



Effect of thermal processing on the performance of the novel single-tube nested real-time PCR for the detection of walnut allergens in sponge cakes



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ABSTRACT

Walnut is commonly included as an ingredient in bakery products, being widely appreciated by many consumers. However, walnut is also classified as an allergenic food, representing a potential risk for the health of sensitised individuals when present in processed foods as a hidden allergen. In the present work, we developed a single-tube nested real-time PCR system to trace walnut in bakery products and assessed the effect of heat treatment on the performance of the technique. The proposed approach, targeting the sequence encoding for the *Jug r 3* allergen, enabled lowering the relative limit of detection (LOD) of the conventional real-time PCR assay from 0.005% to 0.001% of walnut in both batter and sponge cakes. The absolute LOD was 1 pg of walnut DNA (1.6 DNA copies) in both mixtures, evidencing that the performance of the method was not affected by the heat processing. To our knowledge we proposed for the first time a single-tube nested real-time PCR system as a cost-effective, robust and powerful tool for high-throughput DNA-based detection and quantification of walnut allergens in raw and processed foods.

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1. Introduction

Food-induced allergy is, by definition, an abnormal health effect arising from a specific immunological response that occurs reproducibly on exposure to a given food (Boyce et al., 2010). In theory, any food is susceptible of triggering an allergic reaction in sensitised individuals, however about 90% of the total adverse immunological responses are specifically attributed to eight groups of foods (tree nuts, peanuts, soybean, cereals containing gluten, eggs, milk, crustaceans and fish).

Walnut (*Juglans regia*) is included in the tree nut group for which clinical symptoms have been commonly described as mild to potentially fatal adverse reactions (anaphylaxis). Among the tree nuts, walnut is frequently reported as having the highest incidence of reports inducing severe allergic reactions (Fleischer, Conover-Walker, Matsui, & Wood, 2005; Sicherer, Furlong, Muñoz-Furlong, Burks, & Sampson, 2001). In contrast to other foods such as eggs or milk, patients diagnosed with allergies to one or more tree nuts are predicted to suffer from “life persisting” allergy (Sampson, 2003).

The true prevalence of food allergies has been very difficult to establish since the majority of the information about their prevalence is based on self-reported reactions to foods (questionnaires/surveys), rather than using objective assessments as open and double-blind food challenge tests, or determined sensitisation to foods by serum immunoglobulin E (IgE) and skin prick tests (Zuidmeer et al., 2008). In Europe, food induced

allergies related to tree nuts are often common, with hazelnut being reported as a major contributor, whereas in USA, allergies caused by walnut, almond and cashew consumption seem to be more frequent (Ortolani et al., 2000; Sicherer, Muñoz-Furlong, & Sampson, 2003). The precise number of patients with diagnosis of allergy to walnut or other foods is still unknown, although on the basis of a recent study encompassing several western countries, sera from test subjects were scanned for 5 allergen mixes (including walnut) from a total of 24 foods previously defined as priorities. In the referred study, walnut allergy was estimated to an overall incidence of 2.2% among the tested allergic population (Burney, Summers, Chinn, Hooper, Van Ree, & Lidholm, 2010). So far, the only proven preventive treatment for these individuals consists of the elimination of the food allergen and all the cross-reacting allergens from diet. In this sense, when choosing a food product, the allergic patients have to rely on labelling information, especially in the case of processed foods. Once the total avoidance of the allergenic food is almost impossible to accomplish, sensitised individuals may still be at risk of suffering from allergic reactions due to the presence of hidden allergens in foods owing to incorrect labelling or cross-contaminations during food processing. Therefore, to verify labelling compliance (Directive, 2007/68/EC; Regulation (EU) No, 1169/2011), to help the industrial management of food allergens and to ensure consumer's safety, the development of proper and highly sensitive analytical methodologies is of utmost importance (Costa, Oliveira, & Mafra, 2013).

Most of the available analytical tools rely on protein- and/or DNA-based methods for food allergen assessment (Costa, Mafra, Carrapatoso, & Oliveira, 2012). Regarding the specific detection of walnut in raw and

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processed foods, some techniques have been developed targeting the allergenic/marker proteins or the DNA sequences encoding them. Among the protein-based methods, the immunochemical assays such as enzyme-linked immunosorbent assays (ELISA) (Doi et al., 2008; Niemann, Taylor, & Hefle, 2009; Sakai et al., 2010) and more recently, the mass spectrometry analysis (Bignardi, Elviri, Penna, Careri, & Mangia, 2010; Bignardi et al., 2013; Heick, Fischer, Kerbach, Tamm, & Popping, 2011) have been effectively applied for the detection and quantification of walnut in a wide range of food products. As very reliable alternatives, polymerase chain reaction (PCR) techniques have also been successfully employed to target walnut in processed foods (Brezná, Hudecová, & Kuchta, 2006; Köppel, Velsen-Zimmerli, & Bucher, 2012; Wang, Li, Zhao, Chen, & Ge, 2011; Wang et al., 2009). Until now, the few reported qualitative and quantitative PCR methods regarding walnut detection target the amplification of the gene encoding the allergenic protein Jug r 2 (Brezná et al., 2006; Köppel et al., 2012; Wang et al., 2009, 2011).

In this work, we proposed a new molecular method targeting a different gene encoding the allergenic protein Jug r 3 of walnut. Jug r 3 belongs to the lipid transfer proteins (LTP), being classified as a major allergen with severe clinical presentation (systemic reactions) among the walnut allergic individuals (Pastorello et al., 2004). The suggested novel approach is based on the single-tube nested real-time PCR system following its successful application to other tree nuts, namely almond (Costa, Oliveira & Mafra, 2013) and hazelnut (Costa, Mafra, Kuchta, & Oliveira, 2012). Considering that walnut is often incorporated in bakery products, it was also important to exploit the effect of thermal processing on walnut detection. In general, it is well established that thermal processing can lead to conformational changes of proteins, which can affect their IgE-binding epitopes (Sathe & Sharma, 2009). Regarding the effect of food processing on DNA molecules, few information is still available although recent literature seem to indicate a negative effect on DNA detection after severe heat treatment of food (Gryson, 2010; Iniesto et al., 2013; Platteau, De Loose, De Meulenaer, & Taverniers, 2011; Scaravelli, Brohée, Marchelli, & van Helgel, 2009). Using model batter and sponge cakes, this study intended to further evaluate the influence of thermal treatment on the performance parameters of the developed conventional and single-tube nested real-time PCR systems.

