

## Article

# Detection of peanut allergen by Real Time PCR: looking for the suitable detection marker and DNA isolation method.

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## Abstract

Peanut (*Arachis hypogaea*) contains allergenic proteins, which make it harmful to the sensitive population. The presence of peanut in foods must be indicated on label, to prevent accidental consumption by allergenic population. The development of suitable analytical methodologies to detect this allergen in processed foods is advisable. Real Time PCR allowed a specific and accurate amplification of allergen sequences. The optimal genome targets for specific and sensitive detection are those that show interspecific variation and high copy number. Some food processing methods could induce structural and/or conformational changes in proteins and produce the fragmentation and/or degradation of genomic DNA. In this work, we use chloroplast markers for specifically detection of peanut by Real Time PCR, in order to increase the sensitivity. Three different protocols of DNA isolation were evaluated, for total and organelle-DNA extraction. Binary mixtures of raw and processed peanut flour in wheat were performed at concentrations ranging from 100000 to 0.1 ppm. DNA isolation from peanut, mixtures and other legumes was carried out following three protocols for obtaining genomic and chloroplast-enrich DNA. Quantity and quality of DNA was evaluated, obtaining better results for protocol 2. Specificity and sensitivity of the method has been assayed with specific primers for three chloroplast markers (mat k, rpl16 and trnH-psbA) and Ara h 6 peanut allergen-coding region was selected as nuclear low-copy target and TaqMan probes. Efficiency and linear correlation of calibration curves were within the adequate ranges. Moreover, the influence of pressure and thermal processing on the peanut detectability was analyzed.

**Keywords:** Real Time PCR, peanut, food allergen, chloroplast marker, DNA isolation

## 1. Introduction

Peanut (*Arachis hypogaea* L.) is a plant belonging to legume family and its fruit- the peanut- is worldwide consumed. Peanut allergy is one of the most common IgE-mediated reactivity to food because of its severity and lifelong persistence [1]. Considerable effort has been spent in characterizing peanut allergens and several allergenic proteins have been identified until now (up to Ara h 18 has been included in the WHO/IUIS Allergen Nomenclature Sub-Committee Database). The major peanut allergens, Ara h 1 (65 kDa, vicilin) and Ara h 2 (17 kDa, conglutin), are recognized by 70-90 % of sensitized subjects [2] and Ara h 3 (11 S legumin) has been considered to play a lesser allergenic role [3]. The thermal treatment has significant effect on peanut immunoreactivity. Roasting peanut enhances its IgE-binding capacity [4], while boiling decreases its allergenicity [5]. According to Cabanillas et al. [6], IgE immunoreactivity of roasted peanut decreased significantly at extreme conditions of autoclaving (2.6 bar, 30 min). When DIC treatment (instantaneous controlled pressured-drop, based on 6 bar of pressure for 3 min) was applied to raw and roasted peanut proteins compared

to untreated samples resulted into a remarkable decrease in the protein bands of 65 kDa (putative Ara h 1) and no immunoreactive bands lower than 20 kDa were recognized [7]. From the literature, it is apparent that immunoreactivity changes of peanut proteins following thermal treatment may be largely due to modification in structure as well as reactivity of each individual allergen of peanut as well as their interaction with the food matrix.

Nowadays, as no treatment for food allergies is available, sensitized individuals must avoid the consumption of the offending ingredient but the presence of allergens in foods can be a consequence of fraudulent substitution or adventitious contamination in the food facility. Therefore, to develop a reliable and specific tool to detect traces of specific food allergens is indeed essential to improve the quality of life of sensitized individuals, in agreement with the consensus experts [8]. Usually, protein-based assays as enzyme-linked immunosorbent assay (ELISA) are used to detect small amounts of proteins from specific foods. DNA-based methodologies, such as Real Time PCR and genosensors, have been proposed as specific, sensitive and reliable alternatives to ELISA since this molecule preserves its integrity better than proteins [9]. Until the date, several methods have been performed for peanut allergen detection, either protein or DNA-based technology [10–16].

Foods, including peanuts, are usually thermally treated to preserve food safety or even modify allergenic reactivity [17] but maintaining or improving organoleptic and functional properties [18]. The protein solubility can be highly affected by thermal food processing, and subsequent detection with protein-based techniques might be hampered [19]. In contrast to protein-based techniques, DNA-based assays have been proposed as a reliable, sensitive and specific alternative for food allergen identification. Although DNA is a very stable molecule, fragmentation and/or degradation of DNA molecules after severe treatments have been reported by several authors [20,21]. Some studies have analyzed the effect of many different treatments (boiling, High Hydrostatic Pressure HHP, autoclave, frying, roasting) on the detection of different DNA targets in peanut, hazelnut, walnut, almond or pistachio, among others [22–26].

Specificity, sensitivity and potential of quantification of a Real Time PCR method for allergen detection can be compromised by the selected target sequence. Either multi-copy (such as ITS or chloroplast sequences or genes) or single/low-copy genes have been used as targets for Real Time PCR based detection methods for nut analysis [9,15,27,28]. The objective of this study was to set up and validate a Real Time PCR assay for sensitive, specific and reliable peanut detection, using previously confirmed primer pairs for chloroplast marker sequences [29] or novel primers and probe for allergen coding sequences as targets. Additionally, we aimed to analyze the effect of thermal food processing (boiling, autoclaving and DIC) on peanut detection in complex food products by Real Time PCR.

