



Chestnut allergen detection in complex food products: Development and validation of a real-time PCR method

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ABSTRACT

Chestnut is gaining importance as food allergen, but a specific detection method has not been performed so far. For the first time, the development of a real-time PCR assay for chestnut allergen detection in processed foods is described. Initially, three sets of novel primers specific for allergen-encoding genes (Cas s 5, Cas s 9 and Cas s TLP) were designed and evaluated for chestnut detection. Primers for Cas s 9 and a TaqMan probe were finally selected for a sensitive and specific detection of chestnut in binary mixtures with a practical limit of detection (LOD) of 100 ppm. Effect of processing has been evaluated and sensitivity slightly reduced after boiling treatment. Amplification of Cas s 9 was importantly affected when heat and pressure were applied (autoclaving). Applicability of the method was tested by analysis of commercial food products. Consequently, the results support the development and validation of a real-time PCR method for accurate chestnut allergen detection and labelling.

1. Introduction

Castanea sativa Mill. is a tree belonging to Fagaceae family and its fruit-the chestnut - is generally consumed fresh or prepared for products such as chestnut purée and chestnut flour, which finds application in gluten-free diets (Demirkesen, Mert, Sumnu, & Sahin, 2010; Paciulli et al., 2018). Allergy to chestnut has been reported in the individuals with latex-fruit syndrome and chestnut reactivity has been also associated to fruit or other tree nuts allergy (Pantoja-Uceda et al., 2004; Rico, Crespo, Feliu, & Rodriguez, 2004). Three chestnut food allergens have been included in the WHO-IUIS list (<http://www.allergen.org>): Cas s 5 (chitinase), Cas s 8 (LTP) and Cas s 9 (heat shock protein) (Diaz-Perales et al., 1999, 2000; Lopez-Matas et al., 2004). Additionally, a thaumatin like protein, Cas s TLP, has been described as chestnut food allergen (Palacín et al., 2012).

Nowadays, as there is no treatment for food allergies, sensitised 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 sensitised individuals, in agreement with the consensus experts (Pádua, Moreira, Moreira, Melo de Vasconcelos, & Barros, 2019). Usually, protein-based assays as enzyme-linked immunosorbent assay (ELISA) are used to detect small amounts of proteins from specific foods; however, there is no ELISA in the market for chestnut detection. DNA-based methodologies, such as real-time PCR, microarrays and genosensors, have been proposed as specific, sensitive and reliable alternatives to ELISA since this molecule preserves its integrity better than proteins (Linacero, Sanchiz, Ballesteros, & Cuadrado, 2019). To our knowledge, no research has been performed to develop a method for chestnut allergen detection, either protein or DNA-based technology.

Foods, including chestnuts, are usually thermally treated to preserve food safety and, often, to modify allergenic reactivity (Caballillas & Novak, 2017) but maintaining or improving organoleptic and functional properties (Sanchiz et al., 2019). The protein solubility can be highly affected by thermal food processing, and subsequent detection with protein-based techniques might be hampered (Mattison et al., 2016). Methods based on trypsin digestion combined with liquid chromatography/mass spectrometry can be helpful for allergen screening in baked foods (Qi et al., 2019). Fragmentation and/or

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Table 1

DNA sequences of primers used for conventional PCR (in **bold**) and primers and probes used for real time PCR experiments, for chestnut targets detection. Sequence of 18S primers and LNA probe is also included. The final concentration on the PCR reaction for primers and probe, length and expected size of the amplicons are also indicated.

Oligonucleotide	Sequence (5'→ 3')	Length	Final concentration (nM)	Amplicon (pb)
Cas s 5 fw	GCTTGGAACTCAGCAGAAC	20	500	379
Cas s 5 rev	GGTTTGAGCTAAGAAAGCCG	20		
Cas s TLP fw	TTCACCTGCGAGACTGCTGA	20	500	396
Cas s TLP rev	AGCTTGAGGACATTGTTGCTT	20		
Cas s TLP fw	CTGTGCGAGCCAACGTGAAC	20	250	62
Cas s TLP rev	TCGCATCAGACCCCTTCACT	20		
LNA® probe	6-FAM- TTGCCAG –BHQ1	8	100	
Cas s 9 fw	TGTAGCCAACGTTCCCTCCT	20	500	280
Cas s 9 rev	CTGCTCCACCTTGGCATTCT	20		
Cas s 9 fw	TGATAAGTGGCACAGGGTTG	20	250	63
Cas s 9 rev	TCTGGCAACCTAAACCTCCTAA	22		
LNA® probe	6-FAM-AGGAGCTG-BHQ1	8	100	
Cas s 9 fw	TTGAGGTGAGGATGGGAATG	21	250	88
Cas s 9 rev	TCAACCTGTGCCACTTATC	20		
TaqMan® probe	6-FAM-GTGTGCAAATAAGCGGGGAGAGGA-BHQ1	25	150	
18S fw	CGCGAGAAGTCCACTAAACC	20	150	64
18S rev	CCTACGGAAACCTTGTACGA	21		
LNA® probe	FAM- GGAAGGAG-BHQ	8	100	

degradation of DNA molecules after severe treatments have been reported by several authors (Costa, Oliveira, & Mafra, 2013; Gryson, 2010) and some studies have been performed to analyse the effect of different treatments (boiling, High Hydrostatic Pressure HHP, autoclave, frying, roasting) on the detection of different DNA targets in peanut, hazelnut, walnut, almond or pistachio (Iniesto et al., 2013; Linacero et al., 2016; Prieto et al., 2014; Sanchiz et al., 2017; Scaravelli, Brohée, Marchelli, & Van Hengel, 2009).