2. Materials and methods

2.1. Plant foods

Walnut cultivars used in this study were acquired at Portuguese markets, including different geographical origins (USA, France, Chile, Portugal and Spain). A total of 10 different cultivars of walnut, namely “Payne”, “Vina”, “Hartley”, “Lara”, “Chandler”, “Corne”, “Franquette”, “Serr”, “Femor” and “Marbot” were tested.

Other plant foods, totalising 31 different species, namely peanuts and tree nuts (almond, pine nut, macadamia nut, hazelnut, Brazil nut, chestnut, cashew, pistachio and pecan nut), and the plant species (wheat, soybean, lupine, fava bean, maize, oat, rye, barley, rice, pumpkin seeds, rapeseed, sunflower, tomato, peach, nectarine, apricot, plum, cherry, strawberry, blackberry and raspberry) were purchased at local markets.

2.2. Preparation of model mixtures of batter and sponge cakes spiked with walnut

Model mixtures of batter and sponge cakes were prepared containing 50%, 10%, 5%, 1%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005%, 0.001%, 0.0005% and 0.0001% of walnut. A basic sponge cake recipe was used containing 3 eggs, 100 g of sugar and 100 g of wheat flour with baking powder. The first spiked batter, containing 50% of walnut, was prepared by adding 200 g of grounded walnut to 200 g of batter. All the following spiking

levels of walnut were obtained by serial dilution with batter, i.e., by successive additions of batter until the proportion of 0.0001% of walnut.

All procedures were performed similarly to the preparation of cakes in bakeries, being each batter blended using a cake mixer (Tefal Kitchen Machine, model QA401, SEB group, Berkshire, UK) to ensure the correct homogeneity of the model mixtures. According to the basic recipe for sponge cake preparation, eggs and sugar were first mixed for 5 min to allow formation of a creamy mixture. The wheat flour containing baking powder was then added to the mixture in small portions with continuous mixing for 5 min. After adding the grounded walnut, batter was mixed for more 3 min. From each spiked batter, a sample was collected and immediately stored at -20°C to minimise enzymatic activity until analysis. The remaining batter (approximately 350 g) was then baked at 200°C for 20 min. After cooling, a sample of each sponge cake was retrieved. Portions of the model cakes (~ 100 g) and other plant foods were ground and homogenised separately, into a fine powder of approximately 0.3 mm in diameter in a laboratory knife mill Grindomix GM200 (Retsch, Haan, Germany) using different containers and material, previously treated with a DNA decontamination solution. The fruits, namely, tomato, peach, apricot, plum and cherry, were lyophilised before grinding. To avoid accidental cross-contamination among samples, plant foods and standards, all materials were grounded in different days. After grinding, the samples were immediately stored at -20°C until DNA extraction.

2.3. DNA extraction

DNA was extracted from 200 mg of sample using the commercial Nucleospin Food kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions with minor alterations as described by Costa, Mafra, Kuchta and Oliveira (2012).

Yield and purity of extracts were assessed by agarose gel electrophoresis and by UV spectrophotometric DNA quantification on a Synergy HT multi-mode microplate reader (BioTek Instruments, Inc., Vermont, USA), using a Take 3 micro-volume plate accessory. DNA content was determined using the nucleic acid quantification protocol with sample type defined for double-strand DNA in the Gen5 data analysis software version 2.01 (BioTek Instruments, Inc., Vermont, USA).

2.4. Target gene selection and oligonucleotide primers

The DNA sequence corresponding to *Juglans regia*, nonspecific LTP mRNA, encoding for the Jug r 3 allergen was retrieved from the NCBI database (accession no. EU780670.1). For the development of single-tube nested real-time PCR system, two sets of primers with different annealing temperatures (T_a) were designed using the software Primer-BLAST designing tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) (Table 1). The defined parameters for the design of primers were set considering a difference higher than 10°C in the

Table 1

Key data of primers and probe designed to target *Juglans regia*, nonspecific LTP protein mRNA, encoding for Jug r 3 allergen (NCBI accession no. EU780670.1).

Oligonucleotides	Sequence (5'–3')	T_a (°C)	Amplicon (bp)
Outer primers			
Jug3F	TAC GGT TCC TAC AGT CCC TCC AA	62.4	136
Jug3R	AGG GTT GAG TCC GGG GAT GGA A	64.0	
Inner primers			
Jug3FN	CTC CAA GCT GCT GCA AT	52.8	99
Jug3RN	AAC CAG AAG TCT TTT TCA G	50.2	
Sequencing primers			
Jug3FS	TCA GGC ATG GTG CTG CTG TGT ATG	64.4	319
Jug3RS	TGC AGT TAG TGG AGG TGC TGA TCT	62.7	
Probe			
Jug3P	FAM-TCA ACA AAG CCG CCG CTA CCA CAG CT-BHQ1	68.0	

annealing temperatures between the two sets of primers (Costa, Mafra, Kuchta & Oliveira, 2012; Costa, Oliveira & Mafra, 2013). Thus, the first pair of primers (Jug3F/Jug3R) was designed with an optimal T_a of 66 °C, while the second (Jug3FN/Jug3RN) has a T_a of 54 °C. For the application of real-time PCR systems a hydrolysis probe (Jug3P) was also designed (Table 1). To confirm the *in silico* specificity of the designed primers, the basic local alignment search tool BLAST software (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to identify regions of local similarity between the selected nucleotide sequence and homologue sequences of different species.

To evaluate the quality of the extracted DNA, all extracts were amplified by end-point PCR using universal eukaryotic primers (18SRG-F – CTGCCCTATCAACTTTTCGATGGTA and 18SRG-R – TTGGATGTGGTAGCCG TTTCTCA), targeting a conserved region of 18S ribosomal RNA gene (NCBI accession no. HQ873432.1).

Primers and probe were synthesised by Eurofins MWG Operon (Ebersberg, Germany).

2.5. Sequencing

For sequencing purposes, a third set of primers (Jug3FS/Jug3RS) was specifically designed to produce a larger PCR fragment (319 bp), encompassing the target region of 136 bp amplified by the primers Jug3F/Jug3R, using the end-point PCR conditions described below. Therefore, the amplified fragments of ten different walnut cultivars were sequenced in a specialised research facility (STABVIDA, Lisbon, Portugal). Before sequencing, all PCR products were purified with Jetquick PCR purification kit (Genomed, Löhne, Germany) to remove any possible interfering components. Each target fragment was sequenced twice, performing the direct sequencing of both strands in opposite directions, which allowed the production of two complementary sequences with very good quality.