## 2. Materials and Methods

### 2.1. Plant material and treatments

Peanuts (*Arachis hypogaea*) were provided by Productos Manzanares S.L. (Cuenca, Spain). Several thermal and pressure-based treatments were performed on whole peanuts. One hundred grams of peanuts, immersed in distilled water were boiled for 60 min or autoclaved (Compact 40 Benchtop, Priorclave, London, UK) at 121°C (1.20 bar) and at 138°C (2.56 bar) for 15 and 30 min, as previously described [23]. Peanuts were also subjected to controlled Instantaneous Depressurization (DIC) treatment, performed at the La Rochelle University (LaSiE). DIC treatment was carried out following a factorial experimental design previously described. In this experiment, the moistened whole nuts are placed in a processing chamber and exposed to steam pressure (7 bar) at high temperature (up to 170°C), over a short time (120 s). An instant pressure drop towards a vacuum at about 50 mbar follows this high-temperature-short time stage. This abrupt pressure drop, at a rate  $\Delta P/\Delta t$  higher than 5 bar s<sup>-1</sup>, simultaneously provokes an auto-vaporization of a part of the water in the product, and an instantaneous cooling of the products, which stops thermal degradation. After treatments, peanuts

were freeze-dried (Telstar Cryodos, Terrasa, Spain), defatted using n-hexane (34 ml/g) for 4 hours. Flours were passed through a 1mm mill and stored at 4°C until use.

### 2.3. Binary mixtures

Binary mixtures (spiked samples) of untreated (control) and treated (boiled, B60; autoclave 121°C at 15 min (A1) and at 30 min (A2); autoclave 138°C at 15 min (A3) and 30 min (A4) and DIC (DIC) defatted flours were performed in spelt wheat as described elsewhere [25]. Thus, mixtures containing 10, 100, 1000, 10000 and to 100000 mg/kg (0.01%–10%) of peanut in wheat were prepared in a final weight of 25 g. Spiked samples were mixed using a kitchen robot (Thermomix 31-1, Vorwerk Elektrowerke, GmbH & Co. KG, Wuppertal, Germany). The mixture containing 10% of each peanut sample (100000 mg/kg) was prepared by adding 2.5 g of the nut flour to 22.5 g of spelt wheat flour, and followed by 10-fold dilutions, homogenizing with the kitchen robot (Table S1).

### 2.4. DNA isolation and conventional PCR

Isolation of DNA was performed following different protocols. Genomic DNA was obtained using DNeasy Plant Pro kit (Qiagen, Hilden, Germany), with slight modifications. Briefly, 80 mg of binary mixtures and other plant species was homogenized in 1 ml of CD1 buffer with 5 µl of 25 mg/ml RNase using Tissue Lyser (TissueLyser II, Qiagen, Hilden, Germany) for cycles of 2 min at 24 1/s of frequency with a 30s of pause. After centrifugation for 5 min at 11000 rpm, 500 µl of supernatant were collected and 250 µl of CD2 buffer were added. Incubation for 20 min in ice was included after this step, followed by centrifugation at 11300 rpm for 2 min. Five hundred µl of supernatant were mixed with 1 volume of APP buffer before loading the column. Two steps of washing with AW2 buffer were performed, and DNA was eluted in 50 µl of pre-warmed deionized water (protocol 1). With the aim of performing a representative (compared to genomic DNA kit) but chloroplast-focused, faster and/or cheaper DNA isolation protocols, DNA from the same quantity of flour was isolated with two protocols (2 and 3). Protocol 2 was SpeedTools plasmid DNA purification Kit (Biotools, Loganholme, Australia) following the manufacturer instructions. Regarding protocol 3 or in-home designed protocol, 80 mg of peanut or binary mixture's flours were homogenized in 700 µl of solution I (glucose 50 mM, Tris HCl 25 mM, EDTA 10 mM) using tissue lyser at the same conditions described above. Centrifugation at 3000 rpm for 5 min was performed and 500 µl of supernatant was collected and centrifuged at 12000 rpm for 10 min. Pellet was suspended in 500 µl of MLB (NaCl 150 mM, Na<sub>2</sub>EDTA 50 mM, Tris HCl 10 mM), 250 µl of solution II (2% SDS, NaOH 0.4M) and 500 µl of solution III (29.5% acetate, pH 4.8) and centrifuged at 13000 rpm for 5 min at room temperature. Eight hundred µl of supernatant was kindly mixed with 700 µl with cold isopropanol, and centrifuged 15 min at 13000 rpm. Pellet was washed with 500 µl of cold ethanol, centrifuged 5 min at 13000 rpm and completely dried before suspended in 50 µl of pre-warmed deionized water. DNA from food products was isolated using NucleoSpin kit (Macherey-Nagel, Düren, Germany) following the manufacturer instructions with minor modification [30]. Quality and quantity of isolated DNA was analyzed by spectrophotometry using NanoDrop™ One (Thermo-Fisher, Waltham, MA, USA) and electrophoresis by 0.8% agarose-gels. End-point PCR using universal eukaryotic primers for 18S tested positive amplification of all samples (Table 1). These reactions were carried out in 20 µL, containing 25 ng of DNA, 250 nM of each primer and 1XFastStar PCR Master Mix (Biotools, Loganholme, Australia). SensoQuest LabCycler (Progen Scientific Ltd, London, UK) was programmed with an initial denaturation step at 95°C, 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 30 s and elongation at 72°C for 45 s, and a last step at 72°C for 5 min.

### 2.5. Primers, probes and sequencing

Three chloroplast markers from *Arachis hypogaea* were selected based on previous literature [29], named mat K, trnH-psbA and rpl16 (KJ468094.1). Slight modification on one of the primer sequence

for mat K target was done, due to amplification performance reasons. Moreover, Ara h 6 allergen-coding region of peanut was selected as nuclear low-copy target and specific primers and Taqman probe were designed using primer3 software. *In-silico* analysis of these sequences was also performed using BLASTn from NCBI, searching for homologue sequences from other species, especially legumes and tree nuts. Moreover, end-point PCR was performed with peanut and other species DNA using specific primers (Table S2), following the same program that described in 2.4 section. Then, Sanger sequencing of partial rpl16, trnH-psbA and Ara h 6 sequences was performed in an ABI PRISM 3700 sequencer (Applied Biosystems, Foster City, CA, USA) from the Genomics Service (Universidad Complutense de Madrid, Spain). In the case of Ara h 6, resulted amplicons were cloned into the pCR<sup>TM</sup>4-TOPO<sup>®</sup> Vector using TOPO<sup>®</sup> TA Cloning<sup>®</sup> Kit (Invitrogen, Inc., UK) following the manufacturer's instructions. Sequences were analysed using Bioedit Software (Ibis Biosciences, Carlsbad, CA, USA). Final sequences of primers and probes, their final concentration used in Real Time PCR reactions and amplicon sizes are included in Table 1.

