



TaqMan real-time PCR assay for detection of traces of Brazil nut (*Bertholletia excelsa*) in food products

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ABSTRACT

Mislabeling of food products containing Brazil nut may represent a serious threat for allergic consumers. In order to protect sensitized individuals, reliable methods to detect trace amounts of Brazil nut must be accessible to food industry as well as Food Safety authorities. According to this, a TaqMan real-time polymerase chain reaction (PCR) method has been developed for specific detection of Brazil nut in foodstuffs. The method employs Brazil nut specific primers and probe, targeting 2S albumin gene (*Ber e 1*), and a positive amplification control based on 18S rRNA gene. Results obtained on sensitivity with wheat flour spiked with different concentrations of raw and heat treated Brazil nut showed that the limit of detection (LOD) for the technique was 2.5 mg/kg. Applicability of the Brazil nut specific system was assessed through analysis of 66 different commercial food samples. The reported real-time PCR assay provides a useful tool for detection of Brazil nut DNA, and it can be used as a routine analysis to assert accuracy on food labeling.

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1. Introduction

Brazil nut (*Bertholletia excelsa*) is a tree nut belonging to the Lecythidaceae family in the order Ericales. In recent years there has been an increasing demand for delicatessen food products that contain Brazil nut seeds as an ingredient. Not only do these products offer a high economic value, but their consumption is also associated with several health benefits. Brazil nuts have important antioxidant properties thanks to their high content in selenium (Chunhieng et al., 2004; Yang, 2009). Moreover, even though an average of 60% of their total weight is oil, 73% of the fatty acids are heart-protective monounsaturated and polyunsaturated, and it is an excellent dietary source of micronutrients such as tocopherols, phytosterols and squalene (Phillips, Ruggio, & Ashraf-Khorassani, 2005; Robbins, Shin, Shewfelt, Eitenmiller, & Pegg, 2011; Ros, Tapsell, & Sabaté, 2010; Ryan, Galvin, O' Connor, Maguire & O'Brien, 2006). Protein content of Brazil nuts is also remarkable, with 15–17% of protein by fresh weight. The Brazil nut 2S albumin,

named Ber e 1, comprises about 30% of total protein, and it is exceptionally rich in sulfur aminoacids. However, it is well-established that Ber e 1 is able to elicit allergic responses in sensitized individuals (Alcocer, Rundqvist, & Larsson, 2012; Ryan et al., 2006). Moreover, together with peanuts and hazelnuts, Brazil nuts are the most powerful food allergens (Schubert-Ullrich et al., 2009).

Food allergies have become an important health issue in industrialized countries, and they are ranked by the World Health Organization as the sixth problem of human health. It has been estimated that around 1% of adults and 2.5% of children worldwide suffer from food allergy (Jackson, 2003; Kumar, Verma, Das, & Dwivedi, 2012). Clinical experience suggests that most cases of food allergy are elicited by a relatively small number of foods (colloquially known as 'the big eight'). The most frequent food allergens in children are milk, egg, soy, peanut, tree nuts, fish and crustaceans, whereas in adults are peanuts, tree nuts, crustaceans, fish and egg (Jackson, 2003). Although sensitivity to some food allergens tends to decrease in late childhood, allergies to peanuts, tree nuts and seafood tend to persist (Chapman et al., 2006).

In sensitized individuals, even a minute intake of an allergen could trigger a plethora of symptoms, which range from skin irritations to life-threatening anaphylaxis. Furthermore, at the present time the only effective treatment for food allergy is total avoidance

Abbreviations: BNS, Brazil nut specific system; PAC, positive amplification control.

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of the offending food (Monaci & Visconti, 2010). Nevertheless, accidental exposure to a particular allergen frequently happens due to mislabeling of products or cross-contamination during food processing. In several countries, Brazil nut is included in a list of food ingredients which must be indicated on the label of foodstuffs, as they are likely to cause adverse reactions in susceptible individuals. The EU (European Parliament and Council, 2007, 2011), the USA (Food Allergen Labelling and Consumer Protection Act of 2004) and Canada (Regulations Amending the Food and Drug Regulations, 2011) are some of the countries that have regulated labeling of food allergens. To enforce labeling requirements, robust analytical methods to monitor foods or industrial lines where cross-contamination is possible must be accessible for food industry and Food Safety authorities. The new tools must be fast, sensitive (providing low limit of detection), accurate and specific (allowing discriminate between similar species) in order to enable unequivocal identification of the allergens (Kirsch et al., 2009).