2.6. End-point PCR

For sequencing, PCR products were obtained using a 25 µL of total reaction volume containing 2 µL of walnut DNA extract (100 ng), 670 mM of Tris–HCl (pH 8.8), 160 mM of $(\text{NH}_4)_2\text{SO}_4$, 0.1% of Tween 20, 200 µM of each dNTP, 1.0 U of SuperHot Taq DNA Polymerase (Genaxxon Bioscience, Ulm, Germany), 3.0 mM of MgCl_2 and 240 nM of each primer Jug3FS/Jug3RS (Table 1). The assays were carried out in a MJ Mini thermal cycler (BioRad, Hercules, CA, USA), according to the following programme: initial denaturation at 95 °C for 5 min; 40 cycles at 95 °C for 30 s, 65 °C for 45 s and 72 °C for 1 min; with a final extension at 72 °C for 5 min.

Universal PCR products were obtained using the same reaction components as above, but adding 240 nM of 18SRG-F/18SRG-R. The assays were performed according to the following programme of temperatures: initial denaturation at 95 °C for 5 min; 33 cycles at 95 °C for 30 s, 65 °C for 30 s and 72 °C for 30 s; with a final extension at 72 °C for 5 min.

2.7. Conventional and single-tube nested real-time PCR systems

The conventional real-time PCR assays were performed in 20 µL of total reaction volume, containing 2 µL of DNA (100 ng), 1× of SsoFast Probes Supermix (BioRad, Hercules, CA, USA), 300 nM of each outer primer Jug3F/Jug3R and 200 nM of hydrolysis probe Jug3P (Table 1). For single-tube nested real-time PCR amplification, the mix was similar with the addition 300 nM of inner primers Jug3FN/Jug3RN that were specifically designed for this assay (Table 1). All real-time PCR assays were carried out on a fluorometric thermal cycler CFX96 Real-time PCR Detection System (BioRad, Hercules, CA, USA). The conventional real-time PCR amplifications were performed according to the following temperature programme: 95 °C for 5 min, 50 cycles at 95 °C for 15 s and 66 °C for 45 s, with the collection of fluorescence signal at the end of each cycle. The assays of the single-tube nested real-time PCR were

done in two different phases: (1) 95 °C for 5 min, 10 cycles at 95 °C for 15 s and 66 °C for 45 s, without collecting the fluorescence signal; (2) 40 cycles at 95 °C for 15 s, 54 °C for 20 s and 72 °C for 30 s, with the collection of fluorescence signal at the end of each cycle. Data were processed and analysed using the software BioRad CFX Manager 3.0 (BioRad, Hercules, CA, USA). Cycle threshold (Ct) values were calculated using the software at automatic threshold setting. Conventional real-time PCR and nested real-time PCR trials were repeated in two independent assay using eight replicates in each one.

2.8. Statistical analysis

The independent samples' *t*-test with the statistical programme IBM SPSS STATISTICS (21.0 package, IBM Corporation, New York, USA) was performed to evaluate the significance of differences between the Ct values of batter and sponge cakes at the same spiking level. All data were previously assessed for normality by Shapiro–Wilk test. Significant differences were considered when $p < 0.05$.

3. Results

In this study, for the specific detection of walnut in bakery products, the *Juglans regia* nonspecific LTP mRNA, encoding for the Jug r 3 allergen was retrieved from the NCBI database. Based on the recent and successful application of the single-tube nested real-time PCR system to hazelnut (Costa, Mafra, Kuchta & Oliveira, 2012) and almond (Costa, Oliveira & Mafra, 2013), this novel approach was also employed for the detection and quantification of trace amounts of DNA walnut allergen (Jug r 3) in bakery products. Owing to the fact that bakery products are consumed after being submitted to extensive food processing such as thermal treatments, it was considered essential to evaluate its effect on the detection of walnut DNA using the new proposed method.

3.1. Sequencing of PCR products

The expected products of real-time PCR systems were designed to have a length of 136 bp, which is considered a rather small fragment for direct sequencing. To overcome this problem, an easy, reliable and cost-effective strategy consisted on sequencing a larger fragment of 319 bp to allow an accurate sequencing of the contained target region of 136 bp.

The sequencing results of the ten walnut cultivars presented high resolution electropherograms, therefore confirming the adequacy of the chosen strategy. The method allowed sequencing the fragments in all the extension of the target region (Fig. 1), with no differences encountered for almost all the tested walnut varieties, with the exception of Marbot, Corne and Serr cultivars. In these three cases, a single nucleotide difference regarding the presence of a C nucleotide instead of the expected T in position 245 can be observed (Fig. 1). However, the specificity of the chosen target region was not affected by this small difference among cultivars, since all cultivars exhibited the same performance by PCR techniques. The sequenced fragments were also aligned with the *Juglans regia*, nonspecific LTP mRNA sequence from GenBank, exhibiting ~100% homology.

3.2. Specificity

To guarantee the absence of any possible false-negative, the DNA extracts from all plant foods and model mixtures were previously evaluated for their amplifiability using the primers 18SRG-F/18SRG-R targeting a universal eukaryotic fragment. All tested samples and model mixtures amplified positively for the expected product of 113 bp, confirming that the DNA extracts presented the adequate quality and purity for PCR amplification.

In addition to *in silico* analysis, the specificity of the proposed primers for walnut identification was determined experimentally.

	
	61 71 81 91 121 121 131
EU780670.1	TTGCAGAGGC GGTCAATAACA TGTGGGCAGG TGGCTAGCAG CGTGGGGAGT TGCATTGGCT ACCTCAGGGG
Hartley	TTGCAGAGGC GGTCAATAACA TGTGGGCAGG TGGCTAGCAG CGTGGGGAGT TGCATTGGCT ACCTCAGGGG
Chandler	TTGCAGAGGC GGTCAATAACA TGTGGGCAGG TGGCTAGCAG CGTGGGGAGT TGCATTGGCT ACCTCAGGGG
Lara	TTGCAGAGGC GGTCAATAACA TGTGGGCAGG TGGCTAGCAG CGTGGGGAGT TGCATTGGCT ACCTCAGGGG
Franquette	TTGCAGAGGC GGTCAATAACA TGTGGGCAGG TGGCTAGCAG CGTGGGGAGT TGCATTGGCT ACCTCAGGGG
Fernor	TTGCAGAGGC GGTCAATAACA TGTGGGCAGG TGGCTAGCAG CGTGGGGAGT TGCATTGGCT ACCTCAGGGG
Marbot	TTGCAGAGGC GGTCAATAACA TGTGGGCAGG TGGCTAGCAG CGTGGGGAGT TGCATTGGCT ACCTCAGGGG
Vina	TTGCAGAGGC GGTCAATAACA TGTGGGCAGG TGGCTAGCAG CGTGGGGAGT TGCATTGGCT ACCTCAGGGG
Corne	TTGCAGAGGC GGTCAATAACA TGTGGGCAGG TGGCTAGCAG CGTGGGGAGT TGCATTGGCT ACCTCAGGGG
Payne	TTGCAGAGGC GGTCAATAACA TGTGGGCAGG TGGCTAGCAG CGTGGGGAGT TGCATTGGCT ACCTCAGGGG
Serr	TTGCAGAGGC GGTCAATAACA TGTGGGCAGG TGGCTAGCAG CGTGGGGAGT TGCATTGGCT ACCTCAGGGG
	