## 2.6. Real Time PCR

Real Time PCR reactions were performed with 7900HT Fast Real Time PCR (Applied Biosystems, CA, USA), in a volume of 20 µl containing 5 µl of DNA at different concentrations, different final concentration of primers and probes (Table 1) and 10 µl of TaqMan<sup>®</sup> Gene Expression Master Mix (Applied Bio- system, CA, USA). The used program included an initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15s and primer annealing and elongation at 60°C for 1 min.

The cycle threshold (Ct) value, obtained from 10-fold serial dilutions of peanut DNA in deionized water and different points of binary mixtures containing from 100000 to 0.1 mg of peanut per kg of mixture, were used to generate standard curves for Real Time PCR. The efficiency ( $10^{(-1/\text{slope})}-1$ ) of each reaction was calculated from the slope of each standard curve (Ct vs log DNA content or Ct vs log Quantity of peanut nut flour). Sensitivity and limit of detection (LOD) were determined taking into account the lowest amount of target that can be detected in at least 95% of the cases [31]. Specificity of primers was tested Real Time PCR, by means of the amplification of 10 ng of isolated DNA from different species.

**Table 1. DNA sequences of primers and probes.**

Oligonucleotide	Sequence (5'→ 3')	Final concentration (nM)	Amplicon (bp)	Reference
mat K fw	TGGACTCGCCTCTGGTCAT	600		
mat K rv	CTGCATATCCGCAAATACCG		104	[29]*
mat K probe	FAM-CATCCCATTAGTAAGCCCGTTG-BHQ	200		
trnH-psbA fw	AGGAGCAATAGAAACTGCGT	500		
trnH-psbA rv	TTTTTGCTTAAGGGATACGAGT		68	[29]
trnH-psbA probe	6FAM-TGATATTGCTCCTTTACTTTCAAAA-BHQ1	250		
rpl16 fw	GCGATGGGAACGACGAAAAC	250		
rpl16 rv	TTAGTTCGTTCCGCCATCCC		69	[29]
rpl16 probe	6FAM-ACCTAAGATTCATTTGACGGGA-BHQ1	150		
Ara h 6 fw	AGTGCGATAGGTTGCAGGAC	600		
Ara h 6 rv	AAATCGCAACGCTGTGGTG		107	This article
Ara h 6 probe	6FAM-GCAAAATGGTGCAGCAGTTCAAGAG-BHQ1	200		
Universal 18S fw	CGCGAGAAGTCCACTAAACC	250		
Universal 18S rv	CCTACGGAAACCTTGTTACGA		64	[32]

\*Based on that article with slight sequence modification.

## 2.7. Statistical analysis

The significance of differences ( $p < 0.05$ ) between the Ct values of each spiked level for each treatment (boiling, DIC and autoclave processing) compared to untreated control was evaluated by t-student test using GraphPad Prism Programme.

### 3. Results and Discussion

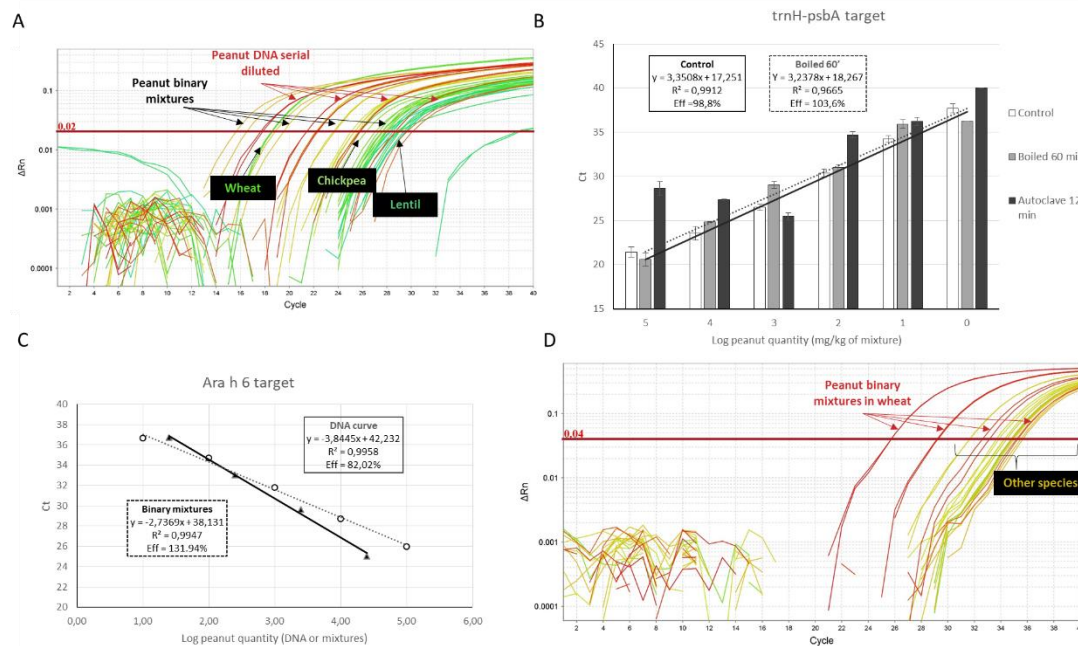
Here we describe the results regarding sensitivity, primer efficiency and specificity of the four probe-based assays. Rpl16, trnH-psbA and Ara h 6 are described in 3.1 section whereas obtained data from mat K assay is included in 3.2, together with the comparison performed after DNA isolation with different protocols.

#### 3.1. Selection of the suitable target

As mentioned in material and methods section, we first reviewed the available literature in DNA-based methods for peanut allergen detection, selecting three chloroplast markers, rpl16, trnH-psbA and mat K because of their better results regarding sensitivity, efficiency of the amplification and specificity [29]. Puente-Lelievre *et al* designed a multiplex assay, based on chloroplast markers, to detect allergenic peanut. Multicopy sequences, such as chloroplast or ITS sequences, have been proposed as very sensitive markers for trace food allergen detection, not only in peanut but in other nuts [15,33–36]. Low/single copy number genes have also provided specific and sensitive results in Real Time PCR [9]. Thus, we designed primers and a Taqman probe for the amplification of the allergen-coding gene Ara h 6, which is major allergen in peanut encoding for a 2S albumin, aimed to detect peanut traces in food.

