JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Comparison of DNA Extraction Methods and Development of Duplex PCR and Real-Time PCR To Detect Tomato, Carrot, and Celery in Food

Simona Pafundo, Mariolina Gullì, and Nelson Marmiroli*

Division of Genetics and Environmental Biotechnology, Department of Environmental Sciences, University of Parma, Viale G. P. Usberti 11/A, 43100 Parma, Italy

Supporting Information

ABSTRACT: Traceability is of particular importance for those persons who suffer allergy or intolerance to some food component(s) and need a strict avoidance of the allergenic food. In this paper, methodologies are described to fingerprint the presence of allergenic species such as carrot, tomato, and celery by DNA detection. Three DNA extraction methods were applied on vegetables and foods containing or not containing the allergens, and the results were compared and discussed. Fast SYBR Green DNA melting curve temperature analyses and duplex PCR assays with internal control have been developed for detection of these allergenic vegetables and have been tested on commercial foods. Spiking food experiments were also performed, assessing that limits of detection (LOD) of 1 mg/kg for carrot and tomato DNA and 10 mg/kg for celery DNA have been reached.

KEYWORDS: allergens, duplex PCR, real-time PCR, SYBR Green, tomato, celery, carrot

INTRODUCTION

Although the prevalence of food allergy is not accurately known, it is believed that 1−2% of the adult population and up to 8% of children suffer from food allergies. If allergies to fruits and vegetables are taken into consideration, a higher prevalence has been estimated in adults. The relevance of this problem is further increased as concomitant allergies to multiple plant-derived foods become common, in particular to fruits and vegetables.[1]

Quality control in food industries traces accidental contaminants or unsafe ingredients with analytical tools. Traceability is also a commercial issue; it complies with food regulations and with the consumers' need for food safety and security.[2−7] Ingredients' traceability is of particular importance in the case of food allergens, because avoidance of the food is, in most cases, the only treatment available to date, thus limiting dietary choices and the quality of life of food-allergic persons. Direct detection of food allergens by the consumer may be difficult, because of product mislabeling or unintentional cross-contamination during food production.[8] To reduce the risk of hidden allergens in foods, several countries have adopted new legislations that require the declaration of the presence of some allergenic ingredients or potential contaminants.[9] Recently, food component analyses have been implemented by the introduction of molecular methodologies[9−11] to tag proteins or DNA. Proteins can be detected by the enzyme-linked immunosorbent assay (ELISA) based on polyclonal or monoclonal antibodies.[12] ELISA has several advantages,[13] but also different drawbacks such as (i) a long developmental time; (ii) matrix effects because of a lower complexity in protein-specific recognition sites; (iii) cross-reactivity; (iv) potential false positive results from noise or matrix;[11] (v) confirmation requirements for positives; (vi) potential false-negative results if the target proteins or the target epitope is affected by food processing and breakdown peptides are not properly detected or because other proteins that are not detected are important allergens for a particular subgroup of individuals; and (vi) the current lack of a multiresidue analysis. Furthermore, immunoassays, although targeting the allergen, should be considered indirect assays because the animal antibodies utilized do not necessarily recognize the same allergenic epitopes that are recognized by the allergic human individuals.[14]

DNA-based tests can be an alternative to immunological methods for several reasons: (i) DNA has a longer resistance and resilience to chemical and physical treatments during processing;[11,15] (ii) the reagents necessary for PCR can be synthesized and manufactured in unlimited amount and at constant quality; (iii) primers and probes can be designed to meet the needs of specificity and sensitivity similarly to monoclonal antibody for ELISA; (iv) specific primers and probes are less susceptible to matrix effects than monoclonal antibody; (v) it can be used to trace specifically many species (animal and plant) including those potentially allergenic; and (vi) it is possible to perform multiresidue analysis.[15]

Polymerase chain reaction (PCR) is the engine of many DNA-based methodologies that allow the presence of even a single DNA molecule from a target species to be detected in a complex food mixture. PCR inhibitors such as polysaccharides, humic acids, and polyphenols are unfortunately common in many food matrices;[10,16] the development of DNA extraction methods that reduce the presence of inhibitors in the reaction mix is therefore required. Real-time PCR with TaqMan probe is a highly specific system of detection,[16,17] which can reach a sensitivity as high as that of immunoassays, but it is more expensive than ELISA. However, real-time PCR combined with the analysis of dissociation curves of amplicons is highly specific for detection as well,[15] but with a lower cost than those claimed for real-time PCR with

Received: February 9, 2011
Revised: September 6, 2011
Accepted: September 6, 2011
Published: September 06, 2011

Table 1. Plant Species and Food Matrices[a]

			18S primers[b]	species-specific primers[b]		
plant material		method[c]		tomato	carrot	celery
leaves of	wheat	CTAB	+	−	−	−
	barley		+	−	−	−
	maize		+	−	−	−
	oat		+	−	−	−
	sunflower		+	−	−	−
	olive		+	−	−	−
	rice		+	−	−	−
seeds of	sesame	CTAB	+	−	−	−
	almond		+	−	−	−
	hazelnut		+	−	−	−
	nut		+	−	−	−
	peanut		+	−	−	−
	cashew		+	−	−	−
	mustard		+	−	−	−
fresh vegetables	carrot	PVP	−	−	−	−
		SDS	+	−	+	−
		G	+	−	+	−
	tomato	PVP	+	+	−	−
		SDS	−	−	−	−
		G	+	+	−	−
	celery	PVP	+	−	−	+
		SDS	+	−	−	+
		G	+	−	−	+
commercial food matrices						
bouillon cube		PVP	−	−	−	−
		SDS	+	−	+	+
		G	+	−	+	+
tomato sauce with hot pepper		PVP	−	−	−	−
		SDS	−	−	−	−
		G	−	−	−	−
pesto food		PVP	+	−	−	−
		SDS	+	−	−	−
		G	+	−	−	−
sesame bars		G	+	−	−	−
pie		G	+	−	−	−
cookies with	cashews	G	+	−	−	−
	nuts	G	+	−	−	−
	chocolate	G	+	−	−	−
	eggs	G	+	−	−	−
homogenized baby food with	kiwi	G	−	−	−	−
	peach	G	−	−	−	−
	apple	G	−	−	−	−

Table 1. Continued

			18S primers[b]	species-specific primers[b]		
plant material		method[c]		tomato	carrot	celery
yogurt with	kiwi	G	±	−	−	−
	peach	G	±	−	−	−
	apple	G	−	−	−	−
spiked pesto	(mg kg^{-1}) of					
	1000 carrot	G	+	−	+	−
	100 carrot	G	+	−	+	−
	10 carrot	G	+	−	+	−
	1 carrot	G	+	−	+	−
	1000 tomato	G	+	+	−	−
	100 tomato	G	+	+	−	−
	10 tomato	G	+	+	−	−
	1 tomato	G	+	+	−	−
	1000 celery	G	+	−	−	+
	100 celery	G	+	−	−	+
	10 celery	G	+	−	−	+
	1 celery	G	+	−	−	−

[a] Table reports results of the specificity test performed by PCR for each primer pair on DNA purified from different matrices with different methods. [b] +, positive result, amplification's product with a high signal intensity; ±, amplification's product with a low signal intensity, compared with the other positive results; −, negative result, no amplification products [c] CTAB, DNA extracted with CTAB method by Doyle and Doyle;[30] PVP, DNA extracted with CTAB PVP method; SDS, DNA extracted with SDS method; G, DNA extracted with GK-resin method.

primers and probe and comparable with the costs of ELISA. In particular, with the use of Fast SYBR Green DNA melting curve temperature analyses (Fast SYBR PCR) an analysis is performed in 20 min, reducing considerably the cost and also the possibility of errors by the operator. In general, PCR has an absolute advantage for species identification, which makes PCR eligible for the detection of allergenic food components of animal or plant origin that may have a genetic or compositional homology to other species used in food manufacturing. In addition, because DNA from various species is extracted simultaneously from a complex food matrix, this can make DNA a multianalyte system and PCR a high-throughput detection method, whereas the available ELISAs are essentially single-analyte tests.