	141 151 161 171 181 191 201
EU780670.1	TACGGTTCCT ACAGTCCCTC CAAGCTGCTG CAATGGGGTC AAGAGCCTCA ACAAAGCGGC CGCTACCACA
Hartley	TACGGTTCCT ACAGTCCCTC CAAGCTGCTG CAATGGGGTC AAGAGCCTCA ACAAAGCGGC CGCTACCACA
Chandler	TACGGTTCCT ACAGTCCCTC CAAGCTGCTG CAATGGGGTC AAGAGCCTCA ACAAAGCGGC CGCTACCACA
Lara	TACGGTTCCT ACAGTCCCTC CAAGCTGCTG CAATGGGGTC AAGAGCCTCA ACAAAGCGGC CGCTACCACA
Franquette	TACGGTTCCT ACAGTCCCTC CAAGCTGCTG CAATGGGGTC AAGAGCCTCA ACAAAGCGGC CGCTACCACA
Fernor	TACGGTTCCT ACAGTCCCTC CAAGCTGCTG CAATGGGGTC AAGAGCCTCA ACAAAGCGGC CGCTACCACA
Marbot	TACGGTTCCT ACAGTCCCTC CAAGCTGCTG CAATGGGGTC AAGAGCCTCA ACAAAGCGGC CGCTACCACA
Vina	TACGGTTCCT ACAGTCCCTC CAAGCTGCTG CAATGGGGTC AAGAGCCTCA ACAAAGCGGC CGCTACCACA
Corne	TACGGTTCCT ACAGTCCCTC CAAGCTGCTG CAATGGGGTC AAGAGCCTCA ACAAAGCGGC CGCTACCACA
Payne	TACGGTTCCT ACAGTCCCTC CAAGCTGCTG CAATGGGGTC AAGAGCCTCA ACAAAGCGGC CGCTACCACA
Serr	TACGGTTCCT ACAGTCCCTC CAAGCTGCTG CAATGGGGTC AAGAGCCTCA ACAAAGCGGC CGCTACCACA
	
	211 221 231 241 251 261 271
EU780670.1	GCTGACCGCC AGGCCGCCTG TGAGTGCCTG AAAAAGACTT CTGGTTCCAT CCCCGGACTC AACCTTGGTC
Hartley	GCTGACCGCC AGGCCGCCTG TGAGTGCCTG AAAAAGACTT CTGGTTCCAT CCCCGGACTC AACCTTGGTC
Chandler	GCTGACCGCC AGGCCGCCTG TGAGTGCCTG AAAAAGACTT CTGGTTCCAT CCCCGGACTC AACCTTGGTC
Lara	GCTGACCGCC AGGCCGCCTG TGAGTGCCTG AAAAAGACTT CTGGTTCCAT CCCCGGACTC AACCTTGGTC
Franquette	GCTGACCGCC AGGCCGCCTG TGAGTGCCTG AAAAAGACTT CTGGTTCCAT CCCCGGACTC AACCTTGGTC
Fernor	GCTGACCGCC AGGCCGCCTG TGAGTGCCTG AAAAAGACTT CTGGTTCCAT CCCCGGACTC AACCTTGGTC
Marbot	GCTGACCGCC AGGCCGCCTG TGAGTGCCTG AAAAAGACTT CTGGTTCCAT CCCCGGACTC AACCTTGGTC
Vina	GCTGACCGCC AGGCCGCCTG TGAGTGCCTG AAAAAGACTT CTGGTTCCAT CCCCGGACTC AACCTTGGTC
Corne	GCTGACCGCC AGGCCGCCTG TGAGTGCCTG AAAAAGACTT CTGGTTCCAT CCCCGGACTC AACCTTGGTC
Payne	GCTGACCGCC AGGCCGCCTG TGAGTGCCTG AAAAAGACTT CTGGTTCCAT CCCCGGACTC AACCTTGGTC
Serr	GCTGACCGCC AGGCCGCCTG TGAGTGCCTG AAAAAGACTT CTGGTTCCAT CCCCGGACTC AACCTTGGTC
	
	281 291 301 311 321 331
EU780670.1	TTGCTGCTGG CCTCCCAGGC AAATGTGGTG TCAGTGTTC TTACAAGATC AGCACCTC
Hartley	TTGCTGCTGG CCTCCCAGGC AAATGTGGTG TCAGTGTTC TTACAAGATC AGCACCTC
Chandler	TTGCTGCTGG CCTCCCAGGC AAATGTGGTG TCAGTGTTC TTACAAGATC AGCACCTC
Lara	TTGCTGCTGG CCTCCCAGGC AAATGTGGTG TCAGTGTTC TTACAAGATC AGCACCTC
Franquette	TTGCTGCTGG CCTCCCAGGC AAATGTGGTG TCAGTGTTC TTACAAGATC AGCACCTC
Fernor	TTGCTGCTGG CCTCCCAGGC AAATGTGGTG TCAGTGTTC TTACAAGATC AGCACCTC
Marbot	TTGCTGCTGG CCTCCCAGGC AAATGTGGTG TCAGTGTTC TTACAAGATC AGCACCTC
Vina	TTGCTGCTGG CCTCCCAGGC AAATGTGGTG TCAGTGTTC TTACAAGATC AGCACCTC
Corne	TTGCTGCTGG CCTCCCAGGC AAATGTGGTG TCAGTGTTC TTACAAGATC AGCACCTC
Payne	TTGCTGCTGG CCTCCCAGGC AAATGTGGTG TCAGTGTTC TTACAAGATC AGCACCTC
Serr	TTGCTGCTGG CCTCCCAGGC AAATGTGGTG TCAGTGTTC TTACAAGATC AGCACCTC

Fig. 1. Alignment of PCR products of 10 walnut cultivars obtained by sequencing. The shadowed region of 136 bp corresponds to the PCR fragments using “outer” primers Jug3F/Jug3R. In position 245, a difference in nucleotide C instead of a T is highlighted for three cultivars (Marbot, Corne and Serr).

