

Detection of allergen coding sequences of kiwi, peach, and apple in processed food by qPCR

Sara Graziano ^a, Mariolina Gulli ^{a,b*} and Nelson Marmioli ^{a,b}

^a Department of Chemistry, Life Sciences and Environmental Sustainability, University of Parma,
Parco Area delle Scienze 11/A, 43124 Parma, Italy

^b Interdepartmental Center SITEIA.PARMA, University of Parma, Parco Area delle Scienze 181/A,
43124 Parma, Italy

*Corresponding author

*Phone: +39 0521905486. E-mail: mariolina.gulli@unipr.it.

ABSTRACT

BACKGROUND: Food traceability becomes lifesaving for persons suffering severe allergy or intolerance, and therefore need a complete avoidance of the immune-trigger food. This paper describes how to fingerprint the presence of some allergenic species (kiwi, peach, and apple) in foods by quantitative Real-Time PCR (qPCR).

RESULTS: Five DNA extraction procedures were tested on fruits and foods. The results were statistically evaluated, and discussed. Analysis by qPCR with SYBR Green was developed to detect traces of these allergenic species in foods. Plasmids containing the target sequences of kiwi, peach and apple were employed as Internal Reference Standard. Analysis of spiked food samples showed a Limit of Detection of 25 mg kg⁻¹ for kiwi, 20 mg kg⁻¹ for peach and 50 mg kg⁻¹ for apple.

CONCLUSION: The qPCR method here developed, combined with the use of Internal Plasmid Reference Standard, represent a specific system for the quick detection of allergenic species in

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/jsfa.8814

complex food matrices, with a Limit of Detection comparable with those reported using more time-consuming methods.

KEYWORDS: Food Allergens; DNA extraction; SYBR Green; plasmid reference material.

RUNNING TITLE: Fruits detection in food by qPCR

INTRODUCTION

Food allergies are considered by the World Health Organization (WHO) among the five most important public health concerns¹⁻³, particularly in industrialized countries, where the affected population represents approximately 2% of adults and up to 4-8% of children.¹ Fruits and vegetables allergies are among the more common: 0.4-6.6% of adults and 2.2-11.5% of children report adverse reaction to fruits.⁴

Food allergy should be distinguished from food intolerance. The first one depends on the stimulation of a specific immune response, whereas the latter represents an adverse non-immunological reaction to foods or food ingredients.¹ The allergenic proteins have been identified and characterised in order to elucidate their properties and their cross-reactivity patterns.⁴ It has been clarified that Pollen-Food Syndrome (PFS) and Lipid Transfer Protein (LTP) syndrome are the most frequent fruit and vegetable allergies.⁴ The only treatment available for the allergic consumers is the avoidance of the allergenic food. Unfortunately, this lowers the dietary choices and the quality of life. The declaration of food allergens on the label may help the correct allergen management. Unfortunately, there is the extensive use of precautionary labelling (i.e. 'may contain' or 'made on the same line as'), and the cross-contamination during food production is frequent.^{5,6}

In order to guarantee the consumers' need for food safety, countries have adopted specific legislation for food labeling. In particular, the presence of some allergenic ingredients or potential contaminants must be declared on the label.⁷⁻¹⁰ Molecular methodologies have been developed to target proteins or DNA of the allergenic species.^{7,11} Proteins can be detected by the enzyme-linked immuno-absorbent assay (ELISA)¹² which has several advantages and disadvantages. The main disadvantages depends on i) the complexity of method development; ii) the quality of the antibody which may differ from

batch to batch, iii) the cross-reactivity, due to homology between the antibody recognition site of allergenic proteins, which may determine false positive results; iv) positives results must be confirmed; v) high frequency of false negative due to matrix effect or to food processing, and viii) analysis in a multiplex format is not yet possible. Furthermore, the ELISA tests use animal antibodies that not necessarily recognize the same allergenic epitopes to which the allergic individuals react, for this reason they should be considered an indirect test.¹²

Over the last years, analytical methods based on High Performance Liquid Chromatography-Tandem Mass Spectrometry (HPLC-MS/MS) or on liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS) have been developed for the determination of food allergens.^{13,14} They demonstrated high sensitivity, and specificity, comparable with ELISA methods, with the advantage to allow a multiplex format.

DNA tests are alternative methods for allergen detection, they are particularly appropriate for the analysis of processed food, since DNA molecules are more resilient to chemical and physical treatments⁷ compared to proteins. The specificity and sensitivity of the reactions are determined by the use of specific primers and probes, and reagents for PCR are easily available with constant quality. Moreover, DNA based tests are less affected by matrix effects than ELISA. The main advantage of DNA tests is the possibility to perform multiplex analyses and for this reason they are widely used to detect specifically both animal and plant species, including those potentially allergenic.¹¹

According to the European Community Respiratory Health Survey, among the different foods tested, human sensitization to fruits is dominated by hazelnut (7.2%), peach (5.4%), apple (4.2%), peanut (2.6%), kiwi (3.6%), and banana (2.5%), followed by walnut (2.2%) and melon (1.6%).^{15,16}

Considering that the most common cross-reaction is between birch pollen and foods (i.e. hazelnut,

peach, and apple), if all birch allergic subjects would be excluded from the population, hazelnut and peach would remain among the four most common food sensitizers. In Italy, apple sensitization is the third highest among the countries studied, even if sensitization to birch is relatively rare.^{17,18}

Allergy to kiwi (*Actinidia deliciosa*) represents the prototype of novel food-induced allergies. Although known as an exotic fruit in the 1980s, kiwi is now among the top 10 food allergy sources according to recent studies in Finland, Sweden, and France.¹⁹ Up to now, the official IUIS Allergen Nomenclature database (www.allergen.org) reports 13 allergens from green kiwi. The papain-like cysteine protease, actinidin Act d 1 (30 kDa), represents a major allergen and is also abundantly expressed in the ripe fruit.²⁰

Peach (*Prunus persica*) is an angiosperm and belongs to the *Rosaceae* family. Allergy to peach is the most prevalent plant food allergy in the Mediterranean area.²¹ Among patients, 80% are sensitized to a nonspecific lipid transfer protein (nsLTP, Pru p 3), which is mainly found in the peel of the fruit²², and more recently Pru p 2 is recognized as the major allergen.²³ In fact, three members of the thaumatin-like protein (TLP) family have been identified, Pru p 2.0101, Pru p 2.0201, and Pru p 2.0301. as immune-triggers of Spanish patients.

Malus domestica is a species of domesticated apple widely cultivated in all continents (except Antarctica), and cultivars have been confirmed to possess various degrees of allergenicity. The fruit is consumed fresh or cooked, and as a spice, it is added in many processed foods, like spice mixtures, soups, broths, and salad dressings.

These allergenic species are items for which labelling is recommended in the Japanese legislation¹⁰, but not in the revised EU Labelling Directive⁹.

Detection methods that can differentiate peach or apple from other *Rosaceae* fruits should be

beneficial to allergic patients by reducing unnecessary avoidance of foods containing *Rosaceae* fruits other than peach or apple. Methodologies based on DNA detection have been developed to trace these fruit species in food. In particular, Watanabe *et al.*²⁴ reported two PCR-based methods for the specific detection of peach (*P. persica*) and apple (*M. domestica*), using as target sequences the trnS-trnG intergenic spacer (trnS-trnG IGS) region and the internal transcribed spacer-1 (ITS-1) region, respectively. The method is based on a PCR amplification followed by electrophoresis on agarose gel and has a sensitivity of about 10 ppm of the ingredient.

Similarly, Taguchi *et al.*²⁵ reported a PCR-based method for the detection of commercially grown *Actinidia* spp. targeting ITS-1 region. Other methods available are based on immunoenzymatic detection of the same allergenic protein.²⁶

ELISA methods for the quantitation of peach LTP (Pru p 3)²⁷, apple LTP (Mal d 1)²⁸, and kiwi (Act d 2)²⁹ exist, but PCR methods should still be useful for confirmation of positive results.²⁴

The quantification of allergens rests on the availability of “Certified Reference Material” (CRM).³⁰ Although, while CRM for many genetically modified organisms (GMO) are commercially available, those for food allergens are lacking. Recombinant plasmids containing species-specific target sequences can be obtained to serve as an “Internal Plasmid Reference Standard” (IPRS). IPRS has been exploited to detect allergens in food.^{31,32} In conjunction with SYBR Green I, they have been used to quantify materials contaminated by almonds (*Prunus dulcis*).³³

This paper reports the development of three qPCR assay targeting genes encoding for the allergenic proteins Act c 2, Mal d 1, and Pru p 2, for the detection of kiwi, peach and apple respectively. Recombinant plasmids containing the target region of these genes were constructed and tested as reference material. Reaction efficiency, linear range, and limit of detection of the method were

evaluated. This type of assay is important when implementing the PCR analytical procedure for industrial application.

MATERIALS AND METHODS

Food materials. All plants and foods were purchased from local markets and stored at 4°C in the dark. The main characteristics of the allergenic species studied in this work are shown in Table S1. A complete list of the sample analysed is given in Table 1. The commercial homogenized baby food contained 25%, 55% and 91% respectively of kiwi, peach and apple, according to what reported on the label; while each yogurt contained the 15% of fruit preparation with the 60%, 63% and 65% of kiwi, peach and apple, respectively.