Allergies to tomato, carrot, and celery constitute a source of growing concern for food producers because the allergenicity is associated with their widespread use. Tomato (*Solanum lycopersicum*) is an angiosperm and belongs to the Solanaceae family. The genome is organized in 12 chromosome pairs, and the haploid genome size is 0.95 pg.[18] Fresh and cooked tomato are basic in many recipes. Sensitivity to tomato is prevalent in the Mediterranean area, with peaks in the pediatric−adolescent population,[19] but frequent also in adults.[20] Celery (*Apium graveolens*) is an angiosperm of the Apiaceae family; the genome is organized in 11 chromosomes pairs, and the haploid genome size is 1.73 pg.[21−23] Celery is consumed raw or as a cooked vegetable. Celery spice is used in many processed foods such as spice mixtures, soups, broths, and salad dressings. Celery, due to its high allergenic potency,[1] is included in the list of foods with mandatory labeling according to the revised EU Labeling Directive.[5] Besides, vegetables belonging to the Apiaceae family are frequent causes of pollen-related plant allergy, particularly in European countries. Carrot (*Daucus carota* L.) is also an important food, with up to 25% of the allergic subjects in Central Europe having carrot allergy.[24] Carrot has a genome organized in nine chromosome pairs, and the haploid genome size is 1 pg.[25] Carrot is consumed raw or as a cooked vegetable. It is often an ingredient in stews and hotpots. Carrot can also be found in some types of cakes.

Methodologies based on DNA detection have been developed to trace allergenic vegetable species in food. Hupfer et al.[23] reported the use of methods for DNA purification from celery stalks and from other food matrices. The limit of detection (LOD) of the methods was, however, determined without a proper statistical analysis, but the conclusion was that a modified CTAB method ranked first in the list. Mustorp et al.[26] described DNA extraction from celery and food, as well as Dovicovicovà et al.,[27] with a mention to DNA extraction from dehydrated bouillons and from meat pâtés, but they did not present any statistical evaluation of the results obtained. The use of CTAB to extract DNA from tomato was suggested also in a work of Chaouachi et al.[28] Turci et al. [29] first presented a comparison between seven DNA extraction methods for tomato, including commercial kits. They analyzed different tomato-based foods, testing the DNA extraction results for different parameters with a fuzzy logic approach. Their conclusion was that the best choice for all of the tested matrices was the commercial Wizard kit. A real-time PCR with TaqMan probes has been reported for *S. lycopersicum*[28] and for *A. graveolens*.[23,26] Dovicovicovà et al.[27] developed an assay based on PCR followed by agarose gel electrophoresis for *A. graveolens*. Zagon et al.[30] reported the development of reverse transcription real-time PCR for the quantification of mRNA transcription of genes encoding for

Table 2. Design of Species-Specific Primers[a]

species	sequence 5′−3′	target gene	accession no.	primer name	amplicon length (bp)	theoretical T_m of amplicon (°C)
carrot (*Daucus carota* L.)	forward, CCAGAGCCATTCACTCGAGATC reverse, ACTGTATCAACATCAAGGACAATGC	Dau c1	Z84376.1	carrot	81	74.5
celery (*Apium graveolens*)	forward, GGGCTTTGTCATTGATGTTGAC reverse, TCCCTTGATTTCGACACTCTTGTA	Api g1	Z48967.1	celery	76	77.1
tomato (*Solanum lycopersicum*)	forward, TGTGGTTTTTTGCATGGTGG reverse, CAGCTCAGTGACTCTGCATGG	Lyc e3	AM051296.1	tomato	51	75.1
almond (*Prunus dulcis*)[b]	forward, GTGACGGAGAATTAGGGTTCGA reverse, CCGGTATTGTTATTTATTGTCACTACCTC	18S rRNA	DQ886376.1	18SR	131	79.4

[a] Target sequences selected for primer's design, for each gene selected its accession number is reported. For each primer pair length and theoretical T_m of the expected amplicon are reported. [b] The primers for 18S rRNA are designed on a target sequence of almond, which is a sequence highly conserved in all plant species tested.

the major allergen isoforms Dau c 1.01 and Dau c 1.02 of *D. carota*. No DNA-based analysis is reported for carrot allergen detection in food.

A detailed comparison (Table S1 of the Supporting Information) of previous achievements and those obtained in this study evidence the following novelties: (i) the internal control proposed in the duplex PCR, in fact, 18SR primer pair, can significantly reduce the false-negative rate, which is a critical point in allergen detection; (ii) the use of Fast SYBR PCR reduces to 20 min the time needed to perform an analysis and reduces also the costs of each analysis; and (iii) it is the first example of a PCR test for carrot DNA detection in processed foods. All of these points are important when considering the implementation of the analytical procedure at an industrial level.

MATERIALS AND METHODS

Food Materials. All plants and foods were purchased from qualified retailers and stored at room temperature in the dark throughout. A complete list is given in Table 1.

Spiking of Pesto Samples with Tomato, Carrot, and Celery. Commercially available pesto food was checked for the absence of carrot, celery, and tomato before spiking. Nine grams of pesto food was spiked with 1 g of each vegetable singularly, to have a final weight of 10 g. The spiked samples were serially diluted (10-fold) to obtain pesto containing 100,000, 10,000, 1000, 100, 10, or 1 mg/kg of carrot, celery, or tomato. The dilutions were obtained by adding 9 g of pesto food to 1 g of the spiked pesto followed by manual mixing and homogenizing.

DNA Extraction from Vegetables and Foods. Vegetables and foods were ground using a Knifetec1095 (Foss Tecator AB, Höganäs, Sweden). DNA was extracted from leaves using the cetyltrimethylammonium bromide (CTAB) method as reported by Doyle and Doyle.[31] DNA was extracted from the food matrix using three different methods. For each method, at least two independent extractions (biological replicates) were performed, with a negative control for each extraction.

GK-Resin Method. This is an extraction procedure based on the protocol described by Meyer et al.,[32] with several modifications. Three hundred milligrams of ground samples was treated with 860 μL of extraction buffer [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), and 1% (w/v) sodium dodecyl sulfate (SDS)]; then 100 μL of 5 M guanidine hydrochloride and 40 μL of proteinase K (20 mg/mL) were added, and the mixture was incubated at 50 °C overnight, by mixing (500 rpm). Samples were stored at room temperature for 2 min and then centrifuged at 17000*g* for 10 min at 4 °C; 500 μL of aqueous phase was added to 1 mL of Wizard Miniprep DNA Purification Resin (Promega, Madison, WI). The samples were mixed by gentle inversion and then processed as reported by the manufacturer's instruction, with the exception of two centrifugation steps instead of one and two incubations at room temperature of the mixture DNA−resin for 30 min. DNA was purified with 50 μL of distilled water at 70 °C.