The specificity was tested using different plant species to evaluate any possible cross-reactivity. From the 31 species investigated, there was no observable cross-reactivity with the designed primers, evidencing the adequate specificity of the assay for the correct identification of walnut.

3.3. Development of analytical method

The real-time PCR systems (conventional and single-tube nested real-time PCR) were optimised using model mixtures of batter and sponge cakes spiked with known amounts of walnut. For the comparison

and the evaluation of the proposed real-time PCR systems, the prerequisites for method performance established in the available documents of MIQE guidelines (Bustin et al., 2009) and of the definition of minimum performance requirements for analytical methods of genetically modified organisms testing (Mazzara et al., 2008) were carefully considered.

3.3.1. Real-time PCR system

The real-time PCR assays were performed using model mixtures of batter and sponge cakes spiked with walnut ranging from 50% to 0.0001%. The amplification results show the detection of walnut in batter and sponge cakes down to 0.001% (10 mg/kg), but only in 4 and 6 out of 16 replicates, respectively (Table 2). Considering that the limit of detection (LOD) is the lowest concentration level with positive identification of the analyte at least in 95% of the times (Bustin et al., 2009; Mazzara et al., 2008), in the present work the LOD was defined when amplification was positive for the total number of replicates. Therefore, the relative LOD determined for both batter and sponge cakes corresponded to 0.005% (50 mg/kg) of walnut since it was the lowest level with positive amplification of all replicates (Table 2, Fig. 2A, B). The relative limit of quantification (LOQ) achieved with the conventional real-time PCR system was equal to the LOD (50 mg/kg of walnut in batter and sponge cakes) since it was within the linear range of the calibration curve.

To evaluate the performance of the real-time PCR method, several parameters have to comply with the acceptance criteria established for this type of assay. Accordingly, the correlation coefficient (R^2) should be above 0.98, the PCR efficiency should range between 90%–110% and the slope should be between -3.6 and -3.1 (Bustin et al., 2009; Mazzara et al., 2008). In the performed real-time PCR runs, the values for R^2 , PCR efficiency and slope were in good agreement with the acceptance criteria. In the case of model mixtures of walnut in batter, mean values for R^2 , PCR efficiency and slope were 0.9939, 109.8% and -3.1072 , respectively (Table 2, Fig. 2A). For the real-time PCR assays carried out with model walnut cakes, mean values for R^2 , PCR efficiency and slope were 0.9964, 106.6% and -3.1724 , respectively (Table 2, Fig. 2B). The Ct mean values at the LOD (50 mg/kg) obtained by both model mixtures of walnut in batter and sponge cakes were 35.95 and 35.38, respectively, presenting a difference of approximately 0.6 cycles that could also be observed for other spiked levels (Table 2). In spite of the variances in Ct values, statistically significant differences ($p < 0.05$) were only found for spiked levels of 5% and 10% of walnut between batter and cake.

The dynamic range and the absolute sensitivity of the real-time PCR assay were established using the extracts of model mixtures of batter and sponge cake spiked with 50% of walnut, which were 10-fold serially diluted to cover 6 orders of magnitude of the analyte (100 ng to 1 pg of walnut). The obtained data show that walnut DNA was amplified until a dilution factor of 10,000, with an absolute LOD of 10 pg for both calibration curves (batter and sponge cakes), corresponding to approximately 16 genomic DNA copies (Table 3, Fig. 3A, B). The copy number was determined according to the available walnut genome size (0.62 pg) retrieved from the Plant DNA C-values database (RBG, Kew), assuming that the targeted sequences are single copy genes. In the case of cakes, the real number of walnut genomic copies detected with this system could be slightly lower considering that some loss of genomic material could have occurred during thermal processing. The Ct mean values at the absolute LOD (10 pg) obtained by real-time PCR runs with the model mixtures of walnut in batter and sponge cake were 36.89 and 35.80, respectively, presenting a difference of approximately 1 cycle that could also be observed for other spiked levels (Table 3). However, variations in Ct values reflected statistically significant differences ($p < 0.05$) only for the absolute walnut DNA of 1000 pg between batter and cake.

All the real-time PCR assays of serially diluted extracts exhibited high performance, with mean values of 96.6% and 97.8% for PCR efficiency, slope of -3.4067 and -3.3766 and corresponding correlation coefficients of 0.9995 and 0.9993, for the calibration curves obtained from the amplification of walnut in batter (Table 3, Fig. 3A) or in sponge cakes (Table 3, Fig. 3B), respectively. In general and for both cases of relative and absolute quantification, DNA extracts from model mixtures of walnut in cakes started to amplify earlier than the corresponded level of walnut in batter.

3.3.2. Single-tube nested real-time PCR system

The development of the single-tube nested real-time PCR system for the detection of Jug r 3 gene was based on the use of DNA extracts of model mixtures of batter and sponge cakes spiked with 50% down to 0.0001% of walnut, similarly to the conventional system. The application of this new approach allowed decreasing the LOD from 0.005% (50 mg/kg) to 0.001% (10 mg/kg) for walnut in batter and sponge cakes, respectively, which were $5 \times$ lower than the same values determined with the conventional real-time PCR system (Table 2, Fig. 2A,B). Additionally, with the nested approach it was

Table 2
Real-time PCR and single-tube nested real-time PCR results for the relative detection of spiked walnut in batter and sponge cakes.