Biscuits samples spiked with kiwi, peach, and apple. Spiked samples were prepared as suggested by Abbott *et al.*³⁴ Commercially available biscuits were checked for the absence of kiwi, apple, and peach before spiking. 30 grams of biscuits were spiked with 600 mg of each dried fruits singularly, to have a final concentration of 20 g kg⁻¹. The level of moisture was 5.3% in fruits and 6% in biscuits. The spiked samples were then serially diluted (ten-fold or two-fold) to obtain biscuits containing 2000, 200, 100, 50, 25, or 20 mg kg⁻¹ of kiwi, peach, and apple. Three independent samples were prepared for each dilution.

DNA extraction. All samples listed in Table 1 were ground using Knifetec™1095 (Foss Tecator AB, Höganäs, Sweden).

Fresh fruits and commercial foods. DNA was extracted using five different methods as described by Pafundo *et al.*³⁵ with some modifications: i) CTAB-SDS (Cetyltrimethylammonium Bromide-

Sodium Dodecyl Sulfate) method was modified using 200 mg of sample and adding 20 mg L⁻¹ of CTAB to the extraction buffer; ii) CTAB –PVP (Polyvinylpyrrolidone) method, using 1 g of sample; iii) GK-resin method, using 300 mg of sample; iv-v) Acetone-SDS (ACSDS) and Acetone-PVP (ACPVP) methods are the CTAB-SDS and CTAB-PVP methods with an Acetone pre-treatment as described by Mehar *et al.*³⁶ To carry out the acetone pre-treatment, samples were ground to a fine powder using liquid N₂. The powder was suspended in pre-cooled 90% acetone, and poured into a glass funnel whose base was covered with a Whatman filter paper (n.1) and inserted into a conical vacuum flask connected to a water vacuum pump. To this mixture, 90% acetone was added continuously until the tissue looked completely white. DNA was then extracted from 1 g of cleared, powdered sample. Two independent extractions (biological replicates) and a negative control were performed for each method.

Leaves, seeds, and nuts. DNA was purified with the CTAB method, as previously described.³⁵

Spiked food samples. DNA was extracted from biscuits and spiked biscuits using the GK-resin method as previously described.³⁵

DNA quantification and determination of purity. DNA was quantified with a spectrophotometer VARIAN Cary® 50 UV-VIS (Agilent Technologies, California, USA), by measuring the absorbance (A) at 260 nm and 280 nm. Quality of DNA was estimated by agarose gel electrophoresis and by evaluation of the A₂₆₀ / A₂₈₀ ratio.

Target Gene Selection and Primer Design. Nucleotide sequence information of kiwi, peach, and apple 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 (Life Technologies, Foster City, CA, USA), and termed: Act, Pru and Mal (Table 2). Primer specificity was preliminarily tested *in silico* using the BLAST program (<http://blast.ncbi.nlm.nih.gov/>) (Table S2). Primers were purchased from Sigma (Sigma Aldrich Corporation, St. Louis, MO, USA).

Cloning and sequencing of PCR amplicons. PCR products obtained with the species-specific primers Act, Pru and Mal were cloned using the pGEM®-T Easy Vector System (Promega, Fitchburg, WI, USA). 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, using M13 universal primers. Sequences were analysed and compared using the program ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>).

PCR, and qPCR Conditions. End-point PCR amplification was carried out in a final volume of 25 µl, containing 50 ng of DNA, and 0.25 µM of each forward and reverse primer, 2X GoTaq® Colorless Master Mix (Promega), in a TC-512 Thermal cycler (Techne, Duxford, Cambridge, UK). The master mix is a commercial premixed solution containing a non-recombinant modified form of Taq DNA polymerase lacking the 5'→3' exonuclease activity, 2X Colorless GoTaq® Reaction Buffer (pH 8.5), 400 µM of each dNTPs and 3 mM MgCl₂. PCR conditions were as follows: 5 min initial denaturation at 95 °C followed by 40 cycles with 50 s denaturation at 95 °C, 50 s annealing at 58 °C, 1 min elongation at 72 °C; 10 min final extension at 72 °C. Amplification products were analysed by electrophoresis on 3% (w/v) agarose gel, stained with GelRed™ Nucleic Acid Gel Stain 10000X (Biotium, Hayward, CA, USA).

qPCR with SYBR Green was carried out using the Applied Biosystems 7900HT Fast Real-Time PCR

System (Life Technologies). Reactions were carried out in a final volume of 25 μ l, using 50 ng of DNA with 1X Sybr[®] Premix Ex Taq[™] II (Takara Bio Inc. Shiga, Japan). Different primer concentrations were tested (0.10, 0.15, 0.25, and 0.40 μ M) to obtain the best performing condition which resulted in 0.25 μ M for Act, 0.40 μ M for Mal and Pru, and 0.10 μ M for 18S primer pairs. Cycling conditions were: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 60 s. The amplification process was followed by a ‘dissociation’ stage, during which the temperature was increased from 60 to 95 °C at a rate of 0.22 °C s⁻¹ to produce a melting curve. For all samples, three technical replicates of each biological replicates, and a negative control were performed. To evaluate primer efficiency, IPRS DNA was serially diluted (10-fold) from 1 x 10⁸ to 1 copy of plasmid. For all samples, three technical replicates of two biological replicates, and no template control were analyzed. For each sample, Ct (threshold cycle) value is obtained as the intersection between each amplification curve in an exponential phase, and the threshold line, using a default setting. The standard curve was represented by a semi-logarithmic regression line plot of Ct value vs. log of input nucleic acid and was generated by SDS 2.3 software (Life Technologies). The standard curve was calculated as $y = -ax + b$. The efficiency of the reaction (E) was calculated as $E = (10^{-1/a})$, and the percentage of Efficiency was $(E - 1) \times 100\%$.³⁷

Statistical analyses. Statistical analyses were performed using the software SPSS 21.0.0 for Windows (© Copyright IBM Corporation 1989, 2012). Non-parametric tests were chosen because of a non-normal distribution of data.³⁸ For the statistical evaluation of the effect of different methods or different matrices on yield and purity of DNA extracted, the non-parametric Kruskal-Wallis test and Mann-Whitney U test were applied, when appropriate. When significant results were obtained with the Kruskal-Wallis test, a pairwise comparison was performed and the p values were adjusted by the

Bonferroni correction for multiple tests.

RESULTS AND DISCUSSION

Comparison of DNA extraction methods and statistical analysis. The DNA extracted from fruits and foods were evaluated using the following parameters: *i*) DNA yield, as ng/100 mg fresh weight (FW); *ii*) DNA purity, as ratio A_{260}/A_{280} , which should be in a range between 1.8 and 2.0, according to Cankar *et al.*³⁹ ; *iii*) amplificability by PCR with 18S or CLOR primers. Yield and A_{260}/A_{280} ratio of the DNA samples were evaluated respect to the extraction method (Table 3). The ACPVP method yielded the highest amount of DNA from all samples, except for homogenised baby food with kiwi. It performed at best on homogenised baby food with apple. This method was, therefore, the most efficient for DNA extraction from fresh fruits and it gave DNA of good quality, as shown by DNA yields and by A_{260}/A_{280} ratio (Table 3).

ACSDS, CTAB-SDS, and CTAB-PVP methods gave a similar yield of DNA, with a range from 49 to less than 1 ng/100 mg of fresh weight (FW). GK-resin did not give good results; in fact, the yield and the quality of DNA were not satisfactory when referred to fresh fruit and commercial food. The use of a resin for DNA isolation but not of organic solvents does not eliminate the polysaccharides and polyphenols, present in high amount in the fruits, which are strong inhibitors of PCR reaction.

The comparison of yield and quality (ratio A_{260}/A_{280}) of DNA purified with the same extraction method from different matrices was made using two approaches. First, an evaluation of arbitrary scores attributed to methods (Table 3) and a non-parametric Kruskal-Wallis test (Figure S1). The following pairwise comparison of samples, grouped according to the extraction method, showed that

the A_{260}/A_{280} ratio obtained with ACPVP method was significantly different from that obtained with GK-resin ($p = 0.000$) and with CTAB-SDS ($p = 0.020$). The DNA yield obtained with GK-resin method was significantly lower compared to that obtained with CTAB-PVP ($p = 0.030$) or with ACPVP ($p = 0.000$), similarly CTAB-SDS resulted significantly different from ACPVP ($p = 0.005$).

The analysis of samples grouped according to food categories (fruit, yogurt and homogenized food) showed significant differences for fresh fruits and homogenized baby food using the Kruskal-Wallis test (Figure S2). In particular, the pairwise comparison showed that the yield of DNA purified from fresh fruits was significantly higher when using ACPVP ($p = 0.035$), and the A_{260}/A_{280} ratio was significantly higher when using ACPVP ($p = 0.024$) on homogenized baby food.

A global evaluation of the performance of the five different methods allowed ranking them in the following decreasing order: ACPVP > CTAB-PVP > ACSDS > CTAB-SDS > GK-resin.

Amplificability was tested with end-point PCR using 18S or CLOR primers, both of which tag highly conserved sequences in eukaryotes. DNA was amplified in all samples tested, with few exception (i.e. homogenized baby food with kiwi or apple extracted with CTAB-PVP and ACPVP, homogenized baby food with peach extracted using CTAB-PVP, ACPVP and ACSDS, and yogurt with apple extracted with ACPVP or ACSDS) (Table 1).