Various assays for detection of Brazil nut in foodstuffs have been reported over the last few years. These methods targeted either the allergen itself, marker proteins or DNA fragments. Protein-based methods involve immunochemical detection protocols. Despite the wide range of immunochemical methods available, only enzyme-linked immunosorbent assay (ELISA) is currently convenient for routine screening and quantification in food industry (Kirsch et al., 2009). In the other hand, DNA-based methods are based on the amplification of a specific DNA fragment by the polymerase chain reaction (PCR). Both methods have their advantages and drawbacks (Poms, Klein, & Anklam, 2004). Immunoanalytical procedures require the employment of animal antibodies raised against the allergenic protein. In addition, in order to select a target allergen, it is important to take into account protein stability during food processing because manufacturing operations can have an impact on allergen structure, affecting conformational epitopes and, therefore, preventing antibody recognition (Sathe & Sharma, 2009). Regarding Brazil nut, Ber e 1 protein presents a high stability toward heat processing as well as pepsin digestion, maintaining the native form, so it becomes a suitable biomarker to detect the presence of Brazil nut in foods (Koppelman et al., 2005). Two immunoanalytical techniques which detect Ber e 1 protein in foodstuffs using polyclonal antibodies have been published. Blais, Omar, and Phillippe (2002), reported a qualitative sandwich enzyme immunoassay, and Clemente, Chambers, Lodi, Nicoletti, and Brett (2004) developed an indirect competitive ELISA, both achieving a limit of detection (LOD) of 1 mg/kg. Other authors have obtained polyclonal antibodies against Brazil nuts extracts, like Sharma, Roux, and Sathe (2009) who described a competitive ELISA also with a LOD of 1 mg/kg, but reporting a significant interference with cinnamon. Immunoassays have also been developed for simultaneous detection of peanut and tree nuts. In this respect, Blais, Gaudreault, and Phillippe (2003) attained simultaneous detection of Brazil nut, peanut and hazelnut by employing a reverse dot blot enzyme immunoassay system, whereas Ben Rejeb, Abbott, Davies, Cl  roux, and Delahaut (2005), developed a competitive indirect enzyme immunoassay to detect Brazil nut, almond, cashew, hazelnut and peanut using rabbit polyclonal antibodies. Nonetheless, neither of those polyclonal antibodies is commercially available. Lately, lateral flow devices have earned importance. These kits are extremely simple to perform, but they have the disadvantage of being only qualitative. AgraStrip[®] Brazil nut (Romer[®] Labs, Cheshire, UK) and Lateral Flow Brazil Nut (R-Bio-farm[®], Darmstadt, Germany) are commercially available kits for Brazil nut detection, both with a LOD at the low mg/kg level.

DNA based methods such as PCR provide an alternative approach to detect allergenic ingredients in foods. These methods have the advantages of being highly sensitive, specific and

reproducible, and can be used in highly processed products. Moreover, when real-time PCR assays are used, they also have the benefits of automation, speed and acquisition of quantitative data. Real-time PCR requires more expensive laboratory equipment, but it is more accurate than other DNA quantification methods (Poms, Anklam, & Kuhn, 2004). Three PCR assays have been recently developed for Brazil nut allergen detection (Brezn  , Dud  sov  , & Kuchta, 2010; Hubalkova & Rencova, 2011; R  der, Filbert, & Holzhauser, 2010), but only that of R  der et al. (2010) has a LOD in the range accomplished by available ELISA methods.

In this study we describe the development and optimization of a TaqMan real-time PCR technique, based on selective amplification of a *Ber e 1* gene fragment, for specific identification of Brazil nut. The applicability of the assay was evaluated through the screening of 66 different commercial food samples, consisting in a wide variety of brands and types of products.

2. Materials and methods

2.1. Sample selection

Samples of Brazil nut and other tree nuts were purchased from a local dealer in Madrid, Spain. They were carefully cleaned and shelled separately to avoid cross-species contamination, and stored at -20°C until used. Plant and animal species analyzed for specificity purposes (Table 1) were acquired in different local markets in Madrid. Finally, a total of 66 commercial food products were purchased from various retail markets and delicatessen shops (Spain).

Table 1
Specificity of the real-time PCR system.