Specificity, sensitivity and potential of quantification of a real-time PCR method for allergen detection are compromised by the selected target sequence. Both multi-copy (such as ITS mitochondrial genes) or single/low-copy genes have been used as targets for real-time PCR based detection methods for nut analysis (Brzezinski, 2006; Demmel, Hupfer, Hampe, Busch, & Engel, 2008; Linacero et al., 2019; Lopez-Calleja et al., 2013). The objective of this study was to set up and validate the first real-time PCR assay for sensitive, specific and reliable chestnut detection, using novel primer pairs for allergen coding sequences as targets and hydrolysis probes. Additionally, we aimed to analyse the effect of thermal food processing (boiling and autoclaving) on chestnut detection in complex food products by real-time PCR.

2. Materials and methods

2.1. Samples

Four varieties of *Castanea sativa* Mill (Cella Ampla, Pere Andreu, Primerenc and Tardà) were provided by IRTA (Institut de Recerca I Tecnologia Agroalimentaries; Mas de Bover, Tarragona, Spain) and an additional commercial variety of chestnut, Miquelencia, was purchased to Cooperativa Secallona (Viladrau, Gerona, Spain). Flour from Miquelencia variety was obtained after grounding with a kitchen robot (Thermomix 31–1, Vorwerk Elektrowerke, GmbH & Co. KG, Wuppertal, Germany) and milled with a sieve of 1 mm. Other plant species used in food industry were also used. Food products were purchased in local stores, ground with the kitchen robot and stored at –20 °C.

Binary mixtures (spiked samples) were performed by mixing known amounts of Miquelencia chestnut flour with spelt wheat (*Triticum spelta* L.) flours at different concentrations (0.5, 1, 5, 10, 100, 1000, 10000, 100000 mg/kg). The first mixture with 10% (100000 mg/kg) of chestnut was prepared by adding 10 g of chestnut flour to 90 g of wheat, and followed by 10 or 5-fold dilutions up to 1 mg/kg. Final

weight was 100 g and mixtures were homogenised with the kitchen robot (Sanchiz, 2018).

2.2. Boiling and autoclaving treatments

One hundred grams of chestnuts of variety Miquelencia were immersed in distilled water (1:5 w/v) for boiling for 30 and 60 min or autoclaving in autoclave (Compact 40 Benchtop, Priorclave, London, UK) at 121 °C (118 kPa) and at 138 °C (256 kPa) for 15 and 30 min. All the chestnut samples (untreated (control) and treated) were freeze-dried (Telstar Cryodos, Terrasa, Spain) and ground as above described. Binary mixtures (spiked samples) of treated chestnut flour in spelt wheat were performed as previously described, containing 10, 100, 1000, 10000 and to 100000 mg/kg (0.01%–10% of chestnut). All the samples were stored at 4 °C.

2.3. DNA extraction

DNA from the five varieties (100 mg) were extracted using DNeasy® Plant Mini Kit (Qiagen) following the manufacturer instructions. DNA from control and treated spiked samples (100 mg) was obtained using the CTAB-PVP 1%-chloroform method (Sanchiz et al., 2018), followed by purification using DNeasy Power Plant Pro isolation kit (Qiagen) and eluted in 100 µl of Tris HCl pH 8.3.

Food samples (200 mg) were homogenised with liquid nitrogen and genomic DNA was obtained using NucleoSpin Food kit (Macherey-Nagel, Düren, Germany) following the manufacturer instructions with some modifications (Sanchiz et al., 2017).

The quantity and quality of DNA were determined spectrophotometrically using a NanoDrop ND-1000 (Thermo-Fisher, Waltham, MA, USA), by measuring absorbance at 230, 260 and 280 nm, and by electrophoresis in 0.8% TBE (Tris-borate EDTA) agarose gels.

Extracted DNA from all samples was amplified using 18S universal primers by conventional PCR or Real-time PCR using a specific LNA (Locked Nucleic Acid; from Universal Probe Library, Roche, Germany), as control of DNA quality for the amplification (Table 1).

2.4. Conventional PCR, cloning and sequencing

Primers for conventional PCR assays were designed using NCBI Primer Blast program, on the Cas s 5 (GenBank accession number



Fig. 1. Alignment of the partial sequence of Cas s 9 allergen coding gene in five chestnut varieties and some plant homologue sequences (peach, apple, cacao, walnut, pear). Forward and reverse primers are squared in a continuous line and probe is squared in a discontinuous line.

U48687.1), Cas s TLP (AJ242828.1) and Cas s 9 (AJ582679.1) allergen coding sequences (**Table 1**), and synthesised by Metabion (Metabion International, Planegg/Steinkirchen, Germany).

Conventional PCR assays were performed in a SensoQuest LabCycler (Progen Scientific Ltd, Germany) in a final volume of 20 μ l, containing 25 ng of DNA from the five chestnut varieties, 500 nM of each primer and 1XFastStar PCR Master Mix (Biotools, Loganholme, Australia). PCR programme included an initial step at 95 °C, 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 40 s (Cas s 9 primers) or at 62 °C for 30 s (Cas s 5 and TLP primers), elongation at 72 °C for 1 min (Cas s 9) or 40 s (Cas s 5 and TLP) and a last step at 72 °C for 5 min. Amplicons were cloned in TOPO TA pCR4 Cloning kit (Invitrogen, Inc., UK) following the manufacturer's instructions. Plasmid DNA was obtained using Plasmid DNA purification kit (Biotools, Loganholme, Australia) and sequenced in an ABI PRISM 3700 sequencer (Applied Biosystems, Foster City, CA, USA) from "Servicio de Genómica (Universidad Complutense de Madrid)". Sequences were edited using Software Bioedit (Ibis Biosciences, Carlsbad, CA, USA) and then compared with those in the GenBank (NCBI) non redundant nucleotide (nr/nt) databases, through BLAST. Multiple alignments were performed using Clustal Omega algorithm, we compared sequences of the allergen coding gene from the five varieties and their homologue sequences from other food plant species, which show an identity level higher than 70% with the target.