PCR inhibitors such as polysaccharides, humic acids, polyphenols, which can affect the efficiency of the reaction, are, unfortunately, common in many food matrices.⁴⁰⁻⁴² Therefore, the development of DNA-extraction methods, which reduce the presence of those molecules in the reaction mix, is required. Indeed, different authors^{7, 40-42} reported the need to identify a DNA extraction method that gives optimal results in term of DNA yield and purity. The extraction procedures here developed yielded DNA in both good quantity and quality, in particular, the use of PVP and SDS combined with

either acetone or CTAB allowed obtaining DNA samples adequate for the following PCR analysis. Therefore, considering the three parameters: DNA yield, purity, and amplificability, the preferred methods for DNA extraction were: ACPVP for fresh fruits and for yogurt with peach; ACSDS for yogurt with kiwi and homogenized baby food with kiwi and apple; CTAB-SDS for homogenized baby food with peach; and CTAB-PVP for yogurt with apple.

Evaluation of Primer's design. Species-specific primer pairs were designed to detect traces of allergenic species: *Actinidia deliciosa*, *Prunus persica*, and *Malus domestica*, in processed foods. The *in silico* specificity showed that the primer sequences had 100% identity only with the selected targets (Table S2). Primers are therefore specific for genes encoding known allergenic proteins.

Primers Act, Pru, and Mal were tested individually on DNA extracted from each target species, using end-point PCR and amplicons were loaded on 3% agarose gel (data not shown). Each amplicon was purified from the gel, cloned, and sequenced. In each case, the sequence obtained was identical to the sequence used for primers' design.

qPCR with SYBR Green, DNA Melting Curve Temperature Analyses.

Use of recombinant plasmids as reference material. The sensitivity of the method was evaluated using plasmid dilution series, to produce the calibration curves by qPCR. The standard curves were in agreement with ENGL⁴³ acceptance criteria (slope values between -3.1 and -3.6 and $R^2 > 0.98$). The Act standard curve was $y = -3.05x + 43.90$ ($R^2 = 0.98$) and the efficiency was 108.9%, the Pru standard curve was $y = -3.48x + 43.98$ ($R^2 = 0.99$) and the efficiency was 90.5% and the Mal standard curve was $y = -3.35x + 45.26$ ($R^2 = 0.99$) and the efficiency was 94.9%. The Limit of Detection (LOD) of the method was set respectively at 10 copies for Act and Mal corresponding to 0.8 and 1.1 ng of tissue, and at

10^2 copies for Pru, corresponding to 0.3 ng of tissue. The Limit of Quantification (LOQ) was set respectively 10^2 copies for Act and Mal, and 10^3 copies for Pru. As shown in Fig. 1, comparing the melting curves, the use of plasmid as the reference standard, allowed the identification of the specific amplicon in complex food samples. In fact, in all cases, a single peak with a specific T_m was identified. Using the standard curves obtained with IPSR it was also possible to quantify the copies of species-specific targets contained in the DNA samples purified from food products. In particular, 173, 103, and 7 copies of kiwi, peach and apple target genes were detected in yogurt, and 28, 15, and 33 copies in homogenised baby food.

Analysis with 18S primers on fresh fruits and foods. The ribosomal 18S RNA gene is ubiquitous in the eukaryotic genomes⁴⁴, therefore primers specific for this target can be used in a PCR reaction to reduce false negatives. The DNA extracted from fresh fruits or food products with the best performing method, was amplified with 18S primers, and the C_t values obtained are reported in Table 4. The reactions had an average C_t of 19.60, with the exception of kiwi (average C_t 23.73). The difference of 4 cycles in C_t in the case of kiwi can be attributed mainly to the ploidy level and to its genome size⁴⁵ (4.40 pg), which is much higher than apple's (1.56 pg), and peach's (0.62 pg) genome (Table S1).

To verify if differences in C_t values were related to the heterogeneity of the tested materials (fruits and foods), data were divided into two groups, named respectively "Fruits" (kiwi, peach and apple) and "Foods" (yogurt and homogenised baby food). Here, using the Mann-Whitney U test, no significant differences were found (Table S3). Therefore, food processing did not significantly influence the amplificability with 18S primers of the DNA extracted. This result may depend also on the fact that 18S primer targets a multicopy sequence.

Analysis with species-specific primers on fresh fruits and foods. The species-specific primers listed

in Table 2 were used to amplify DNA extracted from fresh fruits by qPCR. Primer concentration was 0.25 μ M for Act and Pru, and 0.40 μ M for Mal primer. The T_m of each amplicon was evaluated from the melting curves as described in Materials and Methods section, and the results are reported in Fig. 1 and Table 4. A single peak, with no trace of unspecific products or of primer-dimers was detected in each case. The mean T_m values obtained were: i) 81.65 °C using Act primers on kiwi DNA (Fig. 1A), ii) 77.20 °C using Pru primers on peach DNA (Fig. 1C) and iii) 79.80°C using Mal primers on apple DNA (Fig. 1B and Table 5). DNA extracted from leaves, nuts, and seeds of different plant species were tested as non-target species to confirm primers' specificity. No amplification was observed for non-target species (Table 1).

Act, Pru and Mal primers were also used on DNA purified from yogurt and homogenized baby food containing kiwi, peach, and apple as ingredients. As shown in Fig. 1, primers recognized the specific targets also in processed matrices. In all reactions, the amplification curve reached the exponential phase later than with 18S primers. The average C_t values obtained were: 24.52 ± 0.19 using Act primers on kiwi DNA, 28.08 ± 0.08 using Pru primers on peach DNA, and 30.88 ± 0.09 using Mal primers on apple DNA (Table 4). False positives were not found.

A statistical analysis was performed to assess the presence of a “processing effect” on amplificability with species-specific primers. At this purpose, the samples were divided into two groups, named respectively “Fruits” and “Food” and, using the Mann-Whitney U test, differences in C_t values resulted significantly different ($p=0.024$) (Table S3). The species-specific primers target single copy sequences, making any effect of processing on DNA integrity more evident. At this purpose, it is worth to remind that the processing of yogurt with fruit includes pasteurization at high temperatures, fermentation (low pH), mechanical homogenization, and peeling, chopping and cooking of fruits at

high temperatures. Similarly, preparation of homogenised baby foods with fruits includes mechanical and thermal treatments; all these treatments are known to degrade DNA^{46,47} and consequently affect PCR. Nevertheless, the amplification is not compromised when the target sequences are very short (< 100bp).

Tests on spiked food samples. Biscuits were chosen as reference matrix to prepare spiked food samples. First, biscuits were checked for the absence of kiwi, apple and peach in traces, using species-specific primers and 18S specific primers to verify the amplificability of the DNA (Table 1). In all cases, no amplification was obtained with species-specific primers, but only with 18S primers. Biscuits were then spiked as described, and DNA was extracted using GK-resin protocol, and tested by end-point PCR using species-specific primers (Fig. 2). The GK-resin extraction method was utilized on spiked samples since it performs well when used in commercial baked products such as biscuits, according to previous results.³⁵ Samples were then analyzed by qPCR. The species-specific primers amplified their targets in the spiked food down to 20, 25, and 50 mg kg⁻¹ (respectively for peach, kiwi, and apple, see Table 5). Amplicons' T_m range corresponded to that expected for the fruits analyzed, and the C_t values measured with species-specific primers were correlated with the spiking level. However, the evaluation of reaction efficiency in this set of samples showed that in the case of biscuits spiked with peach no inhibition was present ($y = -3.27x + 42.72$, $R^2 = 0.99$), while in biscuits spiked with kiwi or apple an inhibition effect is evident.

The qPCR here developed is a specific test for the detection of allergenic fruit species in food, with a LOD comparable with those reported using ELISA methods.²⁷⁻²⁹

Conclusions. Kiwi, peach, and apple contain food allergens that constitute a source of growing

concern for consumers and for food producers. Indeed, their allergenicity is an emerging problem. Some authors developed methods for the detection of allergenic proteins based on ELISA^{27,29}. The TLP allergen of kiwi was detected in kiwi extracts as well as in kiwi-containing food products, however, it was not highly specific only for kiwi, since it could detect the TLP of banana, bell pepper, apple, and cherry, due to a certain degree of epitope homology between Act d 2 and other TLP.²⁹ Gao and coauthors²⁷ reported the use of a monoclonal antibody-based sandwich ELISA with a sensitivity threshold of 0.5 ng/ml of Pru p 3. However, when different fruit extracts were tested, also apricot, cherry, and plum extracts showed high reactivity may be due to the amino acids that are conserved in LTP of stone fruit differently from other *Rosaceae* fruits.

Furthermore, some end-point PCR methods were developed for the detection of allergenic fruits species. The methods for the specific detection of peach and apple²⁴ were applied on incurred foods, but no commercial samples were tested, while the method for kiwi detection²⁵ was tested also on commercial products, containing or not containing kiwi as an ingredient. In both cases, the authors used an end-point PCR that had a LOD of 10 ppm, but they could not perform the quantification of the allergenic species.

In this study it is proposed: i) the identification of DNA extraction protocols suited for the matrices analysed; ii) the exploitation of a conserved sequence (18S) as internal control to check for false negatives; iii) the development of a qPCR with SYBR Green combined with the analysis of dissociation curves of amplicons which is not available for the species analyzed. This is a highly specific detection system; it can cut down time of execution and costs of the analysis, compared to endpoint PCR. In fact, there is no need for gel visualization, and it is applicable to both highly processed samples, and to fragmented DNA; iv) the production of IPRS containing the species-

specific amplicon of kiwi, peach or apple, which can be used as reference material.