CTAB-PVP Method. Five milliliters of extraction buffer (20 g/L of CTAB, Tris 0.1 M, 20 mM EDTA, 1.4 M NaCl) and 50 ng of polyvinylpyrrolidone (PVP) were added to 1 g of ground samples. The mixtures were incubated for 30 min at 55 °C. Then, 2 mL of phenol/chloroform/isoamyl alcohol (25:24:1) was added, and the samples were centrifuged at 13000*g* at 20 °C for 10 min. The aqueous phase was recovered and 1.5 mL of chloroform/isoamyl alcohol (24:1) added. The mixtures were centrifuged at 13000*g* at 20 °C for 10 min, and the aqueous phase was recovered. Five milliliters of RNase (10 mg/mL) was added, and the samples were incubated at 37 °C for 30 min; 7.5 M ammonium acetate was added as half of the final volume of samples and then mixed by gentle inversion. Each mixture was subsampled in 1 mL aliquots to which 500 μL of cold isopropanol was added. Each subsample was mixed by gentle inversion, incubated at −20 °C for 1 h, and then centrifuged at 13000*g* for 20 min at 4 °C. Pellets were recovered, washed with 500 μL of 100% cold ethanol, and centrifuged at 13000*g* for 5 min at 4 °C. Pellets were recovered, dried at room temperature for 30 min, then centrifuged at 13000*g* for 5 min at 4 °C, and finally incubated at room temperature for 10 min; 50 μL of distilled water was added to each to resuspend the pellets. All of the obtained subsamples were poured in a single tube.

SDS Method. Eight hundred microliters of extraction buffer [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), and 1% (w/v) SDS] was added to 200 mg of sample. The mixtures were incubated at 55 °C for 1 h and then at room temperature for 10 min. Six hundred and sixty microliters of phenol/chloroform/isoamyl alcohol (25:24:1) was added, and the samples were mixed by gentle inversion; mixtures were centrifuged at 13000*g* for 20 min at 20 °C. The aqueous phase was recovered, and chloroform/isoamyl alcohol (24:1) was added as half of the final volume of samples. Samples were centrifuged at 13000*g* for 10 min at 20 °C, the aqueous phase recovered, and 5 mL of RNase (10 mg/mL) added; the samples were incubated at 37 °C for 30 min. Ammonium acetate (7.5 M) was added as half of the final volume, and samples were mixed by gentle inversion. Cold isopropanol was added as two-thirds of the final volume, and

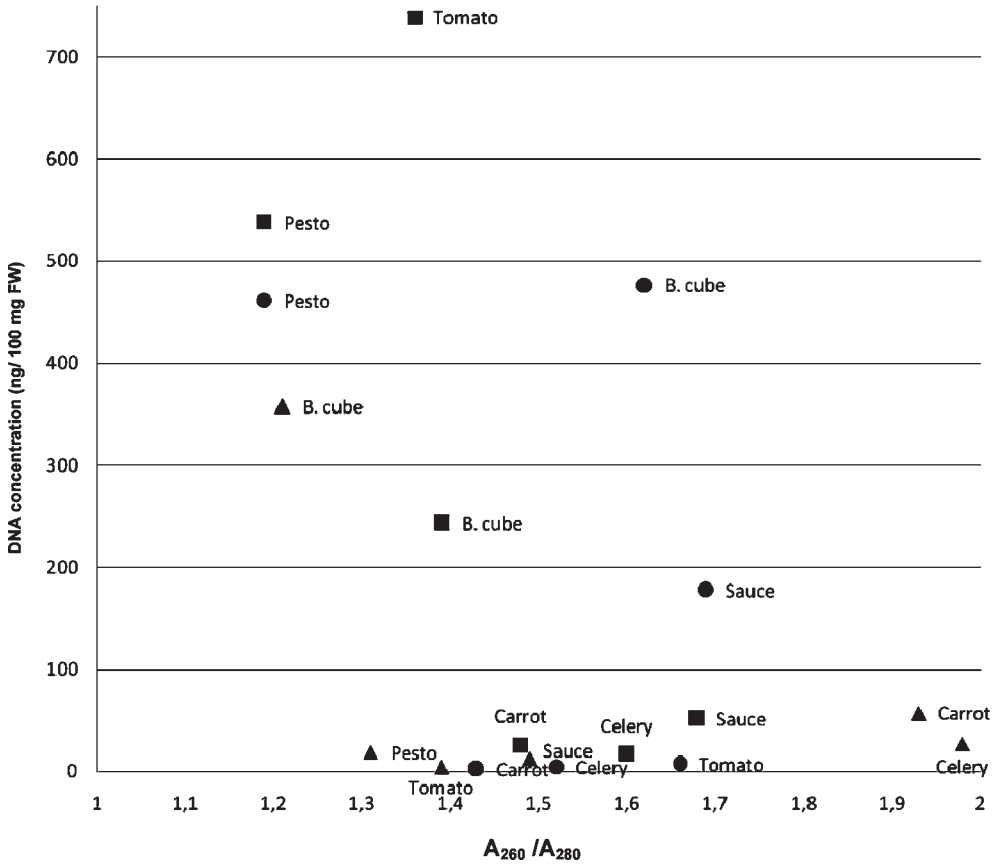

Figure 1. Comparison of DNA extraction methods on vegetables and foods. Genomic DNA was extracted using three methods (●, CTAB-PVP; ■, SDS; and ▲, GK-resin method) from carrot, celery, and tomato and from tomato sauce (sauce), bouillon cube (B. cube), and pesto. The absorbance ratios, A_{260}/A_{280}, and DNA amounts, ng/100 mg of food fresh weight, are reported.

samples were mixed by gentle inversion and incubated for 1 h at 20 °C. Pellets were recovered and washed with 500 μL of 100% cold ethanol. Samples were further centrifuged at 14000*g* at 4 °C for 5 min, and pellets were recovered, dried at room temperature for 30 min, centrifuged at 14000*g* for 2 min at 4 °C, and dried again at room temperature for 10 min; 50−100 μL of distilled water was finally added.

DNA Quantification and Determination of Purity. Extracted DNAs were quantified with a Beckman DU-600 spectrophotometer (Beckman-Coulter, Fullerton, CA), by measuring the absorbance (*A*) at 260 and 280 nm. The quality of DNA was estimated both by agarose gel electrophoresis and by evaluation of the A_{260}/A_{280} ratio.

Target Genes Selection and Primers Design. Nucleotidic sequence information of tomato, carrot, and celery target genes was retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). Specific primers were designed using the software Primer Express v.2.0 (Applied Biosystems Division of Perkin-Elmer Corp., Foster City, CA). Information about genes and primers used in this study is given in Table 2. Primer sequences were compared with plants' sequences present in databases, using the BLAST program (http://blast.ncbi.nlm.nih.gov/). Primers were purchased from MWG (Ebersberg, Germany).

Statistical Analyses. Statistical analyses were performed using the software SPSS v.18. Nonparametric tests were chosen because of a non-normal distribution of data.[33] For the statistical evaluation of DNA concentration and purity, the nonparametric test of Kruskal and Wallis was applied. To evaluate the matrix effect on DNA amplificability in real-time PCR, using the C_t value obtained with 18SR primer pair, the *t* test and the nonparametric *runs* test, also called the Wald−Wolfowitz test, for a sample were used. A study on the possible correlation between C_t value and A_{260}/A_{280} was also performed, using the test of Pearson's correlation and Spearman's rank correlation coefficient. The differences within the group "vegetables" and the group "food" were evaluated with the *t* test for two samples and the nonparametric U test of Mann−Whitney for independent samples. Differences of C_t values obtained with the 18SR primer pair on pesto food and on spiked pesto were determined with the *t* test and the nonparametric *runs* test (Wald−Wolfowitz test). Probability values of significance were considered below 0.05 ($p \leq 0.05$). Standard deviations were calculated for C_t and T_m values.