2.5. Real-time PCR

Specific primers and probes were designed from conserved sequences between all varieties for Cas s 9 and Cas s TLP genes, fitting requirements regarding length and amplicon size (Table 1). Real-time PCR assays were performed with 7900HT Fast Real-Time PCR (Applied Biosystems, CA, USA) in a final volume of 20 µl with 10 µl of TaqMan® Gene Expression Master Mix (Applied Biosystem, CA, USA), 250 nM of each primer, LNA® probe at 100 nM and 5 µl of extracted DNA at

different concentrations. Once selected the target, regarding sensitivity and specificity, TaqMan® probe was designed for Cas s 9 and used at 150 nM in the reaction mix (Table 1).

Reactions were run under following cycling conditions: an initial step at 95 °C, for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and primer annealing and elongation at 60 °C for 1 min. At least two experimental and three technical replicates were analysed, and two non-template controls (NTC) were included in each plate. Standard curves were obtained with the cycle threshold (Ct) from 10-fold serial dilutions of chestnut genomic DNA and from binary mixtures of Miquelencia chestnut flour in wheat. Efficiency of the reaction was calculated from the slope from those standard curves ($10^{(-1/\text{slope})}-1$), either Ct vs log DNA concentration or Ct vs log quantity of chestnut flour.

The effect of boiling and autoclaving processing on *Cas s 9* target amplification has been analysed using standard curves from DNA of binary mixtures made with flour from boiled and autoclaved spiked samples (from 10 to 100000 mg/kg). Applicability of this real-time PCR assay to determine chestnut presence was investigated by analysing 14 commercial foodstuffs.

2.6. Statistical analysis

The significance of differences ($p < 0.05$) between the Ct values of each spiked level for each treatment (boiling and autoclave processing) compared to untreated control was evaluated with analysis of variance (ANOVA) using GraphPad Prism Programme. Previously, the data were assessed for normality by Kolmogorov-Smirnov test.

3. Results and discussion

3.1. Gene target selection and sequence analysis

Coding sequences from Cas s 5, Cas s TLP and Cas s 9 allergens,

deposited in the GenBank database of NCBI, were selected to design primers for conventional PCR (Table 1). Primers were employed for the amplification of partial allergen-coding sequences of five chestnut varieties. Obtained amplicons from Cas s 9 and Cas s TLP showed the expected size (280 and 396 bp, respectively), whereas Cas s 5 amplicons were longer (793 bp or 894 bp) than expected (379 bp) due to the presence of an intron of variable size. Moreover, the sequences obtained for Cas s 5 from the chestnut varieties showed high intraspecific variability (Fig. S1), and consequently, Cas s 5 was not a suitable candidate for Real-time PCR detection. Meanwhile, Cas s 9 and Cas s TLP showed high similarity among the chestnut varieties and therefore, such sequences and the homologue ones from related species, with more than 70% of identity value in GenBank, were aligned, and conserved regions among varieties were used to design the first specific real-time PCR method for chestnut detection (Fig. 1 and Fig. S2).

3.2. Efficiency of amplification, sensitivity and specificity

The selected targets are low copy number genes Cas s 9 and Cas s TLP, being both good candidates to detect a specific ingredient for quantitative purposes (Prado, Boix, & Holst, 2012). False negative results due to intraspecific polymorphism are minimised since a conserved region among several chestnut varieties was selected for primer design. To assess the specificity of Cas s 9 and Cas s TLP primers and probes, 10 ng of DNA from different fruits, tree nuts and other plant species which can be found in foodstuff were tested by Real-time PCR. Cas s TLP assay resulted in positive amplification when DNA from some species was analysed, as peanut, hazelnut, lemon or orange, apart from chestnut with an average Ct of 24.76 ± 0.36 (Table S1). In the case of Cas s 9, specific results were obtained and none of the analysed species was amplified, except chestnut with an average Ct of 25.69 ± 1.08 (Table S1). NTC and wheat DNA were never detected.

The analysis was continued only with Cas s 9 primers and TaqMan probe, whereas Cas s TLP target was discarded because it was not enough specific. Amplification efficiency and sensitivity of the assay were evaluated by the analysis of standard curves. These calibration curves were performed with Ct values obtained from ten-fold dilutions of chestnut DNA (Miquelencia) and from the binary mixtures of different untreated chestnut concentrations made with spelt wheat flour, as previously described by our group (Linacero et al., 2016). Linear correlation, with a R^2 coefficient above 0.99 was obtained between the mean Ct value and the log of the quantity of DNA or chestnut flour, according to the calibration curve (Fig. 2A). Efficiency of the reaction, calculated as indicated before, was 108.95% for DNA curve (slope of -3.12 ; $n = 8$) and 99.70% for binary mixture calibration curve (slope of -3.33 ; $n = 24$), accomplishing with the MIQE guidelines (Bustin et al., 2009).