The method developed is species-specific. Therefore, it will be appropriate for detecting trace amount of kiwi, peach, or apple in processed foods. Further studies would be necessary to apply these results at the industrial level. In particular, a collaborative method validation study would be necessary to find out whether the analytical method performs well, and it is fit for its intended purposes.

ACKNOWLEDGEMENTS

This study was funded by the EuroTransBio project Allergotype E01/0885/02/X21 and by Emilia-Romagna Region (IT) (POR FESR 2007-2013) to NM.

AUTHORS' CONTRIBUTIONS

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

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest

The authors declare that they have no competing interests.

Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent

Informed consent is not applicable for the nature of this study.

Supporting Information

Figure S1: Comparison of DNA extraction methods: statistical analysis.

The five DNA extraction methods were compared considering yield (A) and A_{260}/A_{280} ratio (B) of the DNA purified from all samples (n=45). Graphs show the results of Kruskal Wallis test, and tables show pairwise comparisons. The p values were adjusted by the Bonferroni correction for multiple tests, and significant differences are bolded.

Figure S2: Comparison of DNA extracted from fresh fruits and homogenized baby foods: statistical analysis.

The five DNA extraction methods were compared considering yield (A) of the DNA purified from fresh fruits (n=15) and A_{260}/A_{280} ratio (B) of the DNA purified from homogenized baby foods (n=15). Graphs show the results of Kruskal Wallis test, and tables show pairwise comparisons. The p values were adjusted by the Bonferroni correction for multiple tests, and significant differences are bolded.

Table S1: Allergenic fruit species studied.

Table S2: In silico analysis of primer pairs specificity using BLAST software (last accessed on 30 August 2017). The tables show the accession numbers, descriptions, and statistical parameters of the sequences matching with each primer pair. Accession number and target sequence used for primer design are italicized.

Table S3: Comparison of Ct values obtained from Fruits and Food samples with species-specific primers and with 18S primers

REFERENCES

1. Sicherer SH, Sampson HA. Food allergy: Epidemiology, pathogenesis, diagnosis, and treatment. *J Allergy Clin Immunol* **133**: 291–307 (2014).
2. Muraro A, Agache I, Clark A, Sheikh A, Roberts G, Akdis CA, et al. EAACI food allergy and anaphylaxis guidelines: managing patients with food allergy in the community. *Allergy* **69**: 1008–1025 (2014).
3. Tang MLK, Mullins RJ. Food allergy: is prevalence increasing? *Intern Med J*. doi:10.1111/imj.13362. (2017).
4. Fernandez-Rivas M, Fruit and Vegetable Allergy. Food Allergy: Molecular basis and clinical practice. In: Ebisawa M, Ballmer-Weber B K, Vieths S WRA (ed). *Allergen-based food allergies*. Chem Immunol Allergy, Karger: Basel, pp 162–170 (2015).
5. Boyce JA, Assa'ad A, Burks WA, Jones SM, Sampson HA, Wood RA, et al. Guidelines for the Diagnosis and Management of Food Allergy in the United States: Report of the NIAID-Sponsored Expert Panel. *J Allergy Clin Immunol*; **126**: S1-58 (2010).
6. Ward R, Crevel R, Bell I, Khandke N, Ramsay C, Paine S, A vision for allergen management best practice in the food industry. *Trends Food Sci. Technol.* **21**: 619–625 (2010).
7. Prado M, Ortea I, Vial S, Rivas J, Calo-Mata P, Barros-Velázquez J, Advanced DNA- and Protein-based Methods for the Detection and Investigation of Food Allergens. *Crit Rev Food Sci Nutr* **56**: 2511–2542 (2016).
8. van Hengel AJ, Food allergen detection methods and the challenge to protect food-allergic consumers. *Anal Bioanal Chem* **389**: 111–118 (2007).

9. Commission Directive 2007/68/EC. *Off J Eur Union* **L 310**: 11–14 (2007).
10. Gendel SM, Comparison of international food allergen labeling regulations. *Regul Toxicol Pharmacol* **63**: 279-285 (2012).
11. Holzhauser T, Oliver S, Vieths S, Polymerase chain reaction (PCR) methods for the detection of allergenic foods, *Detecting allergens in food*. Publisher: Woodhead Publishing Limited, Editors: Stef Koppelman, Susan Hefle, pp.125-143 (2006)
12. Yeung J, Enzyme-linked immunosorbent assays (ELISAs) for detecting allergens in foods. In: *Detecting allergens in food*; Koppelman, S.J.; Hefle, S.L. Ed.; CRC Press: Boca Raton, FL, pp. 109-124 (2006).
13. Ahsan N, Rao R S P, Gruppuso P A, Ramratnam B, Salomon A R, Targeted proteomics: Current status and future perspectives for quantification of food allergens. *J Proteomics* **143**: 15-23 (2016).
14. Monaci L, Visconti A, Mass spectrometry-based proteomics methods for analysis of food allergens. *TrAC - Trends Anal. Chem.* **28**: 581–591 (2009).
15. Burney P, Summers C, Chinn S, Hooper R, van Ree R, Lidholm J. Prevalence and distribution of sensitization to foods in the European Community Respiratory Health Survey: a EuroPrevall analysis. *Allergy* **65**: 1182–1188 (2010).
16. Vanga SK, Jain M, Raghavan V, Significance of fruit and vegetable allergens: Possibilities of its reduction through processing. *Food Rev Int*; doi: 10.1080/87559129.2016.1239208 (2016).
17. Fernandez-Rivas M, Bolhaar S, Gonzalez-Mancebo E, Asero R, van Leeuwen A, Bohle B, *et al* Apple allergy across Europe: how allergen sensitization profiles determine the clinical expression of allergies to plant foods. *J Allergy Clin Immunol* **118**: 481–488 (2006).

18. González-Mancebo E, Fernández-Rivas M, Outcome and safety of double-blind, placebo-controlled food challenges in 111 patients sensitized to lipid transfer proteins. *J. Allergy Clin. Immunol.* **121**: 1507–1508 (2008).
19. Ballmer-Weber BK, Hoffmann-Sommergruber K, Molecular diagnosis of fruit and vegetable allergy. *Curr Opin Allergy Clin Immunol* **11**: 229–235 (2011).
20. Pastorello EA, Conti A, Pravettoni V, Farioli L, Rivolta F, Ansaloni R *et al*, Identification of actinidin as the major allergen of kiwi fruit. *J Allergy Clin Immunol*; **101**: 531–537 (1998).
21. Asero R, Antonicelli L, Arena A, Bommarito L, Caruso B, Crivellaro M *et al*, EpidemAAITO: Features of food allergy in Italian adults attending allergy clinics: a multi-centre study. *Clin Exp Allergy* **39**: 547–555 (2009).
22. Sánchez-Monge R, Lombardero M, García-Sellés FJ, Barber D, Salcedo G, Lipid-transfer proteins are relevant allergens in fruit allergy. *J Allergy Clin Immunol*; **103**: 514–519 (1999).
23. Palacín A, Tordesillas L, Gamboa P, Sanchez-Monge R, Cuesta-Herranz J, Sanz ML *et al*, Characterization of peach thaumatin-like proteins and their identification as major peach allergens. *Clin Exp Allergy* **40**: 1422–1430 (2010).
24. Watanabe S, Taguchi H, Temmei Y, Hirao T, Akiyama H, Sakai S *et al*, Specific detection of potentially allergenic peach and apple in foods using polymerase chain reaction. *J Agric Food Chem* **60**: 2108–2115 (2012).
25. Taguchi H, Watanabe S, Hirao T, Akiyama H, Sakai S, Watanabe T *et al*, Specific detection of potentially allergenic kiwifruit in foods using polymerase chain reaction. *J Agric Food Chem* **55**: 1649–1655 (2007).
26. Marzban G, Herndl A, Maghuly F, Katinger H, Laimer M, Mapping of fruit allergens by 2D

- electrophoresis and immunodetection. *Expert Rev Proteomics* **5**: 61–75 (2008).
27. Gao Z, Ma Y, Zhou X, Yang Z, Jia H, Gao L *et al*, Quantification of Peach Fruit Allergen Lipid Transfer Protein by a Double Monoclonal Antibody-based Sandwich ELISA. *Food Anal Methods* **9**: 823–830 (2016).
28. Kiewning D, Schmitz-Eiberger M, Effects of long-term storage on Mal d 1 content of four apple cultivars with initial low Mald1 content. *J Sci Food Agric* **94**: 798–802 (2014).
29. Gavrović-Jankulović M, Spasić M, Ćirković Veličković T, Stojanović M, Inić-Kanada A, Dimitrijević L *et al*, Quantification of the thaumatin-like kiwi allergen by a monoclonal antibody-based ELISA. *Mol Nutr Food Res* **52**: 701–707 (2008).
30. Monaci L, Visconti A, Immunochemical and DNA-based methods in food allergen analysis and quality assurance perspectives. *Trends Food Sci Technol* **21**: 272–283 (2010).
31. Pafundo S, Gulli M, Marmiroli N, Multiplex real-time PCR using SYBR® GreenER™ for the detection of DNA allergens in food. *Anal Bioanal Chem* **396**: 1831–1839 (2010).
32. D'Andrea M, Coisson JD, Locatelli M, Garino C, Cereti E, Arlorio M, Validating allergen coding genes (Cor a 1, Cor a 8, Cor a 14) as target sequences for hazelnut detection via Real-Time PCR. *Food Chem* **124**: 1164–1171 (2011).
33. Pafundo S, Gulli M, Marmiroli N, SYBR®GreenER™ Real-Time PCR to detect almond in traces in processed food. *Food Chem* **116**: 811–815 (2009).
34. Abbott M, Hayward S, Ross W, Godefroy SB, Ulberth F, Van Hengel AJ *et al*, Validation Procedures for Quantitative Food Allergen ELISA Methods: Community Guidance and Best Practices. *J AOAC Int* **93**, 442–450 (2010).
35. Pafundo S, Gulli M, Marmiroli N, Comparison of DNA extraction methods and development