General results of rpl16, trnH-psbA and Ara h 6 targets are summarized in Figure 1, and obtained data using mat k primers and probe are described in paragraph 3.2. DNA used in these experiments was isolated with Protocol 1, described in M&M section. Regarding rpl16 primers, as can be visualized in the amplification plot (Fig. 1A), specificity was much compromised, since Ct<30 were obtained in Real Time PCR for some species different to peanut, such as chickpea, soybean, lentil or beans. Sequencing of partial rpl16 gene using specific primers (table 2SM) revealed that rpl16 sequence from other legumes were identical to the rpl16 peanut partial sequence (data non-shown). In our hands, the rpl16-based protocol was not enough specific for peanut detection, using same and different plant species and trying to reproduce the same Real Time PCR conditions of annealing temperature or starting DNA quantity than the reference paper [29]. Thus, we decided to discard this marker as a target for this protocol.





**Figure 1. Obtained data using different DNA target from peanut.** A) Rpl16 amplification plot of several samples, including a calibration curve (of 10-fold serial diluted DNA; in red), several points of binary mixtures containing spiked peanut (in yellow) and 10 ng of DNA from other species. It can be visualized the amplification of other legumes as chickpea (Ct around 25), lentil (Ct = 29) and even wheat (Ct = 18) (in green tone). B) Real Time PCR detection of peanut targeting trnH-psbA chloroplast marker. Binary mixtures were performed with untreated (control, White columns), boiled for 60 min (grey) or autoclaved at 121°C (1.2b) for 15 min (black), containing known amounts of peanut in wheat (from 100000 to 1 ppm). Reaction efficiency within the accepted range (90-110%) was obtained in control and boiled 60 min-peanut containing mixtures. C) Ara h 6 calibration curves performed from 10-fold serial diluted peanut DNA (solid line, triangles) or from binary mixtures DNA, containing known amounts of peanut in wheat matrix (pointed line, circle). Correlation coefficient ( $R^2$ ) and efficiency is shown. D) Amplification plot of Real Time PCR for Ara h 6 detection in several samples. In red, plots from several points of serial diluted peanut DNA (from 25 ng to 0.025 ng of DNA); in yellow, amplification plots from other species, including spelta wheat, pistachio, chickpea, hazelnut or pea (all of them with Ct > 31).

The trnH-psbA marker shown good results regarding efficiency of the amplification reaction (98.8%), correlation coefficient (0.9912) and sensitivity (at least 1 mg/kg of peanut in binary mixtures was detected in 100% of the replica) (Fig. 1B, white columns). Specificity resulted also adequate after assaying 10 ng of DNA from several species by Real Time PCR, using primers for peanut trnH-psbA sequence (Table 2).

**Table 2. Specificity of the trnH-psbA based Real Time PCR assay for peanut detection.** 10 ng of DNA from different plant species was assayed at least in triplicate. Protocol 1 was used to obtain genomic DNA. N.D. not detected.

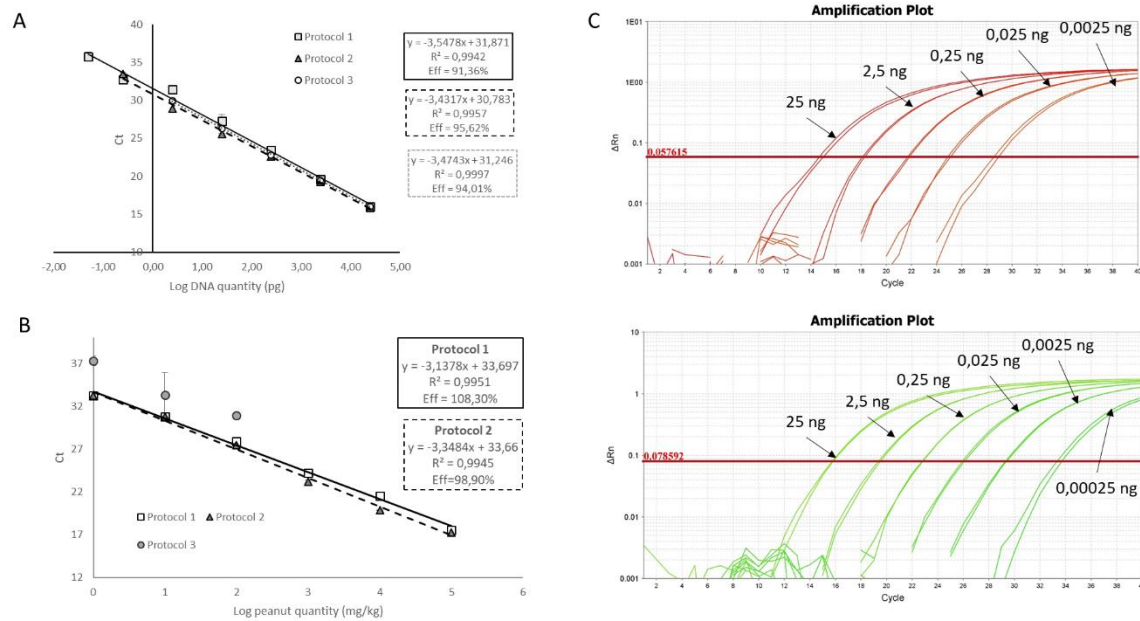
Common name	Scientific name	Ct $\pm$ SD	Common name	Scientific name	Ct $\pm$ SD
Wheat	<i>Triticum spelta</i>	35.70 $\pm$ 0.30	Fababean	<i>Vicia faba</i>	N.D.
Lentil	<i>Lens culinaris</i>	N.D.	Pea	<i>Pisum sativum</i>	N.D.
Chickpea	<i>Cicer arietinum</i>	N.D.	Soy	<i>Glycine max</i>	N.D.
Almond	<i>Prunus dulcis</i>	N.D.	Hazelnut	<i>Corylus avellana</i>	36.97 $\pm$ 0.38
Green bean	<i>Phaseolus vulgaris</i>	N.D.	Cashew	<i>Anacardium occidentale</i>	37.39 $\pm$ 1.48
Walnut	<i>Juglans regia</i>	N.D.	Pistachio	<i>Pistacia vera</i>	35.33 $\pm$ 0.94

Consequently, we decided to test this marker with samples containing thermally processed peanut. This data is presented in 3.3 paragraph of this section.