Sensitivity of this Real-time PCR system was 25 pg of raw chestnut DNA and 100 mg/kg of chestnut flour in binary mixtures with wheat flour. These values were considered the absolute and practical Limit of Detection (LOD) respectively, being the lowest amount of target that could be detected at least in 95% of the replica (Table 2) (European Network of GMO Laboratories, 2015). For the establishment of the practical LOD, at least five different extractions of the nine spiked levels have been performed. As mentioned before, this is the first attempt of a real-time PCR assay for chestnut detection, and until the date, no other DNA or protein-based method has been published for the identification of this nut in foods. Real-time PCR has been chosen by many researchers as a specific and sensitive tool for detecting other food allergens, including nuts. A recent review compiles real-time PCR assays focused on tree nuts detection in foodstuff and processed foods (for almond, hazelnut, pistachio, cashew, Brazil nut, walnut, Macadamia nut or pine nut), using different chemistries and achieving limits of detection in the range of 1000 to 0.1 ppm (Linacero et al., 2019).

3.3. Effect of boiling and autoclaving treatments on Cas s 9 amplifiability

In recent years, flours from some seeds and nuts, as chestnut, rice or legumes, have aroused interest as substitutes of wheat flour for gluten-free formulations (Ling, Zhang, Li, & Wang, 2016; Paciulli et al., 2018; Sanchiz et al., 2019). Many nuts, including chestnut, are part of complex food products (sauces, cookies, pasta, yogurt, ice cream, etc) being extensively processed by heat or/and pressure. Food processing can affect the ability of the food components to trigger an allergic reaction in an unpredictable way and the effect of thermal processing on food allergenicity has been recently reviewed (Cabanillas & Novak, 2017).

Immunoassays involve the binding of a protein from the allergenic food with specific antibodies, being ELISA and Lateral Flow devices (LFD) the most common assays in food industry. In the last years, DNA-based methods are becoming interesting alternatives to immunoassays (de la Cruz et al., 2017), which show some advantages over these protein-based ones, since DNA is more efficiently extracted than proteins from raw and cooked/processed matrices and it is less affected by heating process. Even though, DNA may undergo fragmentation or degradation due to heat and/or pressure methods (Bauer, Weller, Hammes, & Hertel, 2003; Gryson, 2010). Thus, a real-time PCR method for allergen detection should reach suitable sensitivity, not only in raw samples, but also in treated ones (Costa et al., 2013; Sanchiz et al., 2017; Villa, Costa, Gondar, Oliveira, & Mafra, 2018). Several works provided valuable results for detection of allergenic foods by real-time PCR using different chemistries (Brežná, Hudecová, & Kuchta, 2005; Brežná, Píknová, & Kuchta, 2009; Roder, Vieths, & Holzhauser, 2011). Although many of them also test the assay in commercial foodstuff, only a few analyse the effect of technological food treatment on DNA and the performance of the real-time PCR assay for specific allergen detection.

Recently, it has been reported that boiling decreased allergic response (Skin Prick Test), even becoming negative in autoclaved chestnut extracts (Gimenez-Licitra, De La Cruz Martinez, Barranco, Fernandez Crespo, & Dieguez, 2018). In this work, the effect of boiling (30 and 60 min) and autoclave (121 °C/1.18 atm and 138 °C/2.56 atm for 15 and 30 min) on the amplification of Cas s 9 target by real-time PCR was analysed. Binary mixtures were obtained mixing treated chestnut flour in a matrix of wheat (from 100000 to 10 mg of chestnut/kg) as explained with untreated samples. At least three DNA extractions for each treatment and spiked level were performed and analysed by duplicated (Table 2). A bar graph plotting Ct and log of quantity of chestnut (mg/kg) for each treatment is represented in Fig. S3.

Boiling for 30 min did not diminish significantly the ability to amplify Cas s 9 target compared to control (Figure S3). Efficiency of the amplification reaction in these boiled samples was within the range of 90–110%, and slope and linearity (R^2) of the curve was -3.43 and 0.994 respectively, being possible to detect up to 100 mg/kg of boiled chestnut at least in 80% of replicates and consequently, the LOD was increased to 1000 mg/kg (Table 2). A similar result was obtained for boiling during 60 min. In this case, Ct value was delayed up to 2 cycles compared to control and the differences in the mean Ct were significant (Table 2 and Fig. S3). Once temperature and pressure are combined in an autoclave, detection of the target was significantly reduced (Fig. S3). Even in the softest conditions (121 °C/1.18 atm for 15 min), the linearity of the calibration curve is not suitable ($R^2 = 0.933$). As temperature, pressure and time increases, Cas s 9 detection becomes difficult or not possible, and analytical parameters of the curves were not maintained (Table 2). In the 10% chestnut-containing mixtures (100000 mg/kg), Cas s 9 target was amplified in all replica in autoclaved chestnut at 121 °C 30 min, and in 65% of replica when more drastic conditions were applied (AU 138 °C for 15 min). Finally, 30 min of 138 °C/256 kPa treatment did not allow the detection of the chestnut target Cas s 9 in any replica.