- of duplex PCR and real-time PCR to detect tomato, carrot, and celery in food. *J Agric Food Chem* **59**: 10414–24 (2011).
36. Mehar A, Prabodh T, Theophanes S, Mark T Isolation of high-quality RNA from apple (*Malus domestica*) fruit. *J Agric Food Chem* **54**: 5227–5229 (2006).
37. Pfaffl MW, Štebih D, Dreo T, Žel J, Gruden K, Aoki N *et al*, A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**: 45e–45 (2001).
38. Vickers AJ. Parametric versus non-parametric statistics in the analysis of randomized trials with non-normally distributed data. *BMC Med Res Methodol*; **5**: 35-46 (2005).
39. 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* **6**, 37 (2006).
40. Di Pinto A, Forte VT, Guastadisegni MC, Martino C, Schena FP, Tantillo G. A comparison of DNA extraction methods for food analysis. *Food Control* **18**: 76-80 (2007).
41. Marmiroli N, Maestri E, Polymerase Chain Reaction (PCR). In: Picò Y (ed). *Food Toxicants Analysis. Techniques, Strategies and Development*, Elsevier: Amsterdam, pp 147–187 (2007).
42. Demeke T, Jenkins GR, Influence of DNA extraction methods, PCR inhibitors and quantification methods on real-time PCR assay of biotechnology-derived traits. *Anal Bioanal Chem* **396**: 1977–1990 (2010).
43. ENGL Definition of minimum performance requirements for analytical methods of GMO testing. European network of GMO laboratories, Joint Research Center, 2015; EURL; http://gmo-crl.jrc.ec.europa.eu/doc/MPR%20Report%20Application%202010_2015.pdf
44. Zhu L, Altmann SW, mRNA and 18S–RNA coapplication–reverse transcription for

quantitative gene expression analysis. *Anal Biochem* **345**: 102–109 (2005).

45. Loureiro J, Rodriguez E, Dolezel J, Santos C, Two New Nuclear Isolation Buffers for Plant DNA Flow Cytometry: A Test with 37 Species. *Ann Bot* **100**: 875–888 (2007).
46. Peano C, Samson C, Palmieri L, Gulli M, Marmioli N, Qualitative and quantitative evaluation of the genomic DNA extracted from GMO and non GMO foodstuffs with four different extraction methods, *J Agric Food Chem*, **52**: 6962-6968 (2004).
47. Mano J, Nishitsuji Y, Kikuchi Y, Fukudome SI, Hayashida T, Kawakami H, *et al*, Quantification of DNA fragmentation in processed foods using real-time PCR. *Food Chem* **226**: 149-155 (2017).

Table 1. List of plant species, commercial food matrices and spiked foods utilized for DNA extraction.

			Control ^a	Species specific primers ^b		
Plant species		Method	18S/CLOR	Act	Pru	Mal
Leaves of	Wheat	CTAB	+	-	-	-
	Barley		+	-	-	-
	Maize		+	-	-	-
	Oat		+	-	-	-
	Sunflower		+	-	-	-
	Olive		+	-	-	-
	Rice		+	-	-	-
Nuts and Seeds of	Sesame	CTAB	+	-	-	-
	Almond		+	-	-	-
	Hazelnut		+	-	-	-
	Nut		+	-	-	-
	Peanut		+	-	-	-
	Cashew		+	-	-	-
	Mustard		+	-	-	-
Fresh fruit	Kiwi	CTAB-PVP	+	+	-	-
		CTAB-SDS	+	+	-	-
		GK-resin	nt	nt	nt	nt
		ACSDS	+	+	-	-
		ACPVP	+	+	-	-
	Peach	CTAB-PVP	+	-	+	-
		CTAB-SDS	+	-	+	-
		GK-resin	nt	nt	nt	nt
		ACSDS	+	-	+	-
		ACPVP	+	-	+	-
	Apple	CTAB-PVP	+	-	-	+
		CTAB-SDS	+	-	-	+
		GK-resin	+	-	-	+
		ACSDS	+	-	-	+
		ACPVP	+	-	-	+
	Cranberry	CTAB-SDS	+	-	-	-
	Banana		+	-	-	-
	Cherry		+	-	-	-
	Pear		+	-	-	-
	Apricot		+	-	-	-
	Plum		+	-	-	-
	Blueberry		+	-	-	-
	Strawberry		+	-	-	-
	Raspberry		+	-	-	-
Commercial food matrices						
Sesame bars		GK-resin	+	-	-	-
Biscuits		GK-resin	+	-	-	-
Biscuits with	Cashew Nut	GK-resin	+	-	-	-

	Chocolate		+	-	-	-
	Egg		+	-	-	-
	Apple		+	-	-	+
Homogenized baby food with	Kiwi	CTAB-PVP	-	+	-	-
		CTAB-SDS	+	-	-	-
		GK-resin	nt	nt	nt	nt
		ACSDS	+	+	-	-
		ACPVP	-	+	-	-
	Peach	CTAB-PVP	-	-	-	-
		CTAB-SDS	+	-	+	-
		GK-resin	nt	nt	nt	nt
		ACSDS	-	-	-	-
		ACPVP	-	-	-	-
	Apple	CTAB-PVP	-	-	-	-
		CTAB-SDS	+	-	-	+
		GK-resin	nt	nt	nt	nt
		ACSDS	+	-	-	+
		ACPVP	-	-	-	-
Yogurt with	Kiwi	CTAB-PVP	+	-	-	-
		CTAB-SDS	+	+	-	-
		GK-resin	nt	nt	nt	nt
		ACSDS	+	+	-	-
		ACPVP	+	+	-	-
	Peach	CTAB-PVP	+	-	+	-
		CTAB-SDS	+	-	+	-
		GK-resin	nt	nt	nt	nt
		ACSDS	+	-	+	-
		ACPVP	+	-	+	-
	Apple	CTAB-PVP	+	-	-	+
		CTAB-SDS	+	-	-	+
		GK-resin	nt	nt	nt	nt
		ACSDS	-	-	-	-
		ACPVP	-	-	-	-
Spiked food samples						
Biscuits spiked with	Kiwi		+	+	-	-
	Peach	GK-resin	+	-	+	-
	Apple		+	-	-	+

48.

^aAmplificability was tested with 18S on all samples except for cranberry, banana, cherry, pear, apricot plum blueberry, strawberry and raspberry which were analysed with CLOR primers.

^bSpecificity of Act, Pru, and Mal primers were checked by end-point PCR.

+ : positive result, amplification's product with an high signal intensity; -: negative result, no amplification's products; nt: sample not tested by PCR.

Table 2. Target sequences selection and primers design.

Species	Target gene ^a	Accession number	Primers name		Sequence 5'-3'	Amplicon length (bp)	Theoretical T _m of amplicon (°C)
<i>Actinidia deliciosa</i>	Act c2	AJ871175	Act	forward reverse	CCGTGGCCAGAATTGGATC GGGCCATACTCTAGCGCCTT	61	77
<i>Malus domestica</i>	Mal d1	AM283495	Mal	forward reverse	AGGGGATGCCATTTCTGAC GGCTGGTGTTCTTGATGATG	92	75
<i>Prunus persica</i>	Pru p2.01B	EU424259.1	Pru	forward reverse	AGCTCTTCAAGACCCAGTGC TGTGAAGGTGCTGCTCTTGT	68	74.3
<i>Prunus dulcis</i> ^b	18S rRNA	DQ886376.1	18S	forward reverse	GTGACGGAGAATTAGGGTTCTGA CCGGTATTGTTATTTATTGTCACTACCTC	131	79
<i>Prunus persica</i> ^c	trnL	JN102177	CLOR	forward reverse	TATGGCGAAATCGGTAGACG TTCCAGGGTTTCTCTGAATT	96	80

^aFor each selected gene its accession number is reported. For each primer pair sequences, length, and theoretical T_m of the expected amplicon are reported.

^bThe primers for 18S rRNA are designed on a target sequence of almond, which is a sequence highly conserved in all plant species tested.³¹

^cThe primers for trnL are designed on a target sequence of peach, which is a sequence highly conserved in all plant species tested

Table 3. Comparison of five DNA extraction methods applied to fresh fruits, and commercial foods.