Ara h 6 primers and probe was designed based on the sequencing information of some clones (Fig. SM). Calibration curves (with serial diluted DNA and binary mixtures spiked with peanut) using primers for peanut Ara h 6 coding gene (Fig. 1C), did not show acceptable efficiency (within the range of 90-110%), although good linearity was obtained in both cases ( $R^2 > 0.990$ ). Twenty-five nanograms of peanut DNA (first point of the DNA curve) were detected after 25 cycles of amplification, almost 8 cycles after than the same sample assayed with primers targeting chloroplast markers as mat K and rlp16. Moreover, when DNA from other species was analyzed with Ara h 6 primers, positive signal was obtained ( $C_t > 35$ ), and as a consequence of these data, putative LOD was too high (1000 ppm) (Fig. 1D). We decided to discard this marker as a possible target for peanut detection in treated samples and foodstuff.

### 3.2. Mat K-based Real Time PCR assay and comparison of DNA isolation methods

As commented above, we decided to perform the Real Time PCR assay targeting mat K chloroplast gene, based on the primers and probe published by Puente-Lelievre *et al*, with a slight modification on the reverse primer to improve reaction performance. Mat K primers/probe yielded good results regarding amplification efficiency, after analyzing both performed calibration curves (91.36%-108.30%). Linear dynamic range was obtained between 25 and 0.0025 ng with a  $R^2$  coefficient of 0.994 in the 10-fold diluted DNA curve. After representing the mean  $C_t$  value and the log of the quantity of peanut flour in the mixtures,  $R^2$  was 0.995, in the linear range between 100000 and 1 mg/kg (Fig. 2AB, white square, solid line and Fig. 2C). Thus, absolute LOD was established at 0.0025 ng (2.5 pg) of peanut DNA and practical LOD, calculated with standard curves based on binary mixtures spiked with known quantity of peanut, was established at 1 mg of peanut per kg of mixture, with  $C_t$  max of  $33.22 \pm 0.20$  (Figure 2B, also shown in Table 3) [31]. Since the lowest amplified level is within the linear range of the standard curve, 1mg/kg (or 1 ppm) is also considered as the LOQ. Below 1 mg/kg detection was possible but  $C_t$  was not within the calibration curve (Table 3). These results, regarding absolute LOD and curve efficiency obtained from the slope, resulted more promising than those recently reported by Puente-Lelievre and collaborators. In the particular case of mat K, when it was analyzed as a single marker, authors obtained standard curves with correlation coefficient of 0.98 and 88% of efficiency, calculated from the curve slope. With the slight modification on the reverse primer published by these authors, we acquired  $R^2$  and efficiency within the acceptable ranges for this single chloroplast marker. LOD for mat K target was 1 mg/kg in both previously published study and ours, using different food matrices.



**Figure 2. mat K target Real Time PCR method** A) Calibration curves of Real Time PCR assay using mat K specific primers, performed with 10 fold serial diluted peanut DNA, isolated by three different isolation protocols (1, 2 and 3). B) Calibration curves performed with DNA from binary mixtures of peanut in wheat, using DNA obtained with the 3 different protocols. Efficiency and R<sup>2</sup> values are included for the two kit-based DNA isolation protocols. Only Ct values obtained after DNA isolation by protocol 3 are shown as grey circles for comparison purposes (samples containing 100, 10 and 1 mg of peanut/kg of mixture). Mean and standard error are represented. C) Example of amplification plots of 10-fold serial dilution of peanut DNA in deionized water, isolated with Protocol 1 (upper image) or Protocol 2 (lower figure), from 25,000 to 0.25 pg.

Regarding specificity of the mat K-based assay, 10 nanograms of DNA from several species, as legumes, fruits and tree nuts, were analyzed using specific mat K primers and probe, and Ct ranged between  $34.44 \pm 0.27$  for pistachio and  $39.70 \pm 0.42$  for apple (Table 3). This amplification was not considered significant since 10 ng of peanut DNA would be detected at cycle 18 (by DNA curve extrapolation), being even more than 15 cycles earlier than those species. Mat K-based assay was used for two subsequent objectives: to compare among DNA isolation methods and to analyze the effect of different thermal treatments on the target amplification (paragraph 3.3). Positive amplification control using universal 18S primers was performed before Real Time PCR assays.

**Table 3. Specificity of the mat K-based Real Time PCR assay for peanut detection.** 10 ng of DNA from different plant species was assayed at least in triplicate. Protocol 1 was used to obtain DNA. N.D. not detected.

Common name	Scientific name	Ct ± SD	Common name	Scientific name	Ct ± SD
Apple	<i>Malus domestica</i>	39.70 ± 0.42	Fababean	<i>Vicia faba</i>	36.20 ± 1.62
Lentil	<i>Lens culinaris</i>	39.51 ± 0.68	Chickpea	<i>Cicer arietinum</i>	37.73 ± 2.60
Lemon	<i>Citrus x limon</i>	37.24 ± 0.21	Pea	<i>Pisum sativum</i>	38.47 ± 0.88
Lupin	<i>Lupinus albus</i>	37.71 ± 1.22	Wheat	<i>Triticum spelta</i>	N.D.
Almond	<i>Prunus dulcis</i>	37.79 ± 1.24	Hazelnut	<i>Corylus avellana</i>	37.51 ± 0.48