Selection of the target and chemistry in a real-time PCR assay is critical for the development of a specific and sensitive detection method. Thus, thermal and pressure treatments applied to other nuts

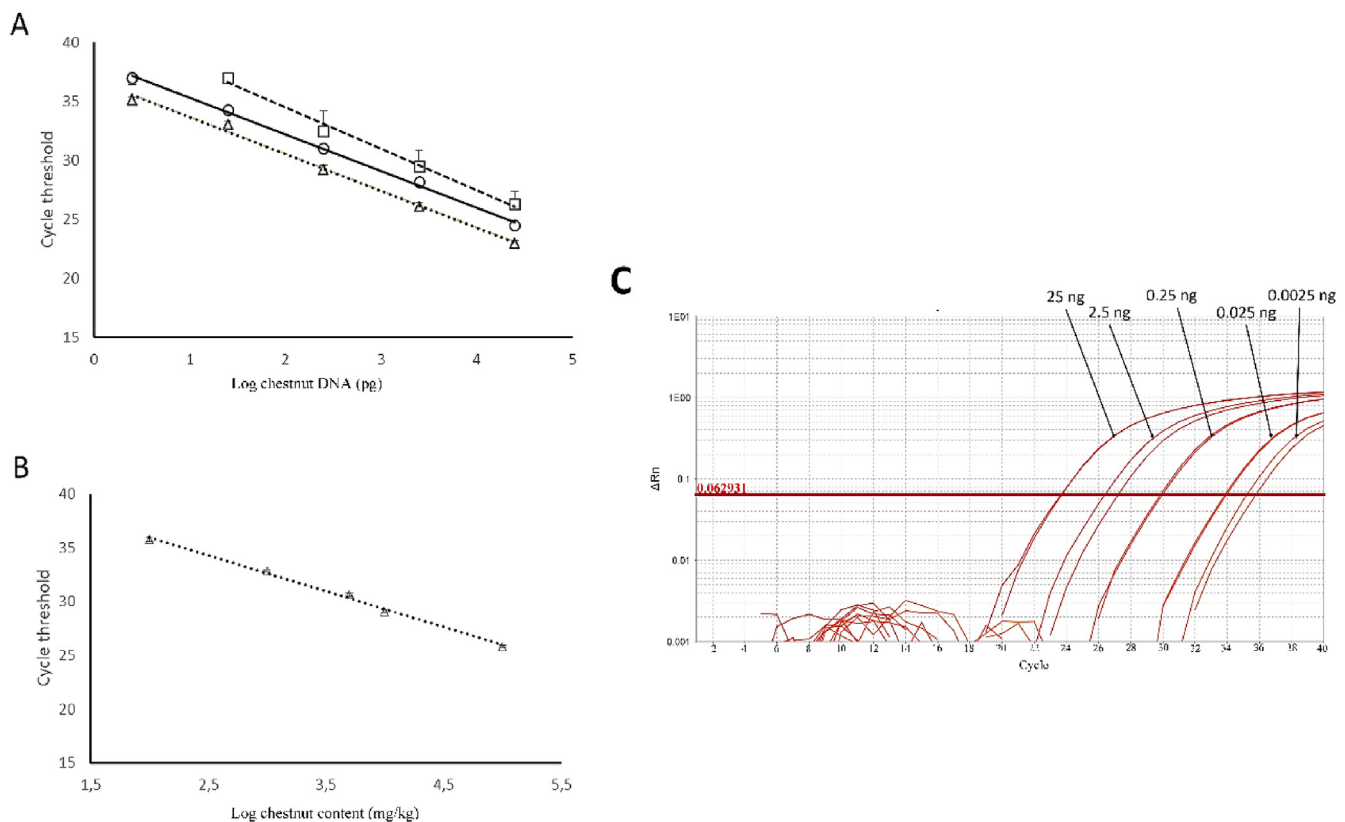


Fig. 2. Cas s 9 standard curves and amplification plot from the Real-time PCR assay. Efficiency and sensitivity of the Real-time PCR assay for chestnut detection was tested with A) Standard curves made with DNA serial dilutions (up to 2.5 pg) from three different chestnut varieties (□: Pere Andreu variety, curve with a slope of -3.52 , efficiency 92.2%, $R^2 = 0.9902$; ○: Primerenc variety, curve with a slope of -3.11 , efficiency 109.5%, $R^2 = 0.9970$; △: Miquelenc variety, curve with a slope of -3.12 , efficiency 108.95%, $R^2 = 0.9944$) and B) Standard curves obtained with binary mixtures containing known amount of untreated chestnut (mg/kg) in wheat flour (curve with a slope of -3.34 , efficiency 99.08% and $R^2 = 0.9950$). Mean Ct values and standard error are shown (at least $n = 8$ for DNA curves and $n = 24$ for binary mixtures for Miquelenc variety). C) Amplification plot from Miquelenc DNA standard curve.

may give different results regarding target detectability by real-time PCR. There are some examples of thermally treated nuts at the same conditions than described in this work. Detection of walnut was drastically reduced in autoclaved samples at 121°C 30 min, when binary mixtures were analysed by Sybr-Green based Real-time PCR, but HHP had no effect on detectability of the allergen coding sequence Jug r 3 target (Linacero et al., 2016). Similar finding was also found in HHP-treated almond, and better DNA extractability for processed samples compared to control was also observed (Prieto et al., 2014). In other study, detection of that target Ana o 1 from cashew was not affected by boiling for 30 and 60 min, and linearity was maintained up to 1000 mg/kg in calibration curves obtained from spiked samples of autoclaved samples at 138°C 15 min (Sanchiz et al., 2018). Some researchers have developed real-time PCR assays for the detection of nuts as walnut, pecan, Brazil nut or Macadamia after roasting at 160°C , resulting in the same performance than control. Real-time PCR is suitable for the detection of allergens, improving the limitations of protein based food analysis in terms of sensitivity, multiplexing capacity, cost and execution time, especially for processed foods (Salihah, Hossain, Lubis, & Ahmed, 2016). In addition, real-time PCR assay offers numerous advantages over ELISA as a sustainable alternative, with less manipulation, less waste, less space and time to get the results.