Matrix	Species	EXTRACTION METHOD									
		CTAB-SDS		CTAB-PVP		GK-resin		ACPVP		ACS-DS	
		Yield ^a (score)	A ₂₆₀ /A ₂₈₀ ^b (score)	Yield (score)	A ₂₆₀ /A ₂₈₀ (score)	Yield (score)	A ₂₆₀ /A ₂₈₀ (score)	Yield (score)	A ₂₆₀ /A ₂₈₀ (score)	Yield (score)	A ₂₆₀ /A ₂₈₀ (score)
Fresh Fruit	Kiwi	1.50 (2)	1.7 (10)	7.91 (2)	1.8 (10)	1.96 (2)	1.1 (2)	83.14 (10)	1.6 (10)	7.84 (2)	1.8 (10)
	Apple	33.87 (5)	1.6 (10)	16.87 (2)	1.8 (10)	1.37 (2)	1.8 (10)	41.86 (5)	1.8 (10)	10.88 (2)	1.6 (10)
	Peach	2.77 (2)	1.4 (5)	8.68 (2)	1.4 (5)	0.47 (2)	0.7 (2)	46.28 (5)	1.8 (10)	BDL (0)	BDL (0)
Yoghurt with	Kiwi	0.61 (2)	1.0 (2)	4.07 (2)	1.4 (5)	BDL (0)	BDL (0)	18.96 (2)	1.6 (10)	28.45 (5)	1.7 (10)
	Apple	3.34 (2)	1.0 (2)	72.78 (5)	1.8 (10)	10.58 (2)	1.4 (5)	99.52 (10)	1.9 (10)	11.24 (2)	1.4 (5)
	Peach	2.28 (2)	1.0 (2)	35.05 (5)	1.7 (10)	BDL (0)	BDL (0)	89.92 (10)	1.8 (10)	24.56 (2)	1.7 (10)
Homogenized baby food with	Kiwi	12.87 (2)	1.6 (10)	36.96 (5)	1.8 (10)	BDL (0)	BDL (0)	16.56 (2)	1.7 (10)	25.36 (5)	1.8 (10)
	Apple	2.24 (2)	1.3 (5)	14.09 (5)	1.6 (10)	2.62 (2)	1.2 (5)	208.60 (10)	1.9 (10)	49.22 (5)	1.8 (10)
	Peach	18.94 (2)	1.6 (10)	7.32 (2)	1.4 (5)	1.27 (2)	1.0 (2)	83.72 (10)	1.9 (10)	14.00 (2)	1.5 (5)
Score		25+56=81		30+75=105		12+26=38		64+90=154		25+70=95	

^aDNA yield is expressed as ng/100 mg fresh weight (FW) of matrices; score 2, from 0 to 25 ng/100 mg FW; score 5, from 26 to 75 ng/100 mg FW; score 10, ≥76 ng/100 mg FW

^bA₂₆₀/A₂₈₀: score 2, less than 1; score 5, from 1.1 to 1.5; score 10 ≥1.6

BDL: below detectable level

Table 4. Results of qPCR with SYBR Green.

Sample		Primer pair	C _t ^a	SD ^b	T _m ^a	SD ^b
Fresh fruits	Kiwi	18S	23.73	0.04	82.15	0.07
		Act	24.52	0.19	78.80	0.28
	Peach	18S	19.05	0.22	82.10	0.42
		Pru	28.08	0.08	73.32	0.21
	Apple	18S	19.71	0.01	81.75	0.21
		Mal	30.88	0.09	76.20	0.26
Homogenized baby food with	Kiwi	18S	28.48	0.11	85.00	0.00
		Act	34.28	0.10	78.56	0.05
	Peach	18S	26.06	0.40	85.10	0.17
		Pru	33.98	0.04	73.25	0.26
	Apple	18S	29.50	0.18	84.93	0.05
		Mal	31.46	0.30	76.16	0.15
Yogurt with	Kiwi	18S	23.70	0.13	85.23	0.11
		Act	31.89	0.10	78.56	0.05
	Peach	18S	22.50	0.11	85.30	0.00
		Pru	31.06	0.17	73.02	0.21
	Apple	18S	28.65	0.53	85.20	0.17
		Mal	33.71	0.15	77.10	0.10

^a C_t or T_m mean and ^b SD of two biological replicates with three technical replicates each

Table 5. Results of qPCR on biscuits spiked with kiwi, peach and apple.

Biscuit sample spiked with	mg kg ⁻¹	Ct ^a	SD ^b	Tm ^a	SD ^b	Efficiency %
Kiwi	2000	30.19	0.18	77.90	0.17	164
	200	33.03	0.37	77.85	0.21	
	100	33.53	0.55	77.60	0.30	
	50	34.19	0.11	77.90	0.00	
	25	34.63	0.49	77.90	0.00	
	20	ND	ND	ND	ND	
Peach	2000	31.78	0.48	73.00	0.00	101
	200	35.45	0.02	73.00	0.00	
	20	38.33	0.47	73.15	0.21	
Apple	2000	29.54	0.00	76.00	0.00	119
	200	32.97	0.16	75.85	0.21	
	100	33.13	0.41	76.15	0.21	
	50	34.32	0.01	76.00	0.00	
	25	ND	ND	ND	ND	
	20	ND	ND	ND	ND	

ND: not detected

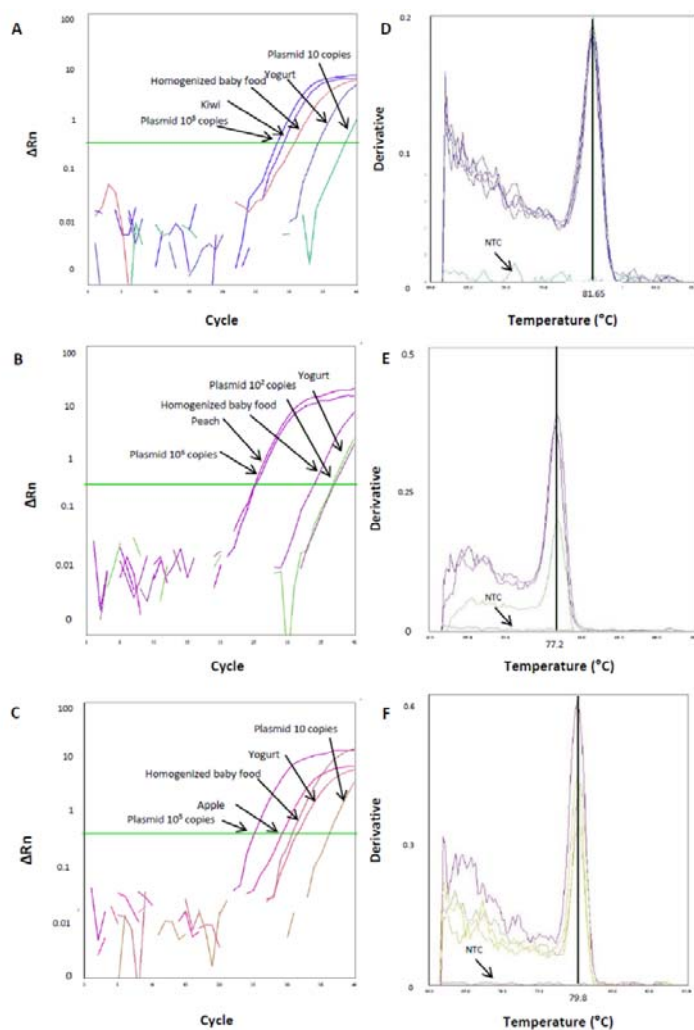
^aCt or Tm mean and ^b SD of three technical replicates of two biological replicates

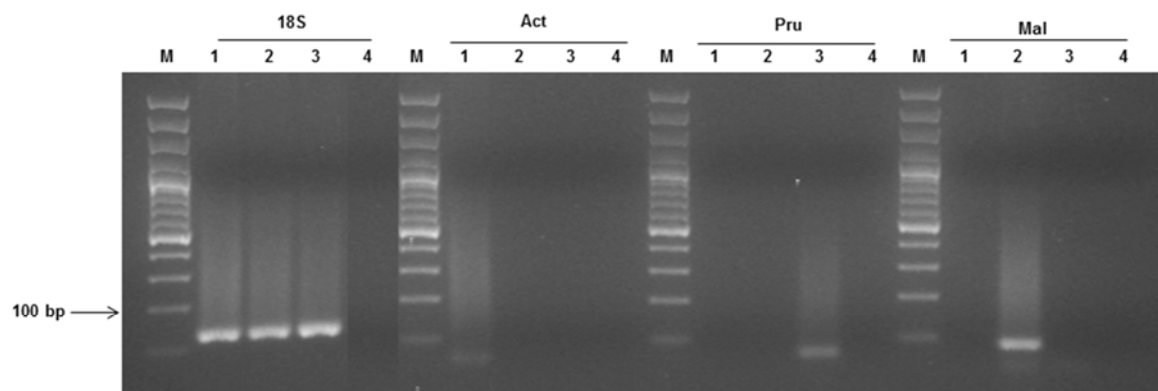
FIGURE CAPTIONS

Figure 1. qPCR with SYBR Green and DNA melting curve analyses on fruits, food products, and on reference plasmids.

Amplification plots, and derivatives of melting curves of amplicons obtained with Act (A, D), Pru (B, E), and Mal (C, F) species-specific primers on fruits, yogurt and homogenized baby foods, and on reference plasmid DNAs. PCRs were performed on Applied Biosystems 7900HT Real-Time PCR System (Life Technologies). NTC: no template control sample used as a negative control.