PCR, Duplex PCR, and Real-Time PCR Conditions. For end-point PCR, amplification was carried out in a final volume of 20 μL containing 50 ng of DNA in the presence of 1× *Taq* buffer (Qiagen, Milan, Italy), 0.25 μM of each forward and reverse primer, 0.2 mM dNTPs, 2 mM $MgCl_2$, and 2.5 U of HotStartTaq DNA polymerase (Qiagen), in a Veriti 96-well thermal cycler (Applied Biosystems).

For duplex PCR optimization two primer pairs were used, combining the primer pair 18SR with the tomato, celery, or carrot specific primers. Amplification was carried out in a final volume of 20 μL containing 50 ng of DNA, 1× *Taq* buffer, different concentrations from 0.20 to 0.40 μM of each forward and reverse primer reported in Table 2, 0.2 mM dNTPs, 2 mM $MgCl_2$, and 2.5 U of HotStartTaq DNA polymerase (Qiagen), in a Veriti 96-well thermal cycler (Applied Biosystems). All samples were run in three technical replicates, with a negative control containing water instead of DNA. PCR conditions were as follows: 2 min initial denaturation at 95 °C followed by 30 cycles with a 30 s denaturation at 95 °C, 40 s of annealing at 58 °C, 1 min of elongation at 72 °C; and 5 min of final extension at 72 °C. Amplification products were analyzed

by electrophoresis on 3% (w/v) agarose gel, stained with GelRed Nucleic Acid Gel Stain 1000× (Biotium, Hayward, CA).

Fast SYBR PCR was carried out using the Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems). For all samples, three technical replicates of each biological replicate and a negative control, with no biological component, but water, were performed. Reactions were carried out in a final volume of 20 μL, using 50 ng of DNA with 1× Fast SYBR Green (Applied Biosystems), and 0.375, 0.5, and 0.7 μM of each forward and reverse primer for carrot and celery primer pairs, but 0.5, 0.7, and 0.9 μM of each forward and reverse primer for tomato primer pairs and 0.2 μM of each forward and reverse primer for 18SR primer pair. PCR conditions were as follows: 20 s at 95 °C followed by 40 cycles of 1 s at 95 °C and 20 s at 60 °C. The PCR program was followed by a "dissociation" stage, during which the temperature was gradually increased from 60 to 95 °C. The melting curves obtained were converted into their negative first derivatives, which showed a maximum corresponding to the melting temperature (T_m) of the amplification product. Two sets of values were obtained for carrot, celery, and tomato DNAs samples: within the same day by one operator and on two different days by two operators.

Cloning and Sequencing of PCR Amplicons. PCR products were cloned using the pGEM-T Easy Vector System (Promega). Plasmid DNA was extracted from transformed recombinant cells, with the Wizard Plus SV Mini-Preps DNA Purification Kit (Promega). Three clones for each fragment were sequenced, with the CEQ 2000 Dye Terminator Cycle Sequencing Quick Start Kit (Beckman-Coulter), using M13 universal primers. Three sequences from each fragment were aligned and compared using the program ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

RESULTS AND DISCUSSION

Evaluation of Primer's Design. Species-specific primer pairs were designed to recover traces of the allergenic species *A. graveolens*, *D. carota* L., and *S. lycopersicum* in highly processed food samples. Primers were designed on genes for known allergenic proteins, according to Pafundo et al.[11] The characteristics of all primers are reported in Table 2. Carrot, celery, and tomato primers were first evaluated individually on DNA extracted from those plant species (data not shown), in an end-point PCR followed by resolution on 3% agarose gel electrophoresis. Each fragment was eluted from the gel, cloned, and sequenced.[11] In all cases, the resulting nucleotide sequence matched the sequence used for primer design.

Comparison of DNA Extraction Methods on Vegetables and Food and Statistical Considerations. To evaluate results of DNA extraction, three parameters were chosen: (i) DNA concentration, ng/100 mg fresh weight (FW); (ii) DNA purity, assessed as the ratio A_{260}/A_{280} (according to Cankar et al.,[34] these values should be in a range between 1.8 and 2); (iii) amplifiability by PCR with 18SR primers. Results in Figure 1 show that concentration and purity of DNA varied independent of the extraction method. With the GK-resin extraction method the amount of DNA recovered was less variable than with other methods; purity was higher, but yield was lower. Using the SDS or CTAB-PVP method the A_{260}/A_{280} ratio was never above 1.7 but the yield was higher, in particular for tomato, pesto, and bouillon cube. The nonparametric Kruskal–Wallis statistical analyses showed that no significant differences were found when using different extraction methods: for DNA extraction amount, $p = 0.368$; for purity, $p = 0.778$. When using different matrices, for DNA extraction amount, $p = 0.292$, and for purity, $p = 0.078$. Quantity and purity of the extracted DNA did not vary significantly

Table 3. Results of Fast SYBR Green DNA Melting Curve Temperature Analyses (Fast SYBR PCR)

sample	primer pair	av C_t[a]	SD	av T_m[c] (°C)	SD
carrot	18SR	15.47	0.12	79.4	0.05
	carrot	21.42	0.05	74.7	0.05
		21.51[b]	0.11[b]		
celery	18SR	20.10	0.08	79.4	0.10
	celery	26.10	0.01	77.3	0.05
		26.15[b]	0.05[b]		
tomato	18SR	15.38	0.11	79.2	0.05
	tomato	25.17	0.03	75.3	0.10
		25.23[b]	0.12[b]		
bouillon cube	18SR	18.30	0.13	79.4	0.05
	carrot	26.46	0.10	74.7	0.05
	celery	27.34	0.11	77.3	0.05
tomato sauce with hot pepper	18SR	30.46	0.09	79.5	0.05
	tomato	30.08	0.13	75.4	0.10
pesto	18SR	16.16	0.09	79.5	0.05
spiked pesto with					
carrot 1000 mg kg^{-1}	18SR	14.45	0.09	79.5	0.10
	carrot	29.45	0.10	74.7	0.05
carrot 100 mg kg^{-1}	18SR	14.42	0.10	79.5	0.05
	carrot	30.30	0.10	74.7	0.05
carrot 10 mg kg^{-1}	18SR	14.45	0.11	79.5	0.05
	carrot	31.41	0.10	74.7	0.05
carrot 1 mg kg^{-1}	18SR	14.45	0.10	79.5	0.10
	carrot	31.70	0.08	74.7	0.05
tomato 1000 mg kg^{-1}	18SR	16.26	0.11	79.5	0.10
	tomato	32.30	0.12	75.3	0.05
tomato 100 mg kg^{-1}	18SR	16.30	0.10	79.5	0.05
	tomato	33.40	0.13	75.3	0.10
tomato 10 mg kg^{-1}	18SR	16.30	0.12	79.5	0.10
	tomato	33.60	0.13	75.3	0.10
tomato 1 mg kg^{-1}	18SR	16.30	0.11	79.5	0.10
	tomato	34.60	0.09	75.3	0.10
celery 1000 mg kg^{-1}	18SR	15.06	0.12	79.5	0.05
	celery	33.68	0.08	77.3	0.05
celery 100 mg kg^{-1}	18SR	15.10	0.10	79.5	0.10
	celery	35.60	0.09	77.3	0.05
celery 10 mg kg^{-1}	18SR	15.10	0.11	79.5	0.10
	celery	35.93	0.08	77.3	0.05
celery 1 mg kg^{-1}	18SR	15.00	0.11	79.5	0.10
	celery	nd[d]		nd	

[a] Values represent C_t mean and SD of three technical replicates of two biological replicates obtained by the same operator in 1 day. [b] Values represent C_t mean and SD of three technical replicates obtained by two operators in 2 days. [c] For comparison with theoretical T_m return to Table 2. [d] Not detected.

for comparison of the vegetables with commercial food matrices. Amplifiability was tested with end-point PCR using 18SR primers, which tag a highly conserved sequence among eukaryotes. Table 1 shows that DNA amplifiability with 18SR rests basically on the extraction method. DNA was amplified when extracted with the GK-resin method from all samples except sauce, with the CTAB-PVP method from tomato, celery, and pesto; and with the SDS method from carrot, celery, bouillon cube, and pesto. DNA was not amplified when extracted with the CTAB-PVP method from carrot, bouillon cube, and sauce and with the SDS method from tomato and sauce.