Green bean	<i>Phaseolus vulgaris</i>	38.25 ± 0.79	Cashew	<i>Anacardium occidentale</i>	37.39 ± 1.48
Kiwi	<i>Actinia deliciosa</i>	N.D.	Grass pea	<i>Lathyrus sativus</i>	37.77 ± 0.89
Carob	<i>Ceratonia siliqua</i>	35.46 ± 2.48	Chestnut	<i>Castanea sativa</i>	N.D.
Walnut	<i>Juglans regia</i>	35.55 ± 0.26	Pistachio	<i>Pistacia vera</i>	34.44 ± 0.27
Pear	<i>Pyrus pyrifolia</i>	N.D.	Soy	<i>Glycine max</i>	39.06 ± 1.11

Protocols 2 and 3 were planned to be faster and cheaper methods to obtain quality DNA from peanut samples, and more focused on the isolation of chloroplast DNA, compared to standard genomic DNA extraction protocol (Protocol 1). Thus, detection of the chloroplast marker, mat K, might be facilitated. Moreover, we aimed to use isolated DNA for a subsequent future use in a specific peanut genosensor, which requires rapid, reproducible and economically worthwhile. General results about the reaction performance using DNA isolated with protocols 2 and 3 are shown in Figure 2. Both calibration curves from 10-fold diluted peanut DNA showed good linearity with correlation coefficients higher than 0.99 and efficiencies of the amplification within the acceptable ranges (90-110%), not significantly different to the performance observed when protocol 1 was applied ( $p > 0.05$ ; Fig. 2A). Similar results were obtained when calibration curves were performed using binary mixtures spiked with known amounts of untreated peanut flour, with DNA obtained by means of Protocol 2 (Fig. 2B, triangle). In contrast, DNA from these samples obtained with protocol 3 or in-home protocol, did not allow detecting peanut with enough reproducibility and feasibility, since each point is obtained from an independent DNA isolation and is not built by serial dilution of the DNA (Fig. 2B).

### 3.3. Effect of thermal treatments on peanut detection

In food industry, processing is frequently applied in order to improve safety and organoleptic properties. In the recent years, many researchers have demonstrated that allergen detection is importantly affected by food processing, not only in protein-based detection methodologies but also in DNA-based ones, being extensively reviewed. It has been defended that food-processing effect on DNA target detection by PCR should be always considered, even although DNA is a very stable molecule, especially compared to proteins. It has been possible to detect DNA markers even in harsh thermal conditions, including autoclave, roasting, boiling, etc. In order to establish the real influence of any processing, many authors have supported the necessity of analyzing the same kind of model mixtures in control and treated samples. For this reason, binary mixtures were also made with treated flours in the same wheat matrix. Here we described the effect of several treatments, based on temperature (boiling) or heat combined with pressure (Despressurized Instant Controlled, named DIC and autoclave at different conditions) on the detection of specific peanut chloroplast DNA markers. A calibration curve has been then performed for each treatment, plotting the average Ct values against the log of the quantity of peanut flour in mixture. NTC (non template control) samples were always not detected (N.D.) and DNA was first isolated with standard Protocol 1. Regarding detection of trnH-psbA marker, accepted parameters (103.6% of efficiency, 0.966 of correlation) were obtained in curves set up from boiled samples, although amplification was delayed 2 cycles compared to untreated control (Fig. 1B, light grey columns). However, when pressure and temperature was applied, in the first autoclave condition (at 121 °C for 15 min), the detection of trnH-psbA was difficult. Linear range was not obtained and Ct > 29 was obtained in samples with 10% w/w of treated peanut (or 100000 mg/kg) in the mixture (compared to Ct ~20 when peanut was kept untreated) (Fig. 1B). Effect of autoclave treatment for longer time was also analyzed, but the tendency was similar to that obtained in softer conditions of autoclave (data non-shown). Amplicon size of trnH-psbA was small, just 68 pb, and this fact usually contributes to better results regarding

sensitivity when DNA integrity is affected. Recently, we designed a Real Time PCR assay to detect cashew presence in food, being possible even in autoclaved samples at 138°C for 15 min, maintaining linearity of the curves up to 1000 ppm. In that case, amplicon size was only 65 pb, targeting partial Ana o 1 allergen coding gene. Nevertheless, amplicon size is not the only important factor influencing the capacity to detect the target in samples with comprised DNA integrity. From our point of view, it becomes essential to analyze, experimentally, the influence of several common food technological treatments on the detection capacity of any specific target. Same approach was tested with specific primers and probe for mat K target, and results are summarized in Table 3 and Figure 1SM. Suitable detection of mat K target was possible in mixtures containing boiled peanut for 1 hour, showing  $R^2 > 0.99$  and 106.73% of amplification efficiency, comparable performance to untreated (Fig. 2SM). Efficiency and correlation coefficient of the curves were within acceptable ranges when binary mixtures were performed with other treated peanut flour as DIC and autoclave at 120°C for 15 and 30 min (Table 4), although detection was significantly delayed several cycles compared to control mixture (Fig. 2SM).

**Table 4. Detection of mat K target by probe-based Real Time PCR in untreated (control) and treated spiked samples.** DNA isolation protocol was DNeasy Plant Pro Kit (Qiagen, Protocol 1) for all samples.