Common name	Scientific name	<i>Ber e 1</i> BNS ^a	18S rRNA PAC ^b
Brazil nut	<i>Bertholletia excelsa</i>	20.50 $\pm$ 0.08 ^c	13.90 $\pm$ 0.02
Almond	<i>Prunus dulcis</i>	– ^d	13.11 $\pm$ 0.02
Cashew nut	<i>Anacardium occidentale</i>	–	13.86 $\pm$ 0.07
Chestnut	<i>Aesculus hippocastanum</i>	–	15.88 $\pm$ 0.02
Hazelnut	<i>Corylus avellana</i>	–	11.37 $\pm$ 0.01
Macadamia	<i>Macadamia integrifolia</i>	–	12.97 $\pm$ 0.04
Peanut	<i>Arachis hypogaea</i>	–	14.56 $\pm$ 0.04
Pecan nut	<i>Carya illinoensis</i>	–	13.43 $\pm$ 0.02
Pine nut	<i>Pinus pinea</i>	–	15.11 $\pm$ 0.06
Pistachio	<i>Pistacia vera</i>	–	13.38 $\pm$ 0.01
Walnut	<i>Juglans regia</i>	–	11.30 $\pm$ 0.01
Barley	<i>Hordeum vulgare</i>	–	15.02 $\pm$ 0.04
Chocolate	<i>Theobroma cacao</i>	–	16.41 $\pm$ 0.04
Chufa sedge	<i>Cyperus esculentus</i>	–	14.79 $\pm$ 0.03
Lupine	<i>Lupinus albus</i>	–	16.52 $\pm$ 0.06
Maize	<i>Zea mays</i>	–	16.38 $\pm$ 0.03
Mung bean	<i>Vigna radiata</i>	–	15.78 $\pm$ 0.01
Oats	<i>Avena sativa</i>	–	14.32 $\pm$ 0.06
Olive	<i>Olea europaea</i>	–	14.01 $\pm$ 0.03
Orange	<i>Citrus sinensis</i>	–	14.23 $\pm$ 0.02
Potato	<i>Solanum tuberosum</i>	–	15.05 $\pm$ 0.05
Rice	<i>Oryza sativa</i>	–	13.87 $\pm$ 0.01
Rye	<i>Secale cereale</i>	–	14.02 $\pm$ 0.02
Sesame	<i>Sesamum indicum</i>	–	16.26 $\pm$ 0.07
Soybean	<i>Glycine max</i>	–	14.12 $\pm$ 0.02
Sunflower	<i>Helianthus annuus</i>	–	15.44 $\pm$ 0.01
Wheat	<i>Triticum aestivum</i>	–	15.72 $\pm$ 0.06
Cattle	<i>Bos taurus</i>	–	16.17 $\pm$ 0.01
Goat	<i>Capra hircus</i>	–	15.02 $\pm$ 0.04
Sheep	<i>Ovis aries</i>	–	13.98 $\pm$ 0.00
Swine	<i>Sus scrofa domestica</i>	–	14.34 $\pm$ 0.07

^a **Bere1 BNS:** Brazil nut specific system on the *Ber e 1* gene (NBRAZIL-FW2/NBRAZIL-REV2, and BexcTM probe).

^b **18S rRNA PAC:** Positive amplification control on the 18S rRNA gene (18SDIR/18SINV, and 18SP probe).

^c Average Cp value $\pm$ SD obtained from triplicate PCR reactions from each DNA extraction.

^d Minus sign indicates no positive signal after 50 PCR cycles.

These products included 18 labeled as containing Brazil nut as an ingredient, 15 labeled as may contain Brazil nut traces, 19 labeled as 'may contain traces of tree nuts', and 14 declaring to contain tree nuts, but not Brazil nut. The commercial food samples were stored at -20°C in airtight containers until analyzed.

2.2. Sample preparation

Experimental binary mixtures of Brazil nut in a wheat flour matrix (10^5 – 1 mg/kg) were prepared to evaluate the test sensitivity. As a first step, 50 g of finely ground Brazil nut was added to 450 g of wheat flour, and the mixture was homogenized for 5 min at high speed using a food processor (Thermomix, Vorwerk, Germany). Then, 50 g of the former mixture was added to 450 g of wheat flour to obtain a concentration of 10^4 mg/kg. Remaining concentrations (up to 1 mg/kg) were obtained following the same procedure. Three additional mixtures containing 5000 mg/kg, 5 mg/kg and 2.5 mg/kg of Brazil nut in wheat flour were also prepared by mixing 250 g of wheat flour with 250 g of the mixtures containing 10^4 mg/kg, 10 mg/kg, and 5 mg/kg, respectively. A pure sample of ground Brazil nut was used as the positive control.

The effect of heat treatment on technique's ability to identify the target species was assessed through the analysis of binary mixtures prepared with ground roasted Brazil nuts. To prepare roasted mixtures, 70 g of ground Brazil nut were processed in an oven at 160°C for 13 min. This roasted sample was used to prepare Brazil nut-wheat flour mixtures containing 10^5 – 1 mg/kg of Brazil nut component, prepared as described above. Binary mixtures were stored at -20°C until use.