Spiked level (%)	Conventional real-time PCR		Single-tube nested real-time PCR	
	Batter	Sponge cakes	Batter	Sponge cakes
	Ct $\pm$ SD ^a	Ct $\pm$ SD	Ct $\pm$ SD	Ct $\pm$ SD
0	nd ^b	nd	nd	nd
0.0001	nd	nd	nd	nd
0.0005	nd	nd	27.86 $\pm$ 0.51 (8/16)	28.26 $\pm$ 1.04 (8/16)
0.001	37.40 $\pm$ 0.29 (4/16) ^c	36.71 $\pm$ 0.87 (6/16) ^c	27.70 $\pm$ 0.86 (16/16)	27.85 $\pm$ 0.99 (16/16)
0.005	35.95 $\pm$ 0.79 (16/16)	35.38 $\pm$ 0.86 (16/16)	26.31 $\pm$ 0.85 (16/16)	25.28 $\pm$ 0.94 (16/16)
0.01	34.75 $\pm$ 0.54 (16/16)	34.85 $\pm$ 0.47 (16/16)	24.90 $\pm$ 0.88 (16/16)	24.50 $\pm$ 0.65 (16/16)
0.05	32.99 $\pm$ 0.36 (16/16)	32.60 $\pm$ 0.38 (16/16)	22.86 $\pm$ 0.46 (16/16)	22.29 $\pm$ 0.31 (16/16)
0.1	31.63 $\pm$ 0.30 (16/16)	31.25 $\pm$ 0.29 (16/16)	21.61 $\pm$ 0.29 (16/16)	20.93 $\pm$ 0.47 (16/16)
0.5	29.21 $\pm$ 0.32 (16/16)	29.18 $\pm$ 0.23 (16/16)	19.37 $\pm$ 0.22 (16/16)	18.86 $\pm$ 0.27 (16/16)
1.0	28.41 $\pm$ 0.15 (16/16)	28.46 $\pm$ 0.23 (16/16)	18.40 $\pm$ 0.21 (16/16)	18.72 $\pm$ 0.31 (16/16)
5.0	26.17 $\pm$ 0.12 (16/16) ^d	26.01 $\pm$ 0.22 (16/16)	16.28 $\pm$ 0.13 (16/16) ^d	16.03 $\pm$ 0.33 (16/16)
10	25.18 $\pm$ 0.15 (16/16) ^d	24.61 $\pm$ 0.32 (16/16)	15.33 $\pm$ 0.23 (16/16)	14.71 $\pm$ 0.30 (16/16)
50	23.97 $\pm$ 0.24 (16/16)	23.21 $\pm$ 0.16 (16/16)	13.80 $\pm$ 0.25 (16/16) ^d	13.13 $\pm$ 0.16 (16/16)
Correlation coefficient (R^2)	0.9939	0.9964	0.9962	0.9962
Slope	-3.1072	-3.1724	-3.1236	-3.1351
PCR efficiency (%)	109.8	106.6	109.0	108.4

^a Mean cycle threshold (Ct) values $\pm$ standard deviation (SD).

^b nd, not detected.

^c (Positive amplifications/total replicates).

^d Significant differences at $p < 0.05$ (t -test) between Ct values of batter and sponge cake at the same spiking level.

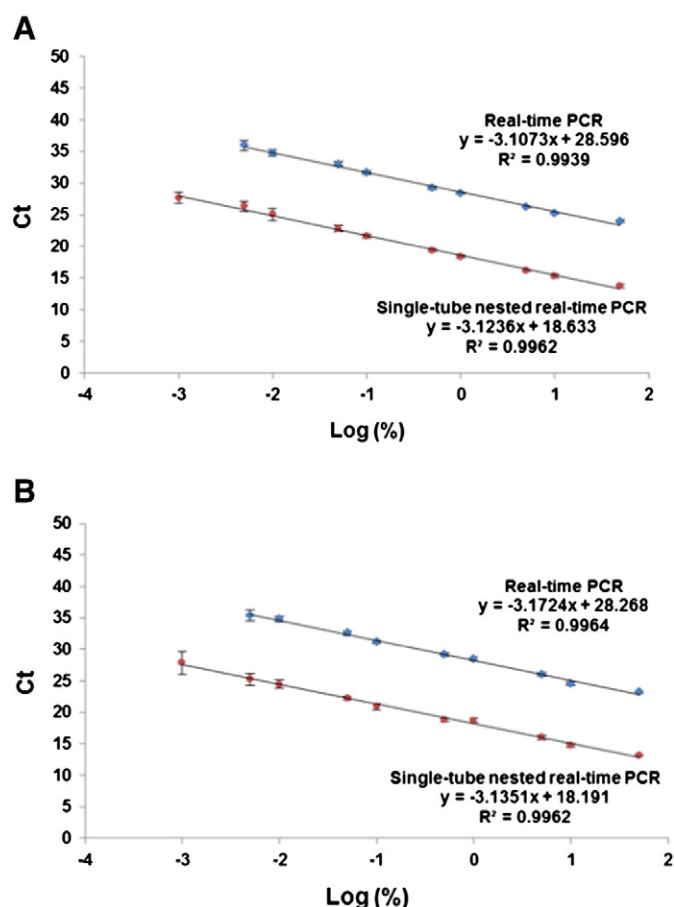


Fig. 2. Comparison of calibration curves obtained with conventional real-time PCR and single-tube nested real-time PCR of model mixtures containing 50% to 0.001% of walnut in batter (A) and sponge cakes (B). Mean values and corresponding standard deviations of $n = 16$ replicates.

also possible to amplify walnut DNA down to 0.0005% (5 mg/kg), but only for half the replicates, not being considered for this reason as the LOD. Within the linear range of the calibration curve, the LOD was also considered the LOQ for this system. All the single-tube nested real-time PCR runs were in good compliance with the acceptance criteria for real-time PCR parameters, presenting mean values for R^2 of 0.9962 and 0.9962, slopes of -3.1236 and -3.1351 and PCR

efficiencies of 109.0% and 108.4%, using the model mixtures of batter (Table 2, Fig. 2A) and sponge cakes (Table 2, Fig. 2B), respectively.

The same dynamic range was used to evaluate the novel system aiming at establishing the absolute LOD. Thus, model mixtures of batter and sponge cake spiked with 50% of walnut were 10-fold serially diluted ranging from 100 ng to 1 pg of walnut. Single-tube nested real-time PCR assays presented positive amplification for all the replicates used over the entire dynamic range, covering six orders of magnitude and allowing the absolute detection of walnut until a dilution factor of 100,000. The absolute LOD was $10 \times$ lower than the conventional system, enabling amplification of walnut DNA down to 1 pg, in both calibration curves (Table 3, Fig. 3A, B). This value of detection corresponds to approximately 1.6 genomic copies of walnut, evidencing the high sensitivity of the developed method. Like for the conventional real-time PCR system, the real value of sensitivity in cakes could be slightly lower due to the probable loss of genomic material during thermal treatment. Considering that the lowest amplified level was within the linear range of the calibration curve, the LOQ was of the same value with the LOD. The performance parameters of single-tube nested real-time PCR assays were also in accordance with the required criteria, presenting mean values for PCR efficiency of 96.5% and 98.4%, slopes of -3.4085 and -3.3609 and correlation coefficients of 0.9966 and 0.9993, for model mixtures of batter (Table 3, Fig. 3A) and sponge cakes (Table 3, Fig. 3B), respectively. As previously noted, with the novel nested real-time PCR system the DNA extracts of walnut in sponge cakes started to amplify earlier than the correspondent level of walnut in batter. As previously highlighted, differences in Ct values between batter and sponge cakes were generally not statistically significant. For the relative detection of walnut, significant differences ($p < 0.05$) were only noted for the levels of 5% and 50%, while in the case of absolute detection it occurred in the level of 10 pg.