3.4. Applicability of the Cas s 9 detection system

The applicability of Cas s 9 as a target to detect chestnut by real-time PCR has been determined analysing several commercial food products, listed in Table 3. Three of them declared chestnut presence in their label (yogurt, jam and pasta), other three did not declared any nut

presence and the rest of them alerted from the presence of traces of nuts. Amplification with 18S primers was performed as DNA quality control; foods with probable harsh DNA fragmentation due to processing provided higher Ct (Table 3). According to our results, practical applicability of the real-time PCR system was possible and adventitious contamination with chestnut did not occur in food facilities. Detection of the allergen was only possible in foods containing chestnut, making evident the presence of this nut, as included in their label. Until the date, any commercial chestnut detection technique is available in the market.

4. Conclusions

In this work, we propose a real-time PCR-based method for the detection of the chestnut allergen in foodstuff using Cas s 9 allergen-coding gene as a target. Cas s 9 has been selected as the best candidate among three allergen coding genes because of highest specificity. The LOD was 25 pg of chestnut DNA and 100 mg of chestnut/kg of a binary mixture with spelta wheat. Additionally, this real-time PCR system has been assayed in boiled (30 and 60 min) and autoclaved samples at four different conditions of temperature/pressure and time ($121^\circ\text{C}/1.18\text{ atm}$ and $138^\circ\text{C}/2.56\text{ atm}$, 15 and 30 min). Detection of Cas s 9 target was slightly reduced by boiling whereas it was not possible when chestnut was subjected to pressured heat (autoclaving). The applicability of the method was demonstrated using commercial food products. According to our results, this real-time PCR methodology has the potential to be used in complex processed foods for the specific and sensitive detection of chestnut. To our knowledge, this is the first study developing an analytical system for chestnut detection in complex

Table 2
Detection of Cas s 9 target by probe-based real time PCR in chestnut DNA and untreated (control) and treated binary mixtures.

Binary Mixtures										
DNA curve										
Amount (ng)	Ct ^a	Boiled 30 min			Boiled 60 min			AU 121 °C 15 min		
		Untreated			Untreated			AU 121 °C 30 min		
		mg/kg	Ct		Ct			Ct		
25000	22.98 ± 0.25 ^(8/8)	100000	25.79 ± 0.11 ^(24/24)	26.78 ± 0.46 ^(10/10)	27.94 ± 0.46 ^(10/10)	31.13 ± 0.12 ^(8/8)	33.17 ± 0.20 ^(6/6)	37.11 ± 0.61 ^(4/6)		
2500	26.12 ± 0.25 ^(8/8)	10000	29.29 ± 0.11 ^(24/24)	30.90 ± 0.25 ^(10/10)	31.85 ± 0.19 ^(10/10)	32.75 ± 0.17 ^(8/8)	36.59 ± 0.42 ^(9/9)	35.69 ± 0.00 ^(3/6)		
250	29.22 ± 0.38 ^(8/8)	1000	32.86 ± 0.17 ^(24/24)	34.22 ± 0.31 ^(10/10)	34.61 ± 0.28 ^(10/10)	36.85 ± 0.90 ^(6/8)	37.34 ± 0.14 ^(2/6)	Undetermined		
25	33.03 ± 0.31 ^(8/8)	100	35.73 ± 0.18 ^(23/24)	37.13 ± 0.41 ^(8/10)	37.35 ± 0.28 ^(7/10)	36.58 ± 0.44 ^(3/8)	Undetermined	Undetermined		
2.5	35.14 ± 0.20 ^(5/8)	10	36.55 ± 0.28 ^(10/24)	36.99 ± 0.47 ^(3/10)	37.60 ± 0.47 ^(4/10)	Undetermined	Undetermined	Undetermined		
Slope	3.124	Slope	-3.33	-3.44	-3.10	-2.88	-	-		
R ^b	0.9945	R ^b	0.9950	0.9936	0.9917	0.9337	-	-		
Eff (%)	108.95	Eff (%)	99.77	95.43	110.17	122.32	-	-		

^a Ct ± SE.

^b Positive amplifications/Total replicates.

Table 3

Detection of chestnut by real time PCR for Cas s 9 allergen coding sequence in commercial food products.

Sample	Label declaration	Cas s 9 ^a	18S rRNA ^a
Cereal bar I	Almond	Negative	17.57 ± 0.46
Cereal bar II	Might contain nuts	Negative	22.35 ± 2.25
Cereal bar III*	Hazelnut	Negative	17.78 ± 0.38
Chocolate with hazelnut	Hazelnut	Negative	18.42 ± 1.67
Black chocolate	Might contain nuts	Negative	18.73 ± 0.03
Cookies cereal and chocolate	Might contain hazelnut	Negative	20.17 ± 1.05
Cookies with fiber	Nuts not declared	Negative	24.92 ± 0.63
Chocolate bar	Nuts not declared	Negative	20.71 ± 1.50
Cookies with chocolate	Nuts not declared	Negative	20.62 ± 0.25
Chocolate with pistachio	Pistachio, almond, hazelnut	Negative	12.06 ± 0.20
Vegetal burger	Might contain nuts	Negative	15.32 ± 0.26
Pesto sauce	5% cashew	Negative	14.73 ± 0.10
Yogurt	8% of chestnut	31.24 ± 0.41	20.43 ± 0.14
Jam	Chestnut ^b	25.36 ± 0.20	16.83 ± 0.00
Chestnut pasta	Contains chestnut ^b	25.00 ± 0.18	16.53 ± 0.37

N.D. = Not determined.

^a Average Cycle threshold (Ct) ± standard error.

^b Nut amount not reported or declared.

foodstuff including a validation of the effect of thermal and pressure processing on Cas s 9 sequence detectability.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.lwt.2020.109067>.

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SUPPLEMENTARY MATERIAL

Table S1. Specificity data for Cas s 9 and Cas s TLP real time PCR assays (10 ng of DNA).