2.3. DNA extraction

Two hundred milligrams of sample were homogenized with 860 μL of extraction buffer (0.010 M Tris, 0.150 M NaCl, 0.002 M EDTA, and 1% SDS, pH 8.0), 100 mL of 5 M guanidine hydrochloride, and 40 μL of 20 mg/mL proteinase K (Merk, Darmstadt, Germany), and incubated overnight at 55°C with shaking at 250 rpm. Then, the samples were cooled down to room temperature. Five hundred microliters of chloroform (Sigma–Aldrich, MO, USA) were added to the lysate before centrifugation at 16 438 g for 10 min. Genomic DNA from the clear aqueous supernatant obtained after the centrifugation (500 μL) was purified using the Wizard DNA Cleanup System Kit (Promega Corp., Madison, WI, USA), with a vacuum manifold, according to the manufacturer's instructions. The DNA was eluted in 50 μL of sterile deionized water. DNA concentration was quantified with a ND-1000 spectrophotometer (NanoDrop Technologies Inc., Montchanin, DE, USA) before storage at -20°C . Three separate extractions of each sample were prepared, and a negative control sample was included within every extraction. DNA extracted from raw and roasted Brazil nut was electrophoresed in a 1% low electroendosmosis D1 (Hispanlab S.A., Torrejón, Spain) agarose gel containing 1 $\mu\text{g/mL}$ ethidium bromide (Sigma–Aldrich)

in Tris-acetic acid-EDTA buffer (0.04 M Tris, 0.02 M acetic acid, 0.001 M EDTA, pH 8.3) (Bio-Rad, Munich, Germany) for 30 min at 110 V. Total DNA was visualized by UV transillumination employing a Molecular Imager ChemiDoc XRS System (Bio-Rad Laboratories, Hercules, CA, USA).

2.4. Primers and probes

Several 2S albumin gene sequences from peanut and tree nut species available in the GenBank-European Molecular Biology Laboratory (GenBank-EMBL) database were aligned with EMBL software package, version 2.2.0. After sequence comparison and analysis, a Brazil nut specific pair of primers (NBRAZIL-FW2 and NBRAZIL-REV2) was designed to amplify a DNA fragment of 131 bp in the *Ber e 1* (Brazil nut 2S albumin) gene, with the aid of Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA). A TaqMan probe (BexcTM) was designed to anneal within the amplicon generated with the Brazil nut specific primers. As a positive amplification control (PAC), universal primers 18SDIR and 18SINV and TaqMan probe 18SP were used, targeting a 77 bp fragment in the 18S rRNA gene from all eukaryotic species (Pegels et al., 2011). The sequences of the primers and probes used in the real-time PCR assay are listed in Table 2. TaqMan probes were designed and synthesized by TibMolBiol (Berlin, Germany).

2.5. Real-time PCR setup

Real-time PCR amplification reactions were performed in a final volume of 10 μL , in a glass capillary tube. Each reaction mixture contained 200 ng of template DNA, 2 pmol of TaqMan probe (TibMolBiol), and LightCycler® TaqMan Master (Roche Diagnostics GmbH, Mannheim, Germany). Primers concentrations were experimentally set to obtain the lowest crossing point (Cp) values with the highest fluorescence. They were 300 nM for the forward primer and 900 nM for the reverse primer. Amplification reactions were run on a LightCycler® 2.0 Instrument (Roche Applied Science, Penzberg, Germany), with a program consisting of 10 min at 95°C (denaturation and Taq polymerase activation), followed by 50 cycles at 95°C for 10 s, 55°C for 30 s, and 72°C for 1 s. Then, samples were cooled to 40°C for 30 s. This program was carried out to amplify the Brazil nut specific system (BNS) and the PAC. Real-time PCR reactions were carried out in triplicate for each DNA extract. The Cp value, which represents the cycle at which increase in fluorescence is the highest and where the logarithmic phase of a PCR begins, were provided automatically by the LightCycler® software as the first maximum of the second-derivative of the curve.

2.6. Construction of the standard curve and data analysis

In order to evaluate the correlation between Cp values obtained in the real-time PCR and the concentration of Brazil nut in the samples, standard curves were obtained using ten-fold dilution

Table 2
DNA sequences and description of the primers and probes used in this study.

Primers	Length (bp)	Sequence (5'→3')	Description	Target gene	Fragment length (bp)
NBRAZIL-FW2	20	GCGAAGGCTTAAGGATGATG	Brazil nut specific forward primer	<i>Ber e 1</i>	131
NBRAZIL-REV2	20	GCATCTCATGGGACTGAGGT	Brazil nut specific reverse primer		
18SDIR	16	TGGTGCCAGCAGCCGC	Positive Control forward primer	18S rRNA	77
18SINV	25	TCCAACACAGAGCTTTTAACTGCA	Positive Control reverse primer		
Probes	Length (bp)	Sequence (5'→3')	Description	Target gene	
BexcTM	22	6FAM-AAGGGATATTCTCGGCCAGCCT-BBQ	Brazil nut probe	<i>Ber e 1</i>	
18SP	22	6FAM-CGCTATTGGAGCTGGAATTACC-BBQ	Universal probe	18S rRNA	

series of Brazil nut DNA (10^2 ng– 10^{-4} ng). Standard curves were also constructed from experimental mixtures containing different concentrations (10^5 –1 mg/kg) of raw and roasted Brazil nut in wheat flour.