4. Discussion

The preparation of foods such as bakery products involves procedures that may alter the native structure of several components. During cooking, processes such as thermal treatments (boiling, roasting, baking), partial hydrolysis, pH variations, high pressure processing and/or radiation are known to affect the integrity of proteins (Mills, Sancho, Moreno, & Kostyra, 2007). This represents one of the major problems associated with the detection of allergenic proteins in processed foods by immunological methods (Cucu, Platteau, Taverniers, Devreese, De Loose, & De Meulenaer, 2011; Cucu, Platteau, Taverniers, Devreese, De Loose, & De Meulenaer, 2013; Platteau et al., 2011). On the other hand, the elevated stability of DNA upon industrial processing has elected

Table 3

Real-time PCR and single-tube nested real-time PCR results for the absolute detection of walnut DNA extracted from model mixtures of 50% of walnut in batter and in sponge cake serially diluted (10-fold) ranging from 100 ng to 1 pg.

Absolute quantity (pg)	Conventional real-time PCR				Single-tube nested real-time PCR			
	50% of walnut in cake batter		50% of walnut in sponge cake		50% of walnut in cake batter		50% of walnut in sponge cake	
	Ct $\pm$ SD ^a	DNA copies ^b	Ct $\pm$ SD	DNA copies	Ct $\pm$ SD	DNA copies	Ct $\pm$ SD ^a	DNA copies
1	40.33 $\pm$ 2.00 (6/16) ^c	1.6	40.70 $\pm$ 2.51 (6/16)	1.6	30.41 $\pm$ 0.98 (16/16)	1.6	29.10 $\pm$ 1.00 (16/16)	1.6
10	36.89 $\pm$ 0.86 (16/16)	16	35.80 $\pm$ 0.74 (16/16)	16	26.42 $\pm$ 0.96 (16/16) ^d	16	25.75 $\pm$ 0.57 (16/16)	16
100	33.62 $\pm$ 0.47 (16/16)	161	32.25 $\pm$ 0.32 (16/16)	161	22.81 $\pm$ 0.27 (16/16)	161	22.31 $\pm$ 0.41 (16/16)	161
1000	30.02 $\pm$ 0.32 (16/16) ^d	1613	28.74 $\pm$ 0.20 (16/16)	1613	19.40 $\pm$ 0.17 (16/16)	1613	18.79 $\pm$ 0.23 (16/16)	1613
10,000	26.53 $\pm$ 0.25 (16/16)	16,129	25.44 $\pm$ 0.28 (16/16)	16,129	16.15 $\pm$ 0.11 (16/16)	16,129	15.40 $\pm$ 0.17 (16/16)	16,129
100,000	23.40 $\pm$ 0.09 (16/16)	161,290	22.32 $\pm$ 0.16 (16/16)	161,290	13.40 $\pm$ 0.20 (16/16)	161,290	12.48 $\pm$ 0.23 (16/16)	161,290
Correlation coefficient (R^2)	0.9995		0.9993		0.9966		0.9993	
Slope	-3.4067		-3.3766		-3.4085		-3.3609	
PCR efficiency (%)	96.6		97.8		96.5		98.4	

^a Mean cycle threshold (Ct) values $\pm$ standard deviation (SD).

^b Number of walnut haploid genome copies (0.62 pg) (RBG, Kew).

^c (Positive amplifications/total replicates).

^d Significant differences at $p < 0.05$ (t -test) between Ct values of batter and sponge cake at the same spiking level.

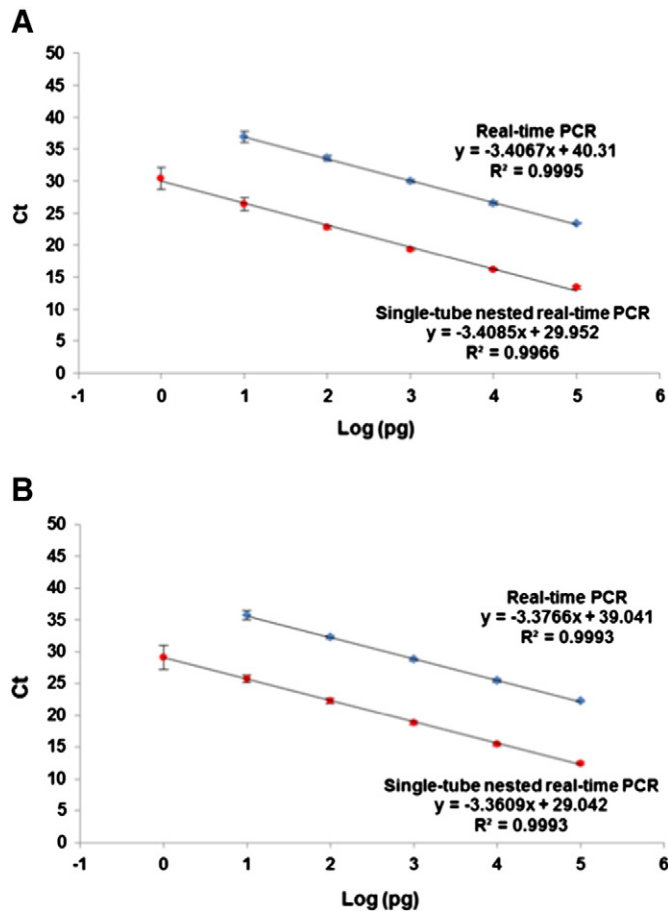


Fig. 3. Comparison of calibration curves obtained with conventional real-time PCR and single-tube nested real-time PCR of walnut DNA 10-fold serially diluted (100 ng to 1 pg) from 50% model mixture of walnut in batter (A) and sponge cake (B). Mean values and corresponding standard deviations of $n = 16$ replicates.