Family	Common name	Scientific name	Cycle threshold (Ct) Cas s 9 ^a	Cycle threshold (Ct) Cas s TLP ^a
Fagaceae	Chestnut	<i>Castanea sativa</i>	25.69 ± 1.08	24.76 ± 0.36
Rosaceae	Almond	<i>Prunus dulcis</i>	Negative	Negative
Anacardiaceae	Cashew	<i>Anacardium occidentale</i>	Negative	Negative
Betulaceae	Hazelnut	<i>Corylus avellana</i>	Negative	34.46 ± 0.57
Juglandaceae	Walnut	<i>Juglans regia</i>	Negative	Negative
Juglandaceae	Pecan	<i>Carya illionensis</i>	Negative	N.D.
Fabaceae	Peanut	<i>Arachis hypogaeae</i>	Negative	33.02 ± 0.55
Anacardiaceae	Pistachio	<i>Pistacia vera</i>	Negative	33.23 ± 0.04
Rosaceae	Strawberry	<i>Fragaria ananassa</i>	Negative	N.D.
Fabaceae	Soy	<i>Glycine soja</i>	Negative	Negative
Actinidiaceae	Kiwi	<i>Actinia deliciosa</i>	Negative	N.D.
Solanaceae	Tomato	<i>Solanum lycopersicum</i>	Negative	N.D.
Rutaceae	Orange	<i>Citrus sinensis</i>	Negative	32.76 ± 0.90
Rutaceae	Lemon	<i>Citrus limon</i>	Negative	34.94 ± 0.56
Rosaceae	Peach	<i>Prunus persica</i>	Negative	N.D.
Anacardiaceae	Mango	<i>Mangifera indica</i>	Negative	32.85 ± 0.38
Moraceae	Fig	<i>Ficus carica</i>	Negative	N.D.
Rosaceae	Apple	<i>Malus domestica</i>	Negative	Negative
Rosaceae	Quirce	<i>Cydonia oblonga</i>	Negative	N.D.
Rosaceae	Pear	<i>Pyrus pyrifolia</i>	Negative	N.D.
Poaceae	Wheat	<i>Triticum spelta</i>	Negative	Negative

Negative means that DNA was not detected after 40 amplification cycles. N.D. Not determined. All samples were first tested with 18S primers as internal amplification control. ^aMean Ct and standard deviation is indicated.


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      *           20           *           40           *           60           *           80           *           100
Miquelencsa : TTCAC TTGCGAGACTGCTGATTGTAGCACC GGTCAGGTTGCATGCAACGGTAATGGTGCAATCCCACCAGCTTCTTTAGTAGAAATCAACATAGCAGCCA : 100
Cella Ampla : TTCAC TTGCGAGACTGCTGATTGTAGCACC GGTCAGGTTGCATGCAATGGTAATGGTGCAATCCCACCAGCTTCTTTAGTAGAAATCAACATAGCAGCCA : 100
Pere Andreu : TTCAC TTGCGAGACTGCTGATTGTAGCACC GGTCAGGTTGCATGCAACGGTAATGGTGCAATCCCACCAGCTTCTTTAGTAGAAATCAACATAGCAGCCA : 100
Primerenc : TTCAC TTGCGAGACTGCTGATTGTAGCACC GGTCAGGTTGCATGCAACGGTAATGGTGCAATCCCACCAGCTTCTTTAGTAGAAATCAACATAGCAGCCA : 100
Tardà : TTCAC TTGCGAGACTGCTGATTGTAGCACC GGTCAGGTTGCATGCAACGGTAATGGTGCAATCCCACCAGCTTCTTTAGTAGAAATCAACATAGCAGCCA : 100

      *           120           *           140           *           160           *           180           *           200
Miquelencsa : ATCGTGGGATGGACTTTTACGATGTTAGCCTTGTAGATGGCTTCAACTTGCCTGTTTCTGTAGCCACCAGAGGCGGCACCTGGTGATTGCAAGGCCACAAG : 200
Cella Ampla : ATCGCGGGATGGACTTTTACGATGTTAGCCTTGTAGATGGCTACAACCTTGCCTGTTTCTGTAGCCACCAGAGGCGGCACAGGTGATTGCAAGGCCACAAG : 200
Pere Andreu : ATCGTGGGATGGACTTTTACGATGTTAGCCTTGTAGATGGCTTCAACTTGCCTGTTTCTGTAGCCACCAGAGGCGGCACCTGGTGATTGCAAGGCCACAAG : 200
Primerenc : ATCGTGGGATGGACTTTTACGATGTTAGCCTTGTAGATGGCTTCAACTTGCCTGTTTCTGTAGCCACCAGAGGCGGCACCTGGTGATTGCAAGGCCACAAG : 200
Tardà : ATCGTGGGATGGACTTTTACGATGTTAGCCTTGTAGATGGCTTCAACTTGCCTGTTTCTGTAGCCACCAGAGGCGGCACCTGGTGATTGCAAGGCCACAAG : 200