Figure 2. Endpoint PCR analysis of gDNA extracted from the spiked samples with kiwi, peach and apple respectively. All gDNA samples were amplified using 18S, Act, Pru and Mal primers, followed by electrophoretic separation of the amplicon through a 3% (w/v) agarose gel. gDNA was extracted from biscuit spiked with: kiwi (1), apple (2), and peach (3); negative control (4); 100 bp ladder (M). Arrows indicate the size of marker.





Detection of allergen coding sequence of kiwi, peach, and apple in processed food by qPCR

Sara Graziano ^a, Mariolina Gulli ^{a,b*} and Nelson Marmiroli ^{a,b}

a Department of Chemistry, Life Sciences and Environmental Sustainability, University of Parma, Parco Area delle Scienze 11/A, 43124 Parma, Italy

b Interdepartmental Center SITEIA.PARMA, University of Parma, Parco Area delle Scienze 181/A, 43124 Parma, Italy

*Corresponding author

*Phone: +39 0521905486. E-mail: mariolina.gulli@unipr.it.

Figure S1: Comparison of DNA extraction methods: statistical analysis. The five DNA extraction methods were compared considering yield (A) and A_{260}/A_{280} ratio (B) of the DNA purified from all samples (n=45). Graphs show the results of Kruskal Wallis test, and tables show pairwise comparisons. The p values were adjusted by the Bonferroni correction for multiple tests, and significant differences are bolded.

A

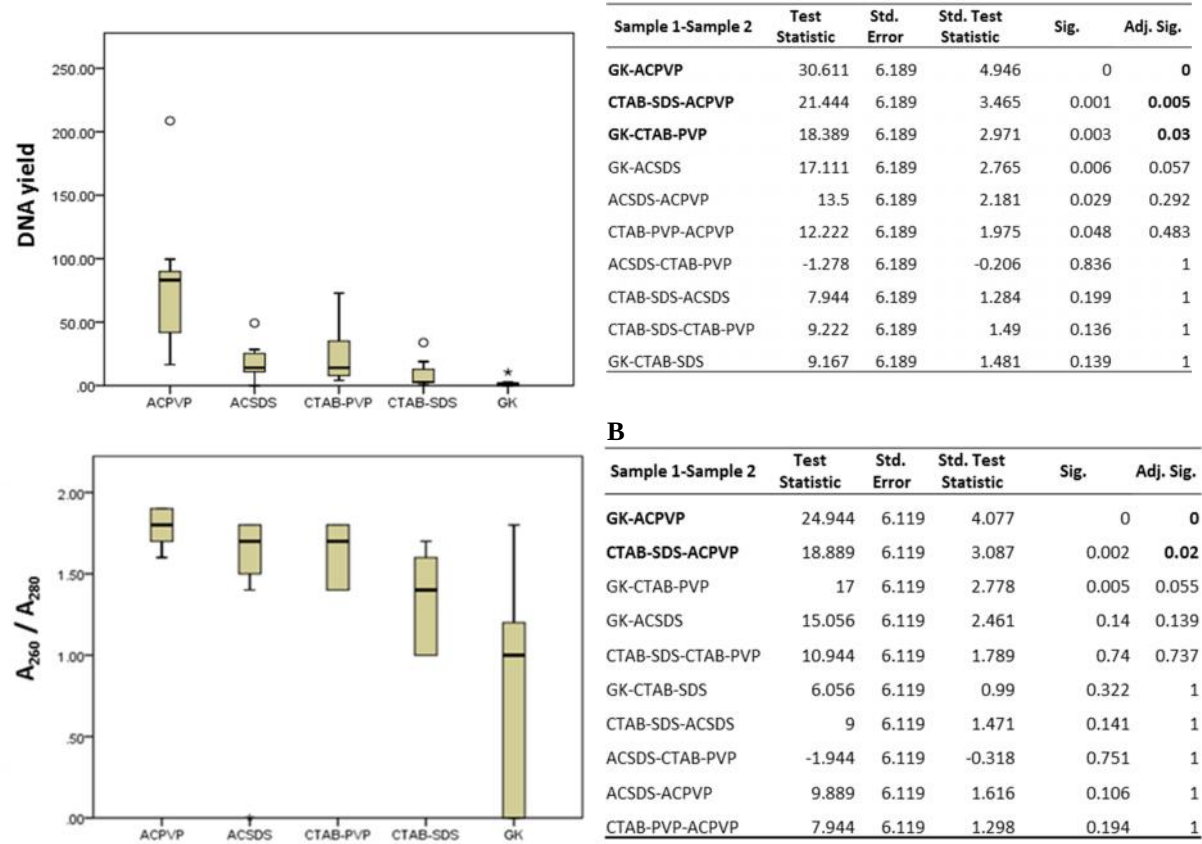
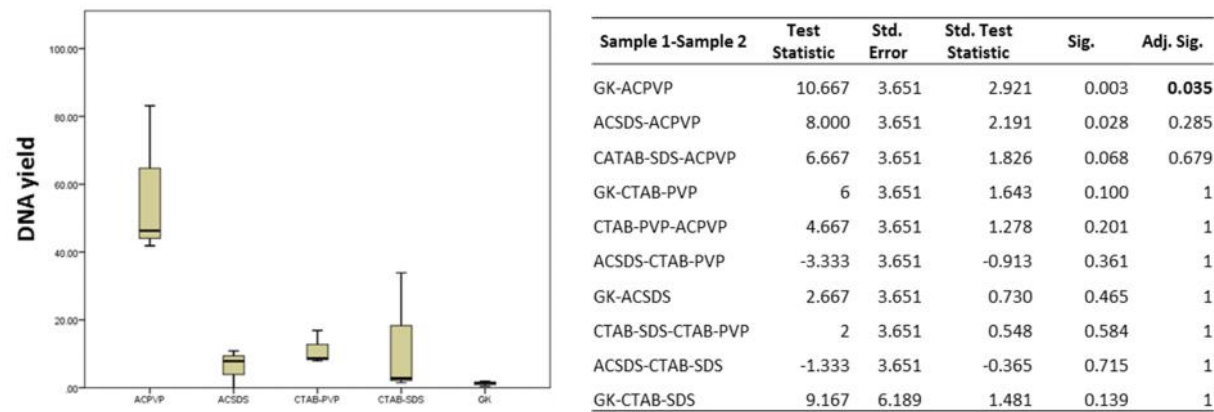


Figure S2: Comparison of DNA extracted from fresh fruits and homogenized baby foods : statistical analysis.
 The five DNA extraction methods were compared considering yield (A) of the DNA purified from fresh fruits (n=15) and A_{260}/A_{280} ratio (B) of the DNA purified from homogenized baby foods (n=15). Graphs show the results of Kruskal Wallis test, and tables show pairwise comparisons. The p values were adjusted by the Bonferroni correction for multiple tests, and significant differences are bolded.

A



B

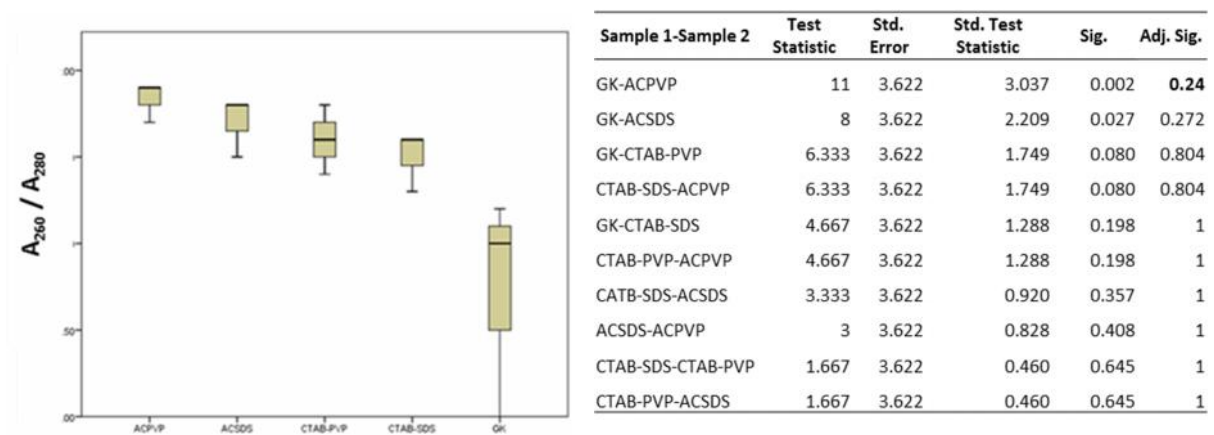


Table S1: Allergenic fruit species studied.