this molecule as a favoured target for allergen evaluation (Costa, Mafra, Carrapatoso & Oliveira, accepted for publication). Therefore, DNA-based methods have been regarded as powerful alternatives for the detection and identification of allergenic food sources in processed foods. Although the higher stability of DNA molecules upon food processing when compared with proteins, they can still be negatively affected by cooking processes such as baking roasting and autoclaving. In this regard, some studies describing the influence of thermal treatment on hazelnut and peanut DNA detection by real-time PCR technique have been reported (Iniesto et al., 2013; Platteau et al., 2011; Scaravelli et al., 2009). Platteau et al. (2011) referred that baking affected the sensitivity of a real-time PCR assay targeting Cor a 8 allergen encoding gene for the identification of hazelnut in cookies. In that study, the sensitivity achieved was 100 mg/kg of hazelnut in dough and 1000 mg/kg of hazelnut in cookies, evidencing a reduced sensitivity after heat treatment. Iniesto et al. (2013) also evaluated the effect of different food processing such as roasting, autoclaving and high pressure treatments to raw hazelnut on DNA yield and amplifiability. The results indicated that roasting and autoclaving reduced the ability to detect hazelnut DNA, which was supported by the low DNA yields and elevated Ct values for the thermally treated samples. Scaravelli et al. (2009) reported an increasing inaccuracy when detecting low levels of peanut DNA (10 mg/kg) in cookies by a real-time PCR assay, as a consequence of prolonging the time of baking.

The described negative effect of heat treatments on the detection of walnut DNA was not verified with both the real-time PCR and single-tube nested real-time PCR systems. In the case of the model sponge

cakes with walnut, heat treatment seems to stabilise those samples. It is important to refer that all cakes were baked using the same combination of temperature (200 °C) and time (20 min), which is commonly used for baking cakes at industrial level. Thus, in order to determine the potentially negative effect on DNA detection using the proposed systems, other temperatures or increasing baking periods should be further investigated. In this study, a basic sponge cake was used to prepare the model mixtures of cakes spiked with walnut. The fact of using fresh instead of lyophilised eggs could have also contributed to the strong enzymatic activity of the batter. In spite of the immediate preservation of the batter mixtures at -20°C , that could not be sufficient to prevent some potential DNA degradation as consequence of the enzymatic activity (Gryson, 2010). All these facts could be considered probable reasons for the herein results concerning the same LOD and LOQ for both processed and unprocessed cakes. Another important issue to emphasise is related to the choice of small walnut amplicons, which enables amplification of highly degraded DNA as a result of heat processing.

From the few reports in literature concerning the specific detection and quantification of walnut, the sensitivity levels for the developed methods based on conventional TaqMan real-time PCR assays ranged from 10 to 100 mg/kg of Jug r 2 DNA in incurred foods (Brezná et al., 2006; Köppel et al., 2012; Wang et al., 2009). Therefore, the obtained LOD of 50 mg/kg of walnut by means of the proposed real-time PCR system targeting the gene encoding the Jug r 3 allergen is in good agreement with those reports.

The development of a novel approach based on single-tube nested real-time PCR system was successfully achieved in the detection of walnut in cakes, as previously succeeded in the cases of hazelnut (Costa, Mafra, Kuchta & Oliveira, 2012) and almond (Costa, Oliveira & Mafra, 2013). Similarly to hazelnut and almond, the sensitivity was increased with the nested system since the relative LOD had a 5-fold reduction down to 10 mg/kg of walnut in batter and sponge cakes. In terms of absolute detection, the present nested system allowed amplifying walnut DNA down to 1 pg, representing approximately 1.6 DNA copies, which is in good agreement with the reported absolute LOD values of 1 and 3.9 DNA copies obtained by single-tube nested real-time PCR detection of hazelnut (Costa, Mafra, Kuchta & Oliveira, 2012) and almond (Costa, Oliveira & Mafra, 2013), respectively. It is important to refer that after thermal processing some walnut genomic material could be lost as a consequence of baking, suggesting that this limit of sensitivity could be lower than the value herein estimated. In this sense, the use of both genomic DNA and plasmid serial dilutions as reference for the calculation of the absolute sensitivity of the novel method could be recommended (D'Andrea, Coisson, Travaglia, Garino, & Arlorio, 2009).

After the heat treatment, sponge cakes presented the same relative and absolute LOD and LOQ of the model mixtures prepared with batter, highlighting an apparent robustness of the method. When compared to the other reports (Brezná et al., 2006; Köppel et al., 2012; Wang et al., 2009), the proposed nested real-time PCR system can be considered a technique with elevated specificity (no cross-reactivity with other plant-foods) and high sensitivity.

Presently, the number of commercial real-time PCR kits for the detection and quantification of allergens in foods is still limited. When compared to the real-time PCR commercial kit available for the detection of walnut allergens in foods, the presented systems show higher relative LOD (10–50 mg/kg) than the 0.4 mg/kg stated by SureFood® allergen walnut (r-Biopharm, Darmstadt, Germany). Regarding the absolute sensitivity, the proposed single-tube nested real-time PCR technique presented lower LOD (1.6 pg DNA copies) than that stated by the referred kit (5 DNA copies). However, this commercial real-time PCR system is not for quantification purposes since no calibration curve is performed in the assay, thus only enabling positive/negative responses until a referred limit of 0.4 mg/kg of walnut. Additionally, both the proposed real-time PCR systems for the specific detection of walnut do not present any cross-reactivity with more than 31 plant species tested, whereas the commercial kit reveals cross-reactivity with pecan nut.

5. Conclusion

In the present work, a molecular approach based on single-tube nested real-time PCR was effectively developed with demonstrated usefulness to trace minute amounts of walnut in bakery products. As expected, the proposed novel system provides advantages in terms of accuracy, sensitivity, specificity, dynamic range and high-throughput capacity over the conventional real-time PCR systems reported herein and in the literature. In addition, the performance of the single-tube nested real-time PCR system was not influenced by the heat processing since the sensitivities attained with both model mixtures of walnut in batter and sponge cakes were similar and Ct values were generally not significantly different. As a result, this novel technique could be widely applied to bakery products ensuring a LOD of 10 mg/kg, with the potential to obtain quantitative information by means of an appropriate calibration curve.

In general terms, this technology has the potential to be used with other model mixtures namely bread, cookies and other types of cakes or commercial food products, as already exploited in the case of hazelnut (Costa, Mafra, Kuchta & Oliveira, 2012). To our knowledge this is the first work evaluating the effect of thermal processing on the detection and quantification of DNA encoding walnut allergens by conventional and single-tube nested real-time PCR systems.

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