      *           220           *           240           *           260           *           280           *           300
Miquelencsa : TTGTCCAGCCAACGTGAACSCGGTTTGCCCACGGAATTGCAAGTGAAGGGTCTGATGGGASCGTGCTTGCTTGCAAGAGCGCTTGACTGCTTTCAAT : 300
Cella Ampla : TTGTCCAGCCAACGTGAACSCGGTTTGCCCACGGAATTGCAAGTGAAGGGTCTGATGGGASCGTGCTTGCTTGCAAGAGCGCTTGACTGCTTTCAAT : 300
Pere Andreu : TTGTCCAGCCAACGTGAACSCGGTTTGCCCACGGAATTGCAAGTGAAGGGTCTGATGGGASCGTGCTTGCTTGCAAGAGCGCTTGACTGCTTTCAAT : 300
Primerenc : TTGTCCAGCCAACGTGAACSCGGTTTGCCCACGGAATTGCAAGTGAAGGGTCTGATGGGASCGTGCTTGCTTGCAAGAGCGCTTGACTGCTTTCAAT : 300
Tardà : TTGTCCAGCCAACGTGAACSCGGTTTGCCCACGGAATTGCAAGTGAAGGGTCTGATGGGASCGTGCTTGCTTGCAAGAGCGCTTGACTGCTTTCAAT : 300

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Figure S2. **Multiple alignment of chestnut varieties for Cas s TLP sequence.** Forward and reverse primers are squared in a continuous line and probe is squared in a discontinuous line.

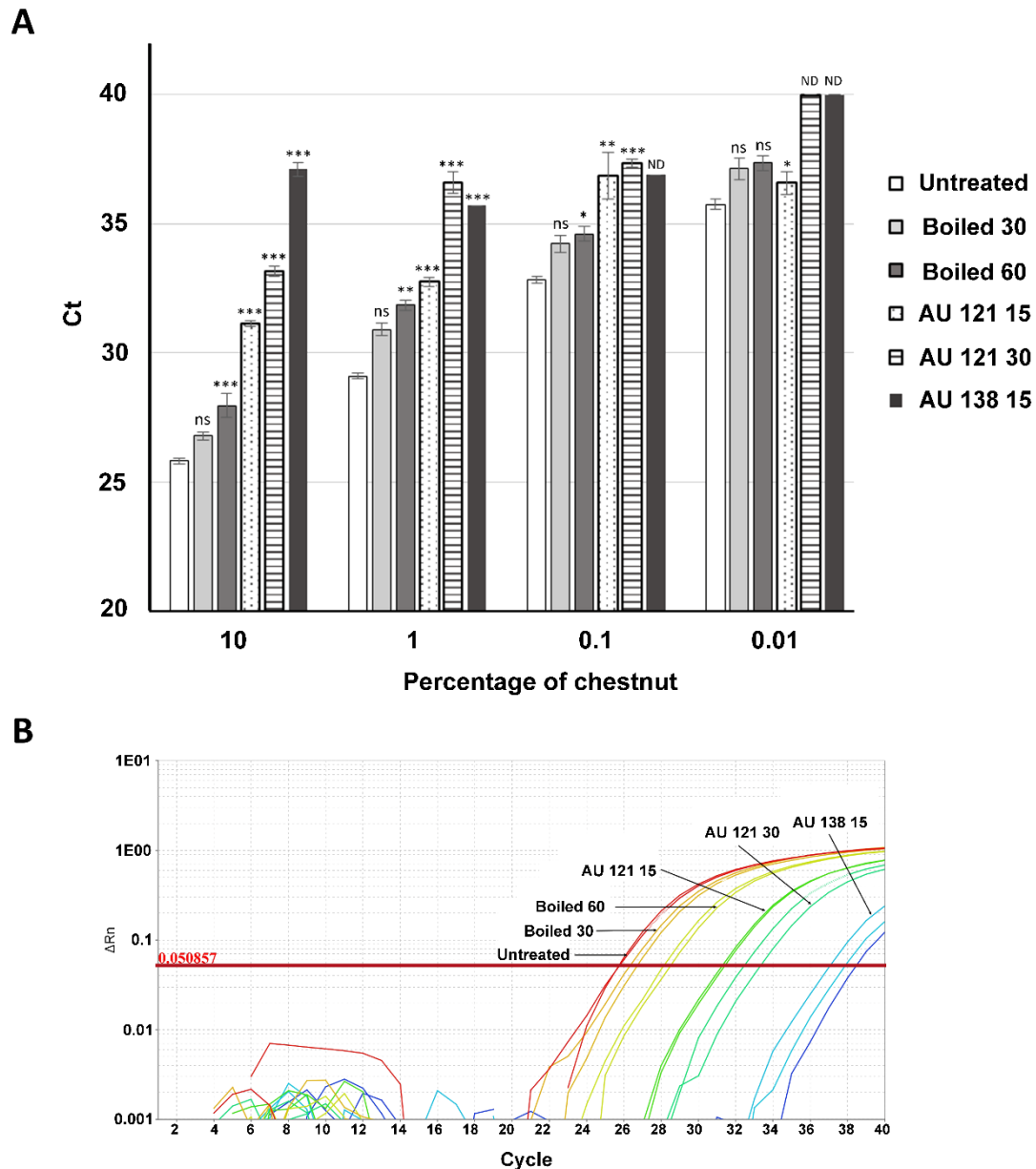


Figure S3. **Cas s 9 detection by Real-time PCR in processed binary mixtures.** A) Calibration curves obtained from different and known spiked levels of untreated or treated chestnut flour in binary mixtures with wheat (from 100 000 to 100 mg/kg). Cas s 9 detection was not possible in autoclaved samples at 138°C 30 min and is not represented. Mean Ct and standard error is showed (at least n=6 for treated samples). Significance of differences between Ct values ($p < 0.05$) of each spiked level (10 to 0.01% of chestnut in the binary mixture) in each thermal treatment compared to the untreated control. ns= not significant; *, **, *** = significant differences with $p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively; N.D. Ct not available B) Amplification plot of 100 000 mg/kg (10%) of untreated, boiled and autoclaved chestnut flour in the binary mixture with wheat.