Variables Cp and Brazil nut concentration are correlated following a semi-logarithmic model:

$$Cp = b \log \text{concentration} + a$$

where b is the slope and a is the intercept.

The amplification efficiency, E , was calculated from the slope of the standard curve using the following formula (Bustin et al., 2009):

$$E = 10^{-1/\text{slope}}$$

$$\% \text{Efficiency} = (E - 1) \cdot 100$$

The LOD of the method was determined with the standard deviation of the response and the slope using the equation $LOD = 3.3\sigma/b$, where σ is the standard deviation of the intercept, and b is the slope of the calibration curve. Moreover, the practical LOD was assessed as the lowest amount of Brazil nut that can be reliably distinguished from the blank matrix, based on sextuplicate analysis of binary mixtures of a wheat flour matrix spiked with decreasing concentrations of raw and roasted Brazil nut (10^5 –1 mg/kg).

Linearity, sensitivity, accuracy and precision parameters of the BNS were determined according to previously described methods (Camacho, Torres, Gil-Alegre, Obregón, & Ruz, 1993; Fajardo et al., 2009; International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, 2005). The following tests were used for linearity analysis: (i) Cochran test, which determined whether the variances of the responses obtained for each concentration of Brazil nut in the binary mixtures were homogeneous for a 0.05 significance level; (ii) Regression analysis, which asserted if existed a statistically significant relationship between Cp and logarithm of the Brazil nut DNA concentration, with a confidence level of 99.0%; (iii) Analysis of variance (ANOVA) with lack of fit, which determined whether the chosen model was suitable to describe the observed data with a confidence level of 95.0%, and (iv) Discriminating capacity, which is the least difference in the logarithm of the target DNA concentration in the sample that the analytical method can discriminate at the specified significance level. In-house validation of the assay was assessed by testing three independent DNA extractions from experimental mixtures containing five different concentrations of Brazil nut, with three replicates per extraction. Precision was evaluated by contrasting the coefficient of variation (CV) values calculated in three different days versus those obtained in the same day, to ponder the influence of the day of analysis in the data obtained from the binary mixtures. Statgraphics Centurion 15.2.14 (XV) (Statpoint Technologies, Inc., Warrenton, Virginia, USA) was used for data analysis.

3. Results and discussion

3.1. Assay development and specificity

The real-time PCR assay developed and evaluated in this work is based on a species-specific primer pair and TaqMan probe designed on the Brazil nut 2S albumin gene, *Ber e 1*. *Ber e 1* has been reported as a major food allergen, demonstrating its ability to bind IgE from the sera of allergic patients, and due to the presence of disulfide bonds, this glycoprotein is resistant to proteolysis and thermal treatment (Moreno & Clemente, 2008; Moreno et al., 2004). In the

present work, the *Ber e 1* gene has been selected because *Ber e 1* protein is the major allergen described in Brazil nut (Alcocer et al., 2012). In addition, availability of different entries for Brazil nut 2S albumin gene in databases, prevents selecting nucleotide regions specific for a particular Brazil nut variety, or regions containing inaccurate nucleotides due to sequencing failures, that could lead to unsuccessful amplifications. Alignment and analysis of 2S albumin DNA sequences available in the GenBank-EMBL database allowed the design of the primer pair NBRAZIL-FW2 and NBRAZIL-REV2, which amplified a DNA fragment of 131 bp in *B. excelsa*, and the BexcTM TaqMan probe (Fig. 1). The selection of a short length DNA fragment is important to avoid lack of amplification of the target, as food processing may cause random cleavage of DNA strands, leading to a reduction in the size of DNA fragments (Jonas et al., 2001). Gel electrophoresis in Fig. 2 shows some degree of DNA fragmentation, even in raw Brazil nut. It may be due to the boiling treatment that is commonly applied to facilitate removing their extremely hard shell. DNA fragmentation increased with heat treatments, and samples roasted at 140 °C/20 min or 160 °C/13 min contained only a smear of low-to-medium molecular weight degraded DNA. Specificity of the BNS was assessed by the analysis of DNA extracted from 11 nuts, and also from several plant and animal species. In the BNS, Brazil nut was amplified with a Cp value of 20.50 ± 0.08 , while no cross-species amplification signal was obtained after 50 cycles on the non-targeted plant and animal species tested, including all the allergenic nuts that must be stated in food labels according to EU Regulation No 1169/2011 (Table 1). To support the absence of PCR inhibition, a PAC system based on the eukaryotic 18S rRNA, was carried out in parallel with BNS for amplification of total eukaryotic DNA. The PAC amplified the specific DNA fragment from all eukaryotic species analyzed with average Cp values of 14.52 ± 1.33 . Inhibition due to the absence of amplifiable DNA in the sample or to the presence of polymerase inhibitors would produce a negative real-time PCR result in the PAC.

