6,298
1	594	252	226	6,340
5	1,240	154	717	5,679
10	2,480	188	1,243	6,223
100	2,767	7	2,118	6,015
Cut-off	100		70	

Entries in italics are wheat MFI values higher than the cutoff

In the same experiments, MFI values of the maize capture probe were much lower than expected, probably due to a degradation of DNA that was much higher for maize than for wheat pasta. To evaluate if the presence of intact maize DNA could affect the amplification efficiency of other templates, we added 10 ng of maize gDNA to each sample and run the assay again. This addition determined a 30- to 35-fold increase of the fluorescence signal detected by the maize bead-set, paralleled by a slight (about 2-fold) decrease in the wheat signal. Nevertheless, wheat DNA detection was still effective and linear, and its limit of detection only increased from 0.5 to 1 %, confirming the robustness of the assay.

Feed and food testing

To determine the applicability of the assay to processed feed and food, a selection of 11 market products of different composition and three compound feed samples were tested in the split assay format (4+1). Considering the heterogeneity of the matrix, at least 1 g of starting material was sampled and grinded for DNA extraction. Each TBP-PCR reaction was performed in duplicate and the two amplification mixtures were pooled. As a positive control, DNA extracted from mixed flour containing equal amounts of all the five detectable species, was also tested.

As ascertained by electrophoresis on agarose gel, TBP-PCR amplification was successful in all the analyzed samples. An exception was represented by the two breakfast cereals, in which only lower bands, up to 500 bp, were visible, indicating relevant DNA degradation (Electronic supplementary material, Fig. S4). These samples were nevertheless processed till the end. For all samples, inhibition tests (inhibition of plasmid PCR amplification by the DNA extract) were also performed and results were negative (not shown).

The results of one of three independent assays done on processed feed and food are reported in Table 5. For each bead set, the cutoff value was set as previously described. MFI values scoring below the established thresholds were considered negative and are reported as 0 in the Table. Samples were also tested for the presence of each of the five plant components, by end-point PCR amplifications carried out with species-specific primer pairs capable of amplifying short fragments (not shown). In most cases, 9 out of 14, the assay results were in agreement with the label. In two cases, multicereal cookies and one of the three feed samples, barley was not detected but a faint band (336 bp) was amplified by barley-specific PCR primers, suggesting that this species was present at a low percentage, below the detection limit, or that barley DNA was more degraded than others. In the two breakfast cereal samples, made of flaked or puffed cereals, the assay was not able to detect any signal except for soybean. In these samples, also species-specific PCR reactions resulted negative. This is likely due to the poor quality of DNA,

Table 5 Multiplex assay on commercial feed and food

Sample	Ingredients on the label		Fourplex				Singleplex	
	Detectable	Undetectable	Barley	Wheat	Maize	Peanut	Soybean	
Gluten-free pasta	✓	M	Rice	0	0	4,035	0	0
Corn with cheese snack	✓	M	—	0	0	5,165	0	0
Multicereal cookie		W, M (5 %), and B (5 %)	Malt	0 ^a	2,328	1,355	0	0
Multicereal flour		W, B, and M	Rice, rye, and oat	93	5,412	0	0	0
Muesli biscuit	✓	W and S	Oat and raisin	0	1,816	0	0	4,465
Organic wafer	✓	S (5 %)	Rice 94 %	0	0	0	0	1,380
Crunchy bread bacon	✓	W	Malt	0	1,288	0	0	0
Corn and peanut snack	✓	M (55 %) and P (32 %)	Palm oil	0	0	1,393	1,563	0
Sponge finger biscuit	✓	W	—	0	1,697	0	0	0
Flaked cereals		M, W, S, and B	—	0	0	0	0	278
Puffed cereals		W (28 %) and B (2.5 %)	Rye and oat 31 %	0	0	0	0	0
Feed 1	✓	M, B, and W	—	2,758	101	6,377	0	0
Feed 2		M, B, and S	—	617	2,539	3,342	0	264
Feed 3		M, B, and S	—	0 ^a	0	6,146	0	540
B-, M-, W-, S-, and P-mixed flour	✓			2,471	1,240	1,597	1,650	3,147
Negative PCR control				43.6	53.4	60.5	80.6	8.8
Wheat				6.5	3,132	2	3.5	208.3
				49.4	61.5	83.8	103.3	251

Checkmarks conform to the label. Entries in italics not in agreement to the label. *Italic*: cutoff values, calculated as described in the text

M maize, W wheat, P peanut, B barley, S soybean

^a False-negative resul: faint-positive signals are visible in specific PCR assay

ascribed to the severe conditions of the flaking and puffing processes, both requiring very high temperatures. It has to be noticed that also feed 3, in which the assay failed to detect barley, contains barley in the flakes form. Maize was below the limit of detection in the multicereal flour, in which also the specific PCR test resulted negative. Conversely, in feed 2, a strong signal for maize, not reported among ingredients, was detected with both the xMAP™ assay and the specific PCR end-point reaction, revealing a mislabeling or a contamination of the product.

Discussion

Once a suitable, short, specific target region is identified, direct DNA-based identification of one single plant component in a food mixture is quite an easy task for a conventional PCR approach. Conversely, the development of new strategies for the simultaneous qualitative identification of multiple ingredients in a complex matrix still represent a complex challenge in food analysis, particularly when it concerns the identification of plant components. In this respect, the TBP-based multi-target amplification strategy we have proposed has released promising results when coupled to the xMAP™ technology. In fact, five different plant species were simultaneously identified in mixed samples

with high sensitivity and specificity, and yet the number of the species can be further increased. The weak cross-reaction occurring between soybean and wheat was overcome by splitting the assay in one fourplex and one singleplex analysis and this adjustment can be taken as a further demonstration of the flexibility of the assay format. Set up on five species, the test is scalable to tens of targets, and the possibility of a customized selection of beads for multiplex assay offer application to many different products. The assay was also able to detect wheat flour contamination in maize pasta with a LOD of 0.5–1 % on a flour mass basis, despite the long high thermal treatment to which the fresh dough is subject for drying. This value is within the same range of other multiplex assays based on padlock probes microarrays [12] or xMAP™ technology [16]. Although not suitable for the detection of allergens, which can induce reactions in very low amounts (1–100 ppm), this limit is sufficient, in example, to detect fraudulent use of less valuable ingredients. As for any DNA-based method, the limit of detection for each component has to be established depending on the product matrix. Once a calibration curve is drawn, quantitative estimations can also be obtained. This could be useful to compare different lots of the same product.

The advantage of the method we have presented mainly relies on the applicability of the TBP-PCR amplification to all

plant species and the availability of a number of intron sequences among which one or more suitable target regions can be chosen. Its specificity is comparable with that of a real-time PCR Taqman assay, being based on the identification of three specific sequences, two primers, and one capture probe.

Among the different food matrixes tested, such as feedstock, biscuits, and snacks, the only unsuccessful samples were those made of puffed or flaked cereal, because of the presence of a highly degraded DNA fraction. To address this specific problem, an improved strategy would surely be to design capture probes targeting shorter intron sequences. Assay efficiency can also be improved by coupling oligoprobes targeted to different introns of the same genome, to the same bead set. This should circumvent problems of poor amplification of some particular intron due to target competition for primers. As shown, sensitivity can be further increased by hybridizing higher amounts of amplified DNA.

After suitable optimization and standardization, the assay could be applied to a wide range of other food and beverages for several purposes, such as quality control during food processing, authenticity test of mixed raw materials (feed, flours, and lyophilized vegetables), or certification of high valuable products, protected by denomination labels (PGI and PDO).

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