Different authors[10,29,34] (see also Table S1 of the Supporting Information) have reported the need to identify a DNA extraction method for vegetables and vegetable-containing foods that gave good results in terms of both DNA yield and purity. Indeed, the presence of inhibitors and DNA degradation may undermine any subsequent analyses, particularly when DNA was extracted from some foods having DNA damaged by processing or when extracted DNA can be contaminated with polyphenols or polysaccharides, as for olive oil.[35]

We have verified that a "good for all" DNA extraction method for the tested matrices does not exist, but rather exists a "case by case" best-performing method such as the GK-resin method for carrot, celery, pesto, and bouillon cube; the SDS method for sauce with hot pepper; and the CTAB-PVP method for tomato. Extraction yielded DNA in relatively good quantity and quality, at variance from previous experience in which DNA was recovered from food in scarce quantity and/or low quality.[35]

Fast SYBR Green DNA Melting Curve Temperature Analyses (Fast SYBR PCR) and Statistical Considerations. *Analysis with 18SR Primers on Fresh Vegetables and Foods.* The 18SR primers were used with Fast SYBR PCR on DNA extracted from fresh vegetables and from food products with the best-performing method for each food sample. The C_t values measured for each sample are reported in Table 3. All of the reactions performed with 18SR reached the exponential phase early as shown by C_t values (average C_t = 15.31), with the exception of celery (average C_t = 20.1), bouillon cube (average C_t = 18.30), and sauce with hot pepper (average C_t = 30.46). A difference of three cycles in C_t in the case of celery cannot be attributed to celery's genome size (1.73 pg),[21−23] which is comparable to carrot's (1 pg),[25] and tomato's (0.95 pg).[18] Statistical analyses (the *t* test and the nonparametrical *runs* test) confirmed that these differences in C_t values were not significant. There was no correlation between the C_t value and the purity of DNA, as A_{260}/A_{280} ratio, according to Pearson's test and Spearman's rank correlation coefficient.

To verify if differences were related with the heterogeneity of the tested materials (vegetables and foods), data were divided into two groups, named, respectively, "vegetables" and "foods". Using the *t* test for two samples and the Mann−Whitney test, no significant differences were found. Therefore, food processing did not significantly influence the amplifiability with 18SR primers of DNA extracted from food samples.

Analysis with Species-Specific Primers on Fresh Vegetables and Foods. The species-specific primers listed in Table 2 were used in Fast SYBR PCR at concentrations of 0.375 μM for carrot and 0.5 μM for tomato and celery to amplify DNA extracted from fresh vegetables listed in Table 1. The T_m of each amplicon was evaluated by the analysis of the melting curves as described under Materials and Methods, and these results are reported in Table 3 and Figure 2. The T_m values observed were (i) 74.7 °C using carrot primers on carrot DNA (Figure 2A), (ii) 77.3 °C using celery primers on celery DNA (Figure 2B), and (iii) 75.3 °C using tomato primers on tomato DNA (Figure 2C), and they corresponded with the theoretical T_m as reported in Table 2. In all samples, and for all of the replicates, there was a single peak, with no trace of unspecific products or primer-dimers. Primer specificity was also confirmed using the DNA extracted from leaves and seeds of different plant species as template in PCR, and in all cases no amplification was observed (Table 1).

The same species-specific primers were used to tag DNA extracted from food products, such as a tomato sauce with hot pepper and a bouillon cube containing celery and carrot as ingredients. As shown in Figure 2, primers recognized the specific targets also in processed matrices, but with some differences depending on DNA extraction method. Carrot- and celery-specific primers could amplify DNA extracted from bouillon cube when each of the three extraction methods was used (Figure 2D,E); the amplification reaction started earlier when DNA extracted with the GK-resin method was used. In the case of tomato primers, the amplification reaction was successful only when DNA was extracted from tomato sauce with the SDS method (Figure 2F). DNA purified from carrot, tomato, and celery was used as positive control in all of the amplifications performed on DNA purified from food matrices. Pesto food, used as a negative control, because of the absence of tomato, celery, and carrot among its ingredients, showed no amplifiability with species-specific primers (data not shown), but its DNA was amplifiable with 18SR primers (Table 1).

The results reported in Table 3 show that all of the reactions performed with species-specific primers reached the exponential phase later than with 18SR primers. The average C_t values obtained were 21.42 using carrot primers on carrot DNA, 26.10 using celery primers on celery DNA, and 25.17 using tomato primers on tomato DNA. For bouillon cube DNA, the C_t values obtained with carrot or celery primers were similar (average C_t = 26.46 for carrot; average C_t = 27.34 for celery). For sauce with hot pepper DNA, the C_t obtained with tomato primers had an average of 30.08; similarly, a high C_t value was measured with 18SR primers (average C_t = 30.46), because DNA extracted from this matrix was scarcely amplifiable. A statistical analysis was performed to establish if there was a correlation between the C_t values obtained with 18SR primers and the species-specific primers. To assess the presence of a "matrix effect" on amplifiability, Pearson's and Spearman's rank correlation coefficient were applied on the C_t values obtained, but the results were not significant. The data were divided into two groups, named, respectively, "vegetables" and "food" and, using the *t* test for two samples and the Mann−Whitney test, a significant difference was found ($p < 0.001$). The conclusion was that species-specific primers can find traces of their target also in samples that have been industrially processed whenever they were present as ingredients in foods. False positives were not found.

Repeatability and Reproducibility for FAST SYBR PCR. Repeatability and reproducibility were evaluated using carrot, celery, or tomato DNAs. The mean and standard deviation (SD) of C_t values of repeatability were calculated from the data obtained by one operator performing three technical replicates of each biological replicate on the same day. As shown in Table 3, we obtained a mean C_t of 21.42 ± 0.05 in the case of carrot primers (CV = 0.23%), a mean C_t of 26.10 ± 0.03 in the case of celery primers (CV = 0.04%), and a mean C_t of 25.17 ± 0.01 in the