3.2. Standard curves and amplification efficiency

To estimate the sensitivity (lowest amount of Brazil nut DNA detected) of the real-time PCR method, standard curves were constructed with ten-fold dilutions of DNA from raw and roasted (160 °C, 13 min) Brazil nut. A good linear correlation ($R^2 > 0.99$) was obtained between the crossing point values and the log of Brazil nut DNA concentration. Linearity was observed over the range between 100 ng and 10 pg of DNA. Sensitivity of the real-time PCR system was 1 pg for raw Brazil nut and 10 pg for roasted Brazil nut. Fig. 3 shows the results of fitting a linear model to describe the relationship between Cp values and logarithm of the raw and roasted Brazil nut DNA concentrations. The Cochran test demonstrated that the variances of the responses obtained for each DNA concentration were homogeneous for a 0.05 significance level, thus allowing the calculation of the regression line parameters by least squares and the discriminating capacity from an average variance of the response (S^2 comb). Since the P -values in the ANOVA tables were lower than 0.01, there was a statistically significant relationship between Cp and log concentration of Brazil nut DNA at the 99% confidence level. Also, since the P -values for lack-of-fit in the ANOVA tables were greater than 0.05, the linear model appeared to be adequate for the observed data at the 95% confidence level. The slope of the log-linear phase of the standard curve reflects the amplification efficiency. The slopes of the linear equations were -3.5 for raw Brazil nut DNA and -3.76 for roasted Brazil nut DNA, which corresponded to amplification efficiencies of 93% and 84.5%, respectively. Efficiencies for an optimal real-time PCR should be between 90% and 100%. This parameter can be affected, among

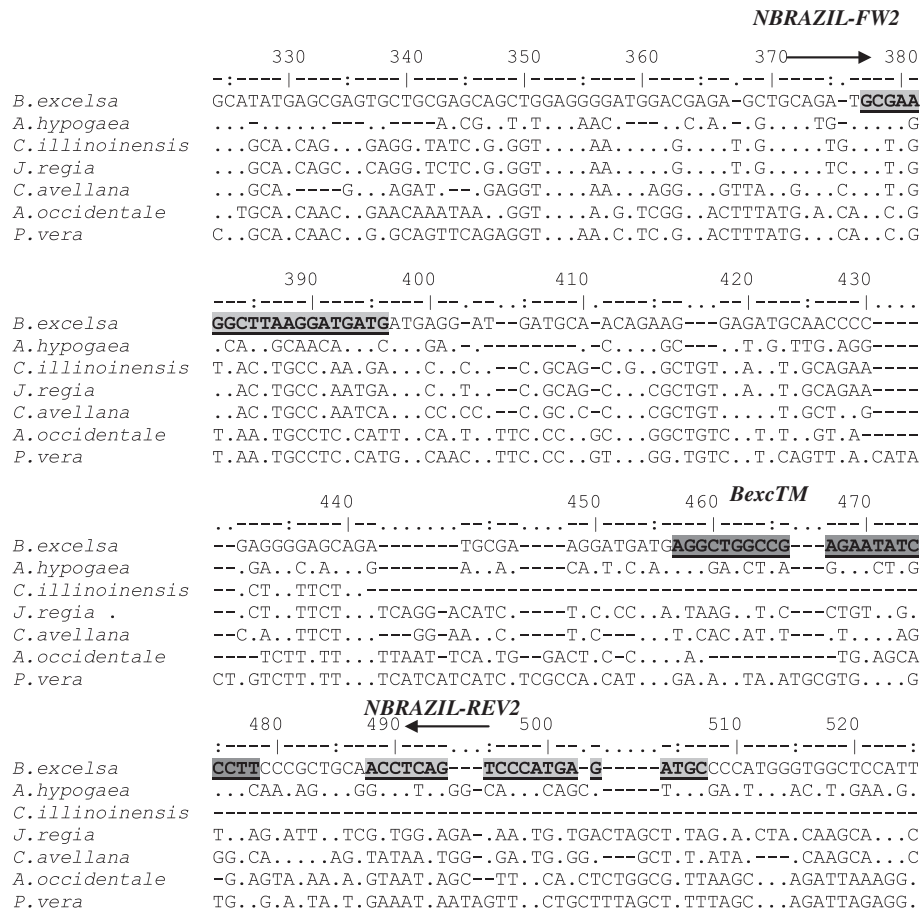


Fig. 1. Alignment of the 2S albumin DNA sequences from Brazil nut (*Bertholletia excelsa* AB044391), peanut (*Arachis hypogaea* FJ713110.1), pecan nut (*C. illinoensis* AY192569.1), walnut (*Juglans regia* 1794251), hazelnut (*Corylus avellana* FJ358504.1), pistachio (*Pistacia vera* DQ631675.1) and cashew nut (*Anacardium occidentale* AY081853.1) available at the GenBank-EMBL database. Primers NBRAZIL-FW2, NBRAZIL-REV2, and BexcTM probe are highlighted.

other things, by the length of the amplicon, secondary structure and primer quality (Dorak, 2006).