Peanut quantity (mg/kg)	Control <sup>1</sup>	Boiling 60 min	DIC 7b 120s
100000	17.55 ± 0.17	18.69 ± 0.25	24.15 ± 0.29
10000	21.52 ± 0.30	23.30 ± 0.30	28.27 ± 0.62
1000	24.17 ± 0.17	26.15 ± 0.28	31.51 ± 1.14
100	27.89 ± 0.14	28.33 ± 0.25	34.62 ± 0.68
10	30.77 ± 0.25	33.36 ± 0.29	38.56 ± 0.33 <sup>†</sup>
1	33.22 ± 0.20	34.17 ± 0.75	39.69 ± 0.25 (50%) <sup>†</sup>
0.5	32.74 ± 0.15 <sup>†</sup>	36.47 ± 0.45	N.A.
0.1	33.87 ± 0.58 <sup>†</sup>	N.A.	N.A.
Slope	-3.14	-3.17	-3.46
Efficiency (%)	108.30	106.73	94.43
R <sup>2</sup>	0.995	0.982	0.995

Peanut quantity (mg/kg)	AU121°C 15 min	AU121°C 30 min	AU138°C 15 min	AU138°C 30 min
100000	20.93 ± 1.14	25.01 ± 0.28	29.50 ± 0.13	38.68 ± 0.89 (50%)
10000	24.84 ± 1.20	29.55 ± 0.33	30.63 ± 0.05	38.92 ± 0.72 (50%)
1000	28.23 ± 0.67	33.28 ± 0.29	35.54 ± 0.06	39.41 ± 0.40 (50%)
100	30.87 ± 0.78	35.39 ± 0.43	37.09 ± 0.33 <sup>†</sup>	39.70 ± 0.34 (25%)
10	32.75 ± 0.21 <sup>†</sup>	37.93 ± 0.49 <sup>†</sup>	39.6 ± 0.32 (75%) <sup>†</sup>	N.D.
1	34.79 ± 1.01 <sup>†</sup>	39.87 ± 0.15 <sup>†</sup> (25%)	N.D.	N.D.
Slope	-3.32	-3.48	-3.02	--
Efficiency (%)	100.05	93.65	114.23	--
R <sup>2</sup>	0.993	0.975	0.885	--

<sup>1</sup>Ct±SE

<sup>2</sup>Percentage of positive amplification

N.A. Not assayed

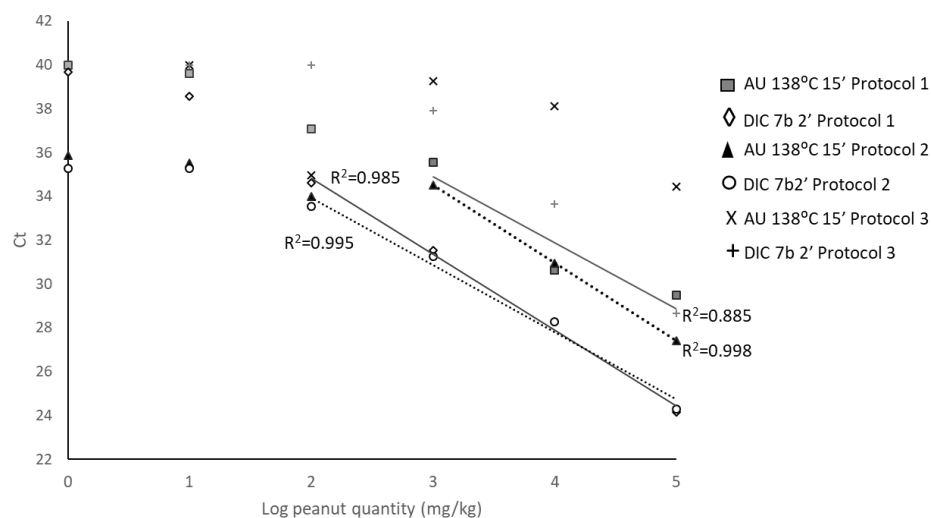
N.D. Signal was not detected after 40 cycles of amplification

<sup>†</sup>Detection is possible but Ct is not in the calibration curve.

According to our results, the influence of DIC treatment, based on high temperature (up to 180°C) and pressure (7b) for a very short time (2 min) [37], on mat K target detectability was similar to the observed effect of autoclave at 121°C for 30 min, with no significant differences among the Ct values in different spiked levels ( $p > 0.05$ ). In both cases, it was possible to detect up to 1 mg/kg of peanut but in less than 50% of the replica (Table 3). Our group recently described for the first time the effect of

this novel thermal treatment on Real Time PCR detection of three tree nuts allergen-coding sequences [38]: Cor a 9 from hazelnut, Pis v 1 from pistachio and Ana o 1 from cashew. In that article, we reported the capacity to detect and quantify DIC-treated samples in mixtures when the allergenic ingredient was around 100000 and 1000 mg/kg. Here, the detection system would allow detecting and even quantifying the presence of treated peanut when it is around 100000 and 100 mg/kg in a mixture. When autoclave at 138°C was applied on peanut and DNA from the mixtures was obtained, linearity of the curves (representing mean Ct of several spiked level *vs* log quantity of peanut in each mixture) was not maintained, and efficiency was slightly higher than the acceptable 110% (Table 4). Detection was not possible in AU 138°C 30 min samples, with Ct > 38 in all the spiked levels.

It resulted interesting to observe that maximum obtained Ct value was higher in samples containing treated peanut compared to those with untreated nut. Thereby, as an example, in samples with 1 mg/kg or ppm (LOD of the system) of untreated/control peanut, Ct was ~33, and then Ct values were not in the calibration curve. However, in boiled peanut containing mixtures, same peanut quantity was detected at cycle 36, within the linear dynamic range of the calibration curve (Table 3). A similar effect can be discussed when we analyzed the results from our reference [29]. Authors prepared several food matrices spiked with known amounts of peanut flour (muffins, cookies, sauces, etc) and generated calibration curves by plotting Ct values against log of peanut concentration. Obtained Ct values for the lowest spiked level were different depending on the matrix (Ct 32-38), and good linearity and efficiency was obtained in some of them. Thus, thermal treatment is contributing to the fragmentation or degradation of DNA molecules affecting the final Ct values.



**Figure 3. Influence of DNA isolation method in the performance of mat K detection in processed matrices.**

Amplification of mat K was analysed in two treated peanut samples, by AU 138°C 15 min and DIC 7b 2 min, which DNA was isolated by Protocols 1, 2 and 3. Curves were performed by plotting mean Ct and log of the peanut quantity.