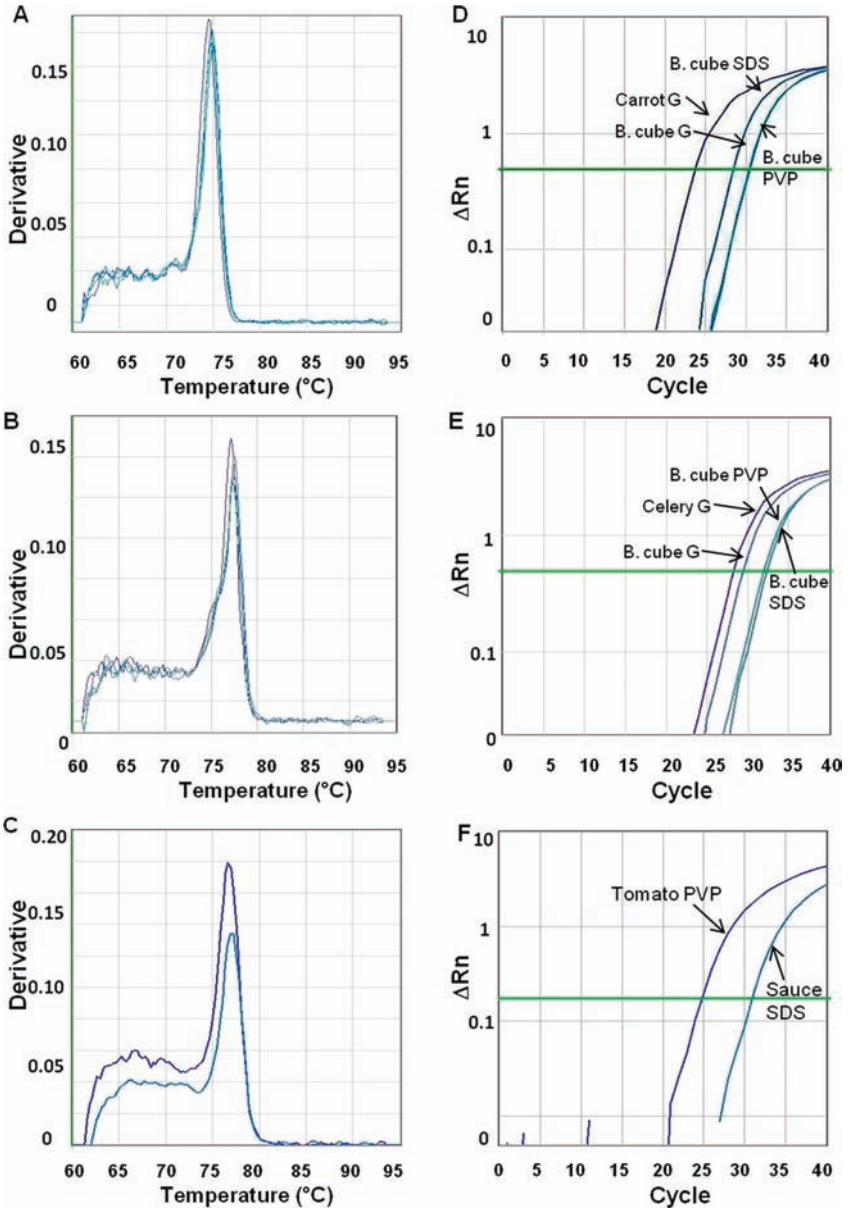

Figure 2. Fast SYBR Green DNA melting curve temperature analyses (Fast SYBR PCR) on vegetables and food products. Derivatives of melting curves and amplification plots of amplicons obtained with (A, D) carrot, (B, E) celery, and (C, F) tomato species-specific primers on vegetable and food DNAs performed on an Applied Biosystems 7900HT Fast Real-Time PCR System. Extraction performed with G, GK-resin method; SDS, SDS method; PVP, CTAB-PVP; or B cube, from bouillon cube.

case of tomato primers (CV = 0.11%). The reproducibility in our case should be better-defined intermediate precision, because it was determined in the same laboratory by two operators working each on three technical replicates, in two days. We obtained a mean C_t of 21.51 ± 0.11 in the case of carrot primers (CV = 0.51%), a mean C_t of 26.15 ± 0.05 in the case of celery primers (CV = 0.19%), and a mean C_t of 25.23 ± 0.12 in the case of tomato primers (CV = 0.47%). These values demonstrate that the results are reproducible and repeatable and thus reliable for routine analysis.

Analysis on Pesto Spiked with Carrot, Celery, or Tomato. Pesto food was chosen as reference matrix to prepare spiked food samples. First, pesto food was checked for the absence of carrot, celery, or tomato also in traces, using species-specific primers, and 18SR primers were used to verify the amplifiability of the DNA (Tables 1 and 3). Because species-specific primers gave negative results in all cases, pesto food could be spiked with decreasing concentrations of each allergenic vegetable individually, from 100 to 0.001 g/kg. DNA was extracted from these spiked samples using the GK-resin method for carrot and celery and the SDS method for tomato. Analyses on these spiked samples were performed in Fast SYBR PCR. A preliminary analysis with 18SR primers confirmed that DNA extracted from all samples could be amplified with the same efficiency. Reactions had an average C_t of 15.5 (Table 3), and statistical analysis confirmed that differences were not significant. Because the samples had the same amplifiability, differences found using the species-specific primers should be attributed to the efficiency of these primers. In fact, all of the species-specific primers amplified their target whenever it was present in the spiked food

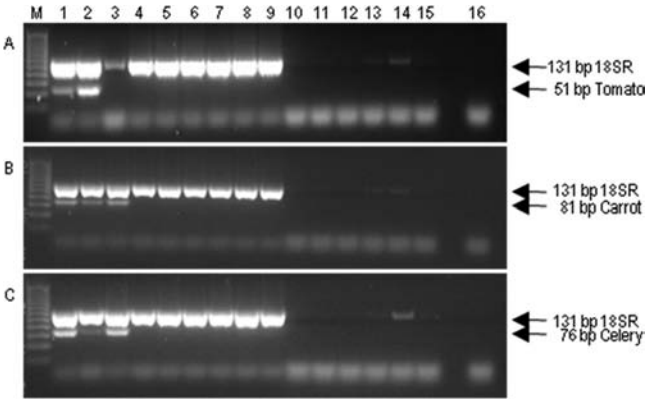

Figure 3. Internal validation and evaluation of specificity in duplex PCRs. Resolution by agarose gel electrophoresis of duplex PCR products was obtained using 18SR primers combined with species-specific primers (A, tomato; B, carrot; C, celery) on the DNA purified from the different plants and foods (lanes 1−16). DNAs were purified from tomato using the (1A) GK-resin, (2A) CTAB-PVP, or (3A) SDS method; from carrot using the (1B) GK-resin, (2B) CTAB-PVP, or (3B) SDS method; and from celery using the (1C) GK-resin, (2C) CTAB-PVP, or (3C) SDS method. DNA was also purified using the GK-resin method from (4) pie, (5) cookies with honey and cashews, (6) cookies with nuts, (7) cookies with chocolate, (8) cookies with eggs, (9) sesame bars, (10) homogenized baby food with kiwi, (11) homogenized baby food with peach, (12) homogenized baby food with apple, (13) yogurt with kiwi, (14) yogurt with peach, and (15) yogurt with apple; (16) negative control. M, 25 base pair ladder. Arrows indicate amplicon sizes.