To test the efficiency and sensitivity of the technique under practical conditions, the real-time PCR amplification was also carried out with experimental mixtures containing different

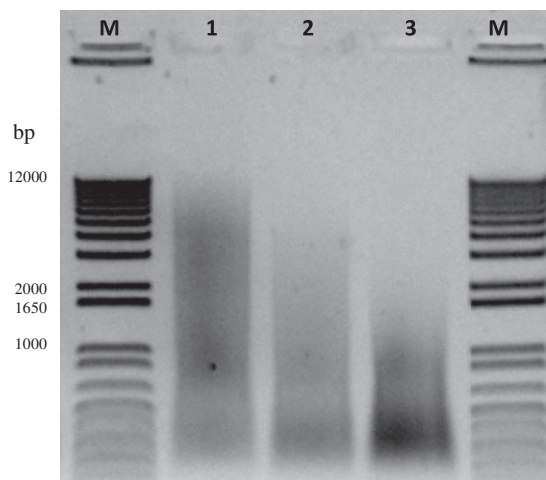


Fig. 2. Gel electrophoresis of total genomic DNA extracted from Brazil nut. Sample in lane 1 was obtained from raw Brazil nut, and samples in lanes 2 and 3 were obtained from air roasted Brazil nut heat-treated at 140 °C/20 min (2), and at 160 °C/13 min (3). M, molecular weight marker 1 Kb Plus DNA ladder (Invitrogen).

concentrations of raw and roasted Brazil nut (1–10⁵ mg/kg) in wheat flour. Fig. 4 summarizes the statistical analysis for linearity of the results obtained. A linear response between the Cp and logarithm of Brazil nut concentration was obtained over the range 10²–10⁵ mg/kg ($R^2 > 0.99$). The slopes of the linear equations were –3.39 for raw Brazil nut mixtures and –3.52 for roasted Brazil nut mixtures, which corresponded to amplification efficiencies of 97.2% and 92.3%, respectively. The LOD of the method based on the standard deviation of the response and the slope was calculated as 0.6 and 0.7 mg/kg for raw and roasted Brazil nut, respectively. Nevertheless, the practical LOD was assessed from analysis of spiked wheat flour samples as the minimum Brazil nut concentration that could be reliably distinguished from the blank matrix. The practical LOD was 2.5 mg/kg for both raw and roasted Brazil nut, based on sextuplicate analysis of spiked samples. All six replicates of samples containing 10, 5, and 2.5 mg/kg raw or roasted Brazil nut were amplified using the BNS, with Cp values of 35.97 ± 0.34, 35.23 ± 0.74, and 36.33 ± 0.72, respectively, for raw Brazil nut, and Cp values of 35.70 ± 0.81, 36.11 ± 0.61, and 36.20 ± 0.52, respectively, for roasted Brazil nut. Nevertheless, only 2 out of 6 mixtures spiked at 1 mg/kg were amplified with average Cp values of 35.83 for raw, and 36.81 for roasted Brazil nut.

Even though it is difficult to establish a threshold dose that would not cause a reaction in any individual within the total population of allergic patients, there is scientific agreement that the detection limits for different allergenic ingredients in foodstuffs must be somewhere between 1 and 100 mg/kg, depending on the