Species	Genome characteristic ¹		Major allergen ^a	Detection method			
				PCR		ELISA	
	2C(pg) ^b	Ploidy		Target	Sensitivity	Target	Sensitivity
<i>Actinidia deliciosa</i>	4.80±0.06	6n (n=29)	Act d 1, Act d 2	ITS-1 ²	1-10 ppm	Act d 2 ⁴	0.1 ng mL ⁻¹
<i>Prunus persica</i>	0.62±0.01	2n (n=16)	Pru p 2, Pru p 3	trnS-trnG ³	10 ppm	rPru p 3 ⁵	0.5 ng mL ⁻¹
<i>Malus domestica</i>	1.56±0.02	2n (n=17)	Mal d 1	ITS- 1 ³	10 ppm	Mal d 1 ⁶	ND

^awww.allergen.org; ^b1pg is equivalent to 978 Mbp; ND, not determined

References

1. Loureiro J, Rodriguez E, Dolezel J, Santos C, Two New Nuclear Isolation Buffers for Plant DNA Flow Cytometry: A Test with 37 Species. *Ann Bot* **100**: 875–888 (2007).
2. Taguchi H, Watanabe S, Hirao T, Akiyama H, Sakai S, Watanabe T *et al*, Specific detection of potentially allergenic kiwifruit in foods using polymerase chain reaction. *J Agric Food Chem* **55**: 1649–1655 (2007).
3. Watanabe S, Taguchi H, Temmei Y, Hirao T, Akiyama H, Sakai S *et al*, Specific detection of potentially allergenic peach and apple in foods using polymerase chain reaction. *J Agric Food Chem* **60**: 2108–2115 (2012).
4. Gavrović-Jankulović M, Spasić M, Ćirković Veličković T, Stojanović M, Inić-Kanada A, Dimitrijević L *et al*, Quantification of the thaumatin-like kiwi allergen by a monoclonal antibody-based ELISA. *Mol Nutr Food Res* **52**: 701–707 (2008).
5. Gao Z, Ma Y, Zhou X, Yang Z, Jia H, Gao L *et al*, Quantification of Peach Fruit Allergen Lipid Transfer Protein by a Double Monoclonal Antibody-based Sandwich ELISA. *Food Anal Methods* **9**: 823–830 (2016).
6. Kiewning D, Schmitz-Eiberger M, Effects of long-term storage on Mal d 1 content of four apple cultivars with initial low Mald1 content. *J Sci Food Agric* **94**: 798–802 (2014).

Table S2: *In silico* analysis of primer pairs specificity using BLAST software (last accessed on 30 August 2017). The tables show the accession numbers, descriptions, and statistical parameters of the sequences matching with each primer pair. Accession number and target sequence used for primer design are in bold.

KIWI

Accession Number	Description	Max score	Total score	Query cover	E value	Identity
AJ871175.2	<i>Actinidia deliciosa</i> mRNA for thaumatin-like protein (tlp gene), allergen Act c 2	40.1	78.3	100%	2.2	100%
JX905282.1	<i>Actinidia deliciosa</i> isolate TLP1-Ad1 thaumatin-like protein (TLP1) mRNA, complete cds	40.1	78.3	100%	2.2	100%
EF417825.1	<i>Actinidia deliciosa</i> thaumatin-like protein gene, partial cds	40.1	78.3	100%	2.2	100%

PEACH

Accession Number	Description	Max score	Total score	Query cover	E value	Ident
EU424259.1	<i>Prunus dulcis</i> x <i>Prunus persica</i> putative allergen Pru p 2.01B (Pru p 2.01B) gene	40.1	80.3	100%	2.4	100%
XM_007215802.2	PREDICTED: <i>Prunus persica</i> thaumatin-like protein 1 (LOC18783179), mRNA	40.1	80.3	100%	2.4	100%
XM_007215809.2	PREDICTED: <i>Prunus persica</i> thaumatin-like protein 1 (LOC18782522), mRNA	40.1	80.3	100%	2.4	100%
XM_007215800.2	PREDICTED: <i>Prunus persica</i> thaumatin-like protein 1 (LOC18784449), mRNA	40.1	80.3	100%	2.4	100%
EU424257.1	<i>Prunus dulcis</i> x <i>Prunus persica</i> putative allergen Pru p 2.01A (Pru p 2.01A) gene	40.1	80.3	100%	2.4	100%
AF362988.1	<i>Prunus persica</i> clone 2 thaumatin-like protein mRNA, complete cds	40.1	72.4	100%	2.4	100%

APPLE

Accession Number	Description	Max score	Total score	Query cover	E value	Ident
AM283495.1	<i>Malus x domestica</i> mal d 1.06A gene for PR-10 protein, exons 1-2, allele 01	40.1	78.3	100%	2.2	100%
XM_008342515.2	PREDICTED: <i>Malus x domestica</i> major allergen Mal d 1-like (LOC103403673), mRNA	40.1	78.3	100%	2.2	100%
JF451364.1	<i>Malus x domestica</i> allergen Mal d 1.06A (Mal d 1.06A) gene, Mal d 1.06A*02.08 allele, complete cds	40.1	78.3	100%	2.2	100%
JF451360.1	<i>Malus x domestica</i> allergen Mal d 1.06A (Mal d 1.06A) gene, Mal d 1.06A*02.07 allele, complete cds	40.1	78.3	100%	2.2	100%
JF451359.1	<i>Malus x domestica</i> allergen Mal d 1.06A (Mal d 1.06A) gene, Mal d 1.06A*02.06 allele, complete cds	40.1	78.3	100%	2.2	100%
JF451357.1	<i>Malus x domestica</i> allergen Mal d 1.06A (Mal d 1.06A) gene, Mal d 1.06A*02.05 allele, complete cds	40.1	78.3	100%	2.2	100%
JF451354.1	<i>Malus x domestica</i> allergen Mal d 1.06A (Mal d 1.06A) gene, Mal d 1.06A*03.03 allele, complete cds	40.1	78.3	100%	2.2	100%
JF682497.1	<i>Malus x domestica</i> isolate 4 Mal d 1.06A (Mal d 1.06A) gene, Mal d 1.06A01.01 allele, complete cds	40.1	78.3	100%	2.2	100%
JF682495.1	<i>Malus x domestica</i> isolate 3 Mal d 1.06A (Mal d 1.06A) gene, Mal d 1.06A03.01 allele, complete cds	40.1	78.3	100%	2.2	100%
JF682494.1	<i>Malus x domestica</i> isolate 2 Mal d 1.06A (Mal d 1.06A) gene, Mal d 1.06A02.04 allele, complete cds	40.1	78.3	100%	2.2	100%
JF682493.1	<i>Malus x domestica</i> isolate 1 Mal d 1.06A (Mal d 1.06A) gene, Mal d 1.06A03.01 allele, complete cds	40.1	78.3	100%	2.2	100%
AM283502.1	<i>Malus x domestica</i> mal d 1.06A gene for PR-10 protein, exons 1-2, allele 01	40.1	78.3	100%	2.2	100%
AM283501.1	<i>Malus x domestica</i> mal d 1.06A gene for PR-10 protein, exons 1-2, allele 02	40.1	78.3	100%	2.2	100%
AM283500.1	<i>Malus x domestica</i> mal d 1.06A gene for PR-10 protein, exons 1-2, allele 01	40.1	78.3	100%	2.2	100%
AM283499.1	<i>Malus x domestica</i> mal d 1.06A gene for PR-10 protein, exons 1-2, allele 02	40.1	78.3	100%	2.2	100%
AM283496.1	<i>Malus x domestica</i> mal d 1.06A gene for PR-10 protein, exons 1-2, allele 01	40.1	78.3	100%	2.2	100%
AM283494.1	<i>Malus x domestica</i> mal d 1.06A gene for PR-10 protein, exons 1-2, allele 02	40.1	78.3	100%	2.2	100%
AM283492.1	<i>Malus x domestica</i> mal d 1.06A gene for PR-10 protein, exons 1-2, allele 02	40.1	78.3	100%	2.2	100%
AM283491.1	<i>Malus x domestica</i> mal d 1.06A gene for PR-10 protein, exons 1-2, allele 01	40.1	78.3	100%	2.2	100%
AY827701.1	<i>Malus x domestica</i> clone M1.07B2 Mal d 1.06A03 (Mal d 1.06A) gene	40.1	78.3	100%	2.2	100%
AY827700.1	<i>Malus x domestica</i> clone M1.07G9 Mal d 1.06A02 (Mal d 1.06A) gene	40.1	78.3	100%	2.2	100%
AY827698.1	<i>Malus x domestica</i> clone 220903E4 Mal d 1.06A02 (Mal d 1.06A) gene	40.1	78.3	100%	2.2	100%
AY827692.1	<i>Malus x domestica</i> clone 220903H2 Mal d 1.06A02 (Mal d 1.06A) gene	40.1	78.3	100%	2.2	100%
AY789249.1	<i>Malus x domestica</i> major allergen Mal d 1.06A (Mal d 1.06A) gene	40.1	78.3	100%	2.2	100%
AY789250.1	<i>Malus x domestica</i> major allergen Mal d 1.06A01 (Mal d 1.06A) gene	40.1	78.3	100%	2.2	100%
AY789248.1	<i>Malus x domestica</i> major allergen Mal d 1.06A02 (Mal d 1.06A) gene	40.1	78.3	100%	2.2	100%
NM_001300878.1	<i>Malus domestica</i> major allergen Mal d 1-like (LOC103403702), mRNA	40.1	78.3	100%	2.2	100%
AY428580.1	<i>Malus x domestica</i> clone 1e Mal d 1-like mRNA, complete cds	40.1	78.3	100%	2.2	100%

Table S3: Comparison of Ct values obtained from Fruits and Food samples with species-specific primers and with 18S primers

Primers	Fruits		Food	
	Ct mean	SD	Ct mean	SD
Species-Specific*	27.82	3.18	32.73	1.41
18S	20.83	2.53	26.48	2.88

* Significantly different ($p < 0.05$) according to Mann-Whitney U test