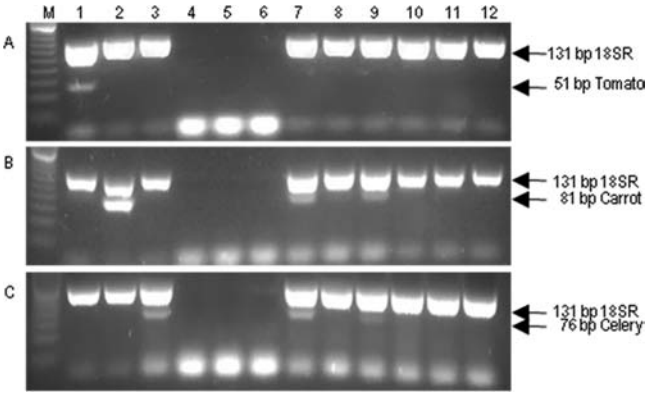

Figure 4. Analysis on food products with duplex PCRs. Resolution by agarose gel electrophoresis of duplex PCR products was obtained using 18SR primers combined with species-specific primers (A, tomato; B, carrot; C, celery) on the DNA purified from the different plants and foods (lanes 1−12). DNAs were purified from tomato using the CTAB-PVP method (1); from carrot (2) and from celery (3) using the GK-resin method; from tomato sauce with hot pepper using the (4) GK-resin, (5) CTAB-PVP, or (6) SDS method; from bouillon cube using the (7) GK-resin, (8) CTAB-PVP, or (9) SDS method; from pesto using the (10) GK-resin, (11) CTAB-PVP or (12) SDS method; and from (13) negative control. M, 20 base pair ladder. Arrows indicate amplicon sizes.

up to 10 mg/kg, whereas carrot and tomato primers were able to detect their targets in pesto food when it was present at 1 mg/kg (Tables 1 and 3). Amplicons' T_m range was within the limit of confidence for acceptability (±0.2 °C) (Table 3). Therefore, the optimized Fast SYBR PCR combined with the analysis of melting curves is a highly specific system of allergenic species detection in complex food matrices, with a LOD comparable with those reported in the literature.[9,12,17,23]

Optimization of Duplex PCRs. Three duplex PCRs were developed, combining the primer pair 18SR, as an internal control, with primer pairs for the specific detection of the allergenic species: tomato, carrot, and celery.

The ribosomal 18S RNA gene is ubiquitous in the eukaryotic genome;[36] primers specific for this target can be used in a PCR reaction to limit the case of false negatives. Primer pairs specific for the allergenic species, on the other hand, will give an amplification product only when the DNA of that species is present in the template. The first step in optimizing duplex PCR regarded the primers' concentration: 0.20, 0.25, 0.30, and 0.40 mM were tried for all combinations of the primers. Good results were found for (i) tomato duplex PCR, 0.20 mM 18SR primers and 0.40 mM tomato primers; (ii) celery duplex PCR, 0.25 mM 18SR primers and 0.25 mM celery primers; (iii) carrot duplex PCR, 0.20 mM 18SR primers and 0.30 mM carrot primers. The optimized duplex PCRs were then tested on DNA extracted from each target matrix with the three methods, as shown in Figure 3. It was evident that for tomato no amplification was found when DNA was extracted with SDS method; only a weak band corresponding to the amplicon of 18SR primers was visible. When DNA was extracted with the CTAB-PVP and GK-resin methods, the amplicons of 18SR (131 bp) and of tomato (51 bp) were generated (Figure 3A). For carrot, DNAs extracted with the three methods gave a positive signal either with 18SR (131 bp) or with carrot (81 bp) primers (Figure 3B). For celery, the amplicon of 18SR (131 bp) was present in all samples; the amplicon of celery (76 bp) was also present in all samples, but when DNA was extracted with the CTAB-PVP method, the amplicon was less abundant (Figure 3C). Duplex PCRs were tested on DNA extracted from several commercial food matrices (Table 1) for which the list of ingredients did not include the allergenic species of this study. For DNA samples extracted from pie and cookies (Figure 3, lanes 4−9) there was an intense band corresponding to the 18SR amplicon (131 bp), whereas no amplicon was obtained with species-specific primers, as expected.

For DNA samples extracted from homogenized baby food and yogurt the amplicon obtained with 18SR primers (131 bp) had a faint intensity, as compared with the positive control, whereas the species-specific primers gave no amplification (Figure 3, lanes 10−15). In all of these cases, the internal control of duplex PCR ensured the correct interpretation of results.

Duplex PCRs were applied on DNA extracted according to the three methods from the foods reported in Table 1, and the amplification results are shown in Figure 4. In the case of commercial sauce with hot pepper (Figure 4, lanes 4−6) the amplification products (18S and tomato amplicon) were not detectable. In the case of bouillon cube (Figure 4, lanes 7−9), the amplification product of 18SR (131 bp) was present in all samples, whereas the amplicon of carrot (81 bp) was present only when DNA was extracted using the GK-resin and SDS methods (Figure 4B); a similar result was obtained for celery (76 bp) (Figure 4C). DNA purified from pesto was amplified only with 18SR (131 bp), as expected (Figure 4, lanes 10−12).

Carrot and celery duplex PCRs can be used to detect their targets also in highly processed food products, whereas for the detection of tomato, more reliable results could be obtained using Fast SYBR PCR. With PCR-based analytical methods, there is certainly the possibility of false-positive and -negative

results. False positives can be recognized and limited by performing the amplification with a negative control. False negatives can be discovered only by using an internal standard, such as primer pairs able to amplify a highly conserved region of the eukaryotic genome. In this work the 18S rRNA gene has been used as an internal control. Figure 4A reports an example of this approach. Duplex PCR performed on DNA from tomato sauce with hot pepper showed no amplification when using both tomato-specific primers and 18SR primers as internal control, indicating that the DNA sample was not properly purified or that the duplex PCR was not sensitive enough for this food sample.

As outlined in Table S1 of the Supporting Information this study goes beyond the existing literature, at least in some aspects: (i) the use of 18S as internal control to reduce false negatives; (ii) the development of a Fast SYBR PCR to cut time of execution and costs; (iii) the successful use of PCR to tag carrot DNA in processed foods. The methods developed are species-specific and therefore will be useful in detecting trace amounts of tomato, celery, or carrot in processed foods. Of course, the implementation of these results at a level of industrial applicability requires further studies. First, on the validation of the methods in a ring test involving different laboratories, we have only performed an interlaboratory test. Second, a stringent evaluation of costs and comparison with existing technologies have not been thoroughly performed yet.

■ ASSOCIATED CONTENT

Supporting Information. Table S1 presents a comparison from the relevant literature and the present study for the plant species described in this paper in terms of: types of samples analyzed, methods for DNA extraction, methods of species detection, and the determined parameters. This material is available free of charge via the Internet at http://pubs.acs.org

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +39 0521905606. Fax: +39 0521905402. E-mail: nelson.marmiroli@unipr.it.

Author Contributions

All authors contributed equally to this work. All authors discussed the results and implications and commented on the manuscript at all stages.

■ ACKNOWLEDGMENT

Project supported by the University of Parma Local Funds (FIL) to NM, by Regione Emilia-Romagna (IT) to SITEIA.PARMA Technopole (POR FESR 2007-2013), and by EU project TRACE-BACK (FP6-2005-FOOD-036300) also to NM. TRACE-BACK is a project supported by European Commission Sixth Framework Programme. The content of this paper does not necessarily reflect the Commission of the European Communities views and in no way anticipates the Commission's future policy in this area.

■ REFERENCES

(1) Vieths, S.; Lüttkopf, D.; Reindl, J.; Anliker, M. D.; Wüthrich, B.; Ballmer-Weber, B. K. Allergens in celery and zucchini. *Allergy* **2002**, *57*, 100–105.

(2) Hernández, M.; Esteve, T.; Pla, M. Real-time polymerase chain reaction based assays for quantitative detection of barley, sunflower, and wheat. *J. Agric. Food Chem.* **2005**, *53*, 7003–7009.

(3) Carcea, M.; Brereton, P.; Hsu, R.; Kelly, S.; Marmiroli, N.; Melini, F.; Soukoulis, C.; Wenping, D. Food authenticity assessment: ensuring compliance with food legislation and traceability requirements. *Quality Assur. Saf. Crops Foods* **2009**, *1*, 93–100.

(4) Commission Directive 2000/13/EC. *Off. J. Eur. Union* **2000**, *L109*, 29−42

(5) Commission Directive 2003/89/EC. *Off. J. Eur. Union* **2003**, *L308*, 15−18.