As described before with untreated samples, DNA from several points of binary mixtures prepared with processed peanut flour (DIC 7b 120 s and AU 138°C 15 min) was isolated using protocols 2 and 3, and compared to standard protocol 1 (Fig. 3). Standard curves built with DNA from mixtures containing from 100000 mg/kg to 1 mg/kg of DIC-treated peanut, obtained from protocols 1 and 2, showed a linear dynamic range extended 4 log<sub>10</sub>, up to 100 mg/kg of treated with R<sup>2</sup> > 0.99 (Fig. 3). In the case of autoclave-treated spiked samples, DNA obtained with protocol 2 allowed to build a curve with a better correlation coefficient than the one with protocol 1 (R<sup>2</sup> 0.998 vs 0.885). With DNA obtained with protocol 3, nevertheless, it was not possible to achieve consistent and reliable data, being complicated to obtain enough DNA from so processed samples. With these data, together with

those described in section 3.2, it can be concluded that protocol 2 allows the peanut DNA isolation in a cheaper and faster manner than standard genomic DNA isolation protocol (here protocol 1), even in processed matrices. Performance of the reactions is not affected, although it is not improving chloroplast marker detection compared to the standard protocol. DNA from other species, isolated with this protocol, should be analysed by Real Time PCR; moreover, this protocol might be established as the routine isolation method in the future, for the sample analysis by a specific genosensor for peanut detection.

As mentioned, Real Time PCR methods for peanut detection have been performed before targeting different sequences, but only a few analyzed the effect of processing on DNA detectability, compared to untreated samples [15, 16, 39]. Among them, usually peanut-containing foodstuff which requires some kind of thermal treatment, such as cookies, sauces or doughs, are prepared and analysed. Here, we incorporated peanut samples processed by a plethora of conditions regarding temperature, pressure and time, observing the influence on the target detectability.

### 3.4. Applicability of the peanut mat K-based detection assay

Finally, we have confirmed the possible applicability of the Real Time PCR assay, based on mat K target amplification, for the detection of peanut traces in foodstuff. All food samples were amplified with 18S primers as false negative control, confirming the presence of isolated DNA and the absence of putative co-isolated PCR inhibitors together with the DNA. A 2-fold dilution of the isolated DNA was used, and conventional end-point PCR using Universal eukaryotic 18S primers was performed with DNA from food before specific peanut detection. We analyzed thirteen commercial food samples by mat K based Real Time PCR assay and results are shown in Table 5. One of them is the cereal bar I, which according to the label contents around 35% of peanut, together to hazelnut, obtaining a mean Ct near to 18. Some of them showed Ct values higher than 36, which might be considered as negative results regarding peanut detection. Cereal bar III declared the presence of peanut/almond traces and 10% of hazelnut content, resulting in a mean Ct of 33. Possible peanut contamination should be considered in food named Chocolate bar I, whose label indicates tree nut traces. Interestingly, 3 out of 13 foods, named chocolate bar II, cookies with fiber and cookies with chocolate, did not declare presence of any allergen on their label; however, Ct 31-32 were obtained and possible contamination should not be discarded. Even although these Ct values are high, are included in the linear range of the standard curve, and those foods might be containing between 10 and 1 mg/kg of peanut, or even more if DNA has been damaged during processing, as described above. Comparison with commercial qPCR and/or ELISA tests would be interesting in order to determine the feasibility of this assay.

**Table 5. Detection of mat K target in several commercial food products by Real Time PCR.** Mean Ct and standard deviation is shown. Measurements of the same sample were performed at least twice in two different DNA extractions. N.D. means that non signal after 40 cycles of amplification. All samples were first assayed for amplification inhibitor presence using eukaryotic universal primers.

Food	Food allergen declaration	Ct $\pm$ SD
Cereal Bar I	Peanut (35%), Hazelnut (24%)	17.97 $\pm$ 0.26
Cereal Muesli	Tree nut and peanut traces	38.73 $\pm$ 0.76
Cereal Bar II	Almond and tree nuts	32.40 $\pm$ 0.29
Cereal Bar III	Hazelnut (10%), Almond and Peanut traces	33.74 $\pm$ 0.29
Cereal Bar IV	May content tree nut traces	35.90 $\pm$ 0.94
Chocolate with pistachio	Pistachio (5%), Almond, Hazelnut, tree nut traces	36.80 $\pm$ 0.43
Vegetal Burger	May content tree nut traces	39.17 $\pm$ 0.96
Sausage with walnut	Walnuts	N.D.
Chocolate	Almond and Hazelnut traces	37.99 $\pm$ 0.33
Chocolate Bar I	Tree nut traces	32.42 $\pm$ 0.16

Chocolate Bar II	Not declared	31.32 ± 0.21
Cookies with fiber	Not declared	31.02 ± 0.30
Cookies with chocolate	Not declared	32.25 ± 0.46

#### 4. Conclusions

In this study, a Real Time PCR assay addressed to detect peanut in complex food samples has been performed. Several gene targets and DNA isolation methods have been proposed and compared in this work. Moreover, different conditions of heat, pressure and time (as boiling, autoclave and D.I.C. processing) were applied to peanut nuts and its influence on the amplification of selected targets has been determined. As markers, three chloroplast markers (trnH-psbA, rpl16 and mat K) and one nuclear marker (Ara h 6 allergen coding sequence) were analysed regarding sensitivity, efficiency and specificity of each Real Time PCR method. In our hands, the assay based on mat K target detection was the most specific, reliable and sensitive enough for the detection of peanut in several matrices, including binary mixtures containing strongly treated peanut, submitted to high temperatures and pressure. For DNA isolation, kits based on silica membranes resulted more adequate for the obtaining of quality DNA from complex food matrices, named in this work protocol 1 for the isolation of total DNA and 2 for the obtainment of plasmid-enrich DNA. These two protocols have been compared, showing same amplification performance for mat K target. According to our results, mat K-based Real Time PCR method is suitable for reliable and specific detection of peanut in complex mixtures, processed samples and commercial food products.

**Supplementary Materials:** The following supplementary material is available online at [www.mdpi.com/xxx/s1](http://www.mdpi.com/xxx/s1), Table S1: Preparation of binary mixtures of peanut in spelt wheat flours, Table S2: Primers used for sequencing purposes; Figure 1SM: Sequence alignment of two clones of partial Ara h 6-allergen coding gene; Figure 2SM. Amplification of mat K marker in treated and untreated binary mixtures.

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