(6) Commission Directive 2006/142/EC. *Off. J. Eur. Union* **2006**, *L368*, 110−111.

(7) Commission Directive 2007/68/EC. *Off. J. Eur. Union* **2007**, *L 310*, 11−14

(8) Holzhauser, T.; Stephan, O.; Vieths, S. Detection of potentially allergenic hazelnut (*Corylus avellana*) residues in food: a comparative study with DNA PCR-ELISA and protein sandwich-ELISA. *J. Agric. Food Chem.* **2002**, *50*, 5808–5815.

(9) van Hengel, A. J. Food allergen detection methods and the challenge to protect food-allergic consumers. *Anal. Bioanal. Chem.* **2007**, *389*, 111–118.

(10) Di Pinto, A.; Forte, V. T.; Guastadisegni, M. C.; Martino, C.; Schena, F. P.; Tantillo, G. A comparison of DNA extraction methods for food analysis. *Food Control* **2007**, *18*, 76–80.

(11) Pafundo, S.; Gullì, M.; Marmiroli, N. SYBR GreenER real-time PCR to detect almond in traces in processed food. *Food Chem.* **2009**, *116*, 811–815.

(12) Poms, R. E.; Klein, C. L.; Anklam, E. Methods for allergen analysis in food: a review. *Food Addit. Contam.* **2004**, *21*, 1–31.

(13) Pafundo, S.; Gullì, M.; Marmiroli, N. Multiplex real-time PCR using SYBR GreenER for the detection of DNA allergens in food. *Anal. Bioanal. Chem.* **2010**, *396*, 1831–1839.

(14) Yeung, J. Enzyme-linked immunosorbent assays (ELISAs) for detecting allergens in foods. In *Detecting Allergens in Food*; Koppelman, S. J., Hefle, S. L., Eds.; CRC Press: Boca Raton, FL, 2006; pp 109−124.

(15) Holzhauser, T.; Stephan, O.; Vieths, S. Polymerase chain reaction (PCR) methods for the detection of allergenic foods. In *Detecting Allergens in Food*; Koppelman, S. J., Hefle, S. L., Eds.; CRC Press: Boca Raton, FL, 2006; pp 125−143.

(16) Marmiroli, N.; Maestri, E. Polymerase chain reaction (PCR). In *Food Toxicants Analysis. Techniques, Strategies and Development*; Picò, Y., Ed.; Elsevier: Amsterdam, The Netherlands, 2007; pp 147−187.

(17) Kirsch, S.; Fourdrilis, S.; Dobson, R.; Scippo, M.-L.; Maughuin-Rogister, G.; De Pauw, E. Quantitative methods for food allergens: a review. *Anal. Bioanal. Chem.* **2009**, *395*, 57–67.

(18) Stack, S. M.; Royer, S. M.; Shearer, L. A.; Chang, S. B.; Giovannoni, J. J.; Westfall, D. H.; White, R. A.; Anderson, L. K. Role of fluorescence in situ hybridization in sequencing the tomato genome. *Cytogenet. Genome Res.* **2009**, *124*, 339–350.

(19) Petersen, A.; Vieths, S.; Aulepp, H.; Schlaak, M.; Becker, W. M. Ubiquitous structures responsible for IgE cross reactivity between tomato fruit and grass pollen allergens. *J. Allergy Clin. Immunol.* **1996**, *98*, 805–815.

(20) Crespo, J. F.; Rodriguez, J. Food allergy in adulthood. *Allergy* **2003**, *58*, 98–113.

(21) Yang, X.; Quiros, C. F. Characterizing the celery genome with DNA-based genetic markers. *J. Am. Soc. Hortic. Sci.* **1995**, *120*, 747–751.

(22) Yang, X.; Quiros, C. F. Construction of a genetic linkage map in celery using DNA-based markers. *Genome* **1995**, *38*, 36–44.

(23) Hupfer, C.; Waiblinger, H.-U.; Bush, U. Development and validation of a real-time PCR detection method for celery in food. *Eur. Food Res. Technol.* **2007**, *225*, 329–335.

(24) Ballmer-Weber, B. K.; Wüthrich, B.; Wangorsch, A.; Fötisch, K.; Altmann, F.; Vieths, S. Carrot allergy: double-blinded, placebo controlled food challenge and identification of allergens. *J. Allergy Clin. Immunol.* **2001**, *108*, 301–307.

(25) Bennett, M. D.; Smith, J. B. Nuclear DNA amount in angiosperms. *Philos. Trans. R. Soc. London B: Biol. Sci.* **1991**, *B334*, 309–345.

(26) Mustorp, S.; Engahl-Axelsson, C.; Svensson, U.; Holck, A. Detection of celery (*Apium graveolens*), mustard (*Sinapis alba*, *Brassica juncea*, *Brassica nigra*) and sesame (*Sesamum indicum*) in food by real-time PCR. *Eur. Food Res. Technol.* **2008**, *226*, 771–778.

(27) Dovîĉovîĉová, Ľ.; Olexová, L.; Pangallo, D.; Siekel, P.; Kuchta, T. Polymerase chain reaction (PCR) for the detection of celery (*Apium graveolens*) in food. *Eur. Food Res. Technol.* **2004**, *218*, 493–495.

(28) Chaouachi, M.; El Malki, R.; Berard, A.; Romaniuk, M.; Laval, V.; Brunel, D.; Bertheau, Y. Development of a real-time PCR method for the differential detection and quantification of four solanaceae in GMO analysis: potato (*Solanum tuberosum*), tomato (*Solanum lycopersicum*), eggplant (*Solanum melongena*), and pepper (*Capsicum annuum*). *J. Agric. Food Chem.* **2008**, *56*, 1818–1828.

(29) Turci, M.; Savo Sardaro, M. L.; Visioli, G.; Maestri, E.; Marmiroli, M.; Marmiroli, N. Evaluation of DNA extraction procedures from traceability of various tomato products. *Food Control* **2010**, *21*, 143–149.

(30) Zagon, J.; Jansen, B.; Knoppik, M.; Ehlers, A.; Kroh, L. W.; Holzauser, T.; Vieths, S.; Broll, H. Gene transcription analysis of carrot allergens by relative quantification with single and duplex reverse transcription real-time PCR. *Anal. Bioanal. Chem.* **2010**, *396*, 483–493.

(31) Doyle, J. J.; Doyle, J. L. Isolation of plant DNA from fresh tissue. *Am. J. Bot.* **1988**, *75*, 1238.

(32) Meyer, R.; Chardonnens, F.; Hübner, P.; Lüthy, J. Polymerase chain reaction (PCR) in the quality and safety assurance of food: detection of soya in processed meat products. *Z. Lebensm. Unters. Forsch. A* **1996**, *203*, 339–344.

(33) Vickers, A. J. Parametric versus non-parametric statistics in the analysis of randomized trials with non-normally distributed data. *BMC Med. Res. Methodol.* **2005**, *5*, 35–46.

(34) Cankar, K.; Štebih, D.; Dreo, T.; Žel, J.; Gruden, K. Critical points of DNA quantification by real-time PCR – effects of DNA extraction method and sample matrix on quantification of genetically modified organisms. *BMC Biotechnol.* **2006**, *6*, 37.

(35) Pafundo, S.; Agrimonti, C.; Marmiroli, N. Traceability of plant contribute in olive oil by AFLPs. *J. Agric. Food Chem.* **2005**, *53*, 6995–7002.

(36) Zhu, L.-J.; Altmann, S. W. mRNA and 18S-RNA coapplication-reverse transcription for quantitative gene expression analysis. *Anal. Biochem.* **2005**, *345*, 102–109.