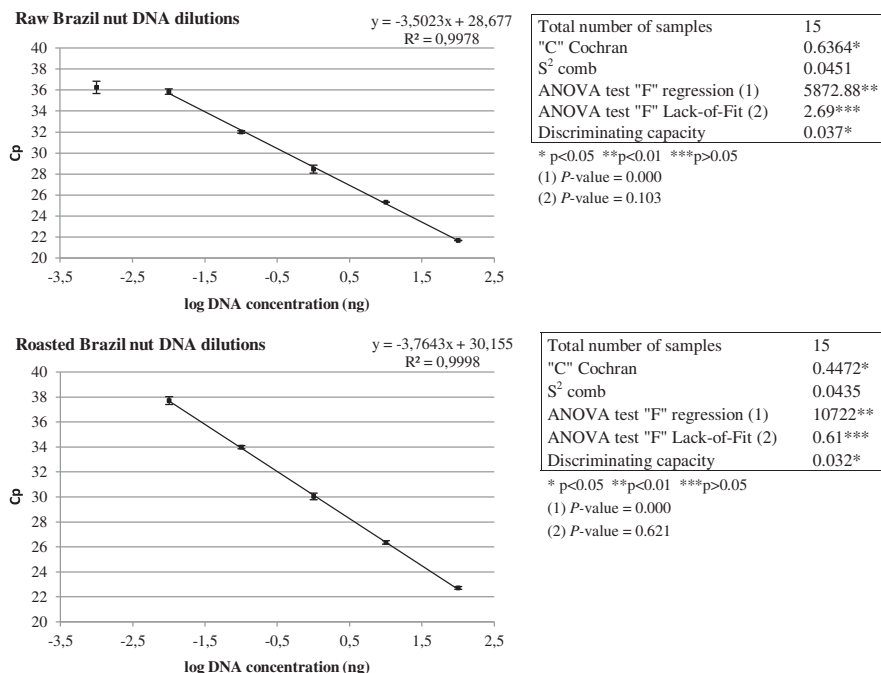


Fig. 3. Cp values plotted versus the logarithm of the DNA concentration (ng) of raw and heat treated (160 °C/13 min) Brazil nut. Linearity test, regression line, and sensitivity parameters were calculated for the BNS. Error bars indicate the standard deviation of mean from three independent experiments.

respective food (Poms, Klein, et al., 2004; Taylor, Crevel, Sheffield, Kabourek, & Baumert, 2009; Threshold Working Group, 2008). Thus, the LOD of the real-time PCR method developed herein is appropriate to protect allergic consumers.

Accuracy is defined as the ability of the analytical method to give results as close as possible to the theoretical value (Camacho et al., 1993). This parameter was assessed analyzing wheat flour samples

spiked with known amounts of Brazil nut (100, 1000, 5000, 10 000 and 100 000 mg/kg). Mean percent recovery values were 103.19 ± 12.3 for raw Brazil nut and 100.57 ± 10.3 for roasted Brazil nut. The Student's *t*-test demonstrated the accuracy of the method, since there were no significant differences between 100% and the mean recovery values at the 95.0% confidence level. The influence of the day of analysis on the assay precision was evaluated through

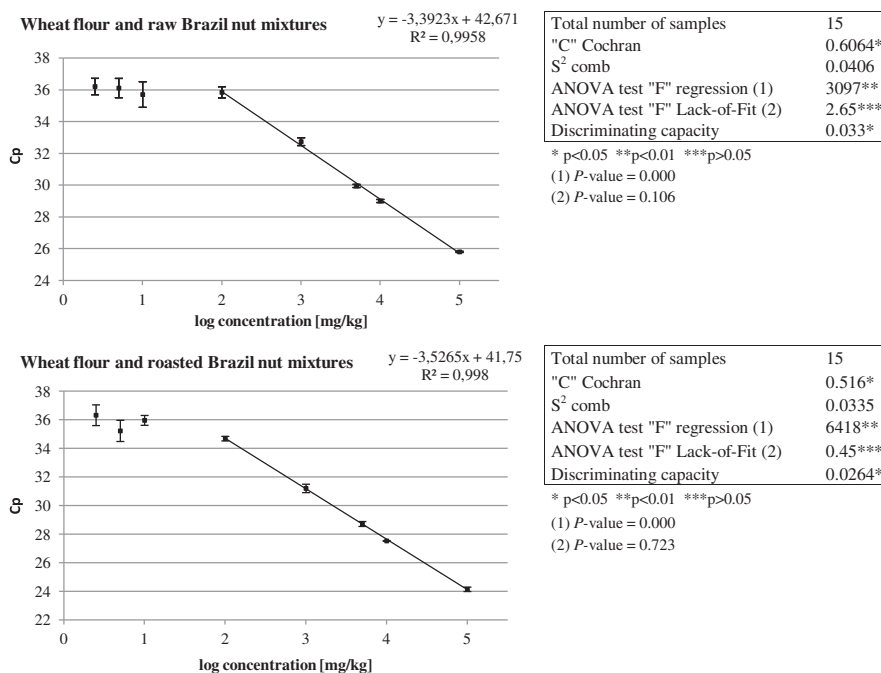


Fig. 4. Cp values plotted versus the logarithm of Brazil nut concentrations (10^5 –2.5 mg/kg) in wheat flour for raw Brazil nut mixtures, and heat treated (160 °C/13 min) Brazil nut mixtures. Linearity test, regression line, and sensitivity parameters were calculated for the BNS. Error bars indicate the standard deviation of mean from three independent experiments.

Table 3

Results of the Brazil nut real-time PCR analysis of 66 commercial food products.

	Number of samples analyzed	<i>Ber e 1</i> (BNS)	18S rRNA (PAC)
Commercial food products that declared Brazil nut as an ingredient			
Chocolate	5	+(2)/(–3)	+
Food bar	6	+	+
Muesli	2	+	+
Cereal	4	+	+
Biscuit	1	+	+
Commercial food products that declared traces of Brazil nut			
Chocolate	15	+(1)/(–14)	+
Commercial food products that declared to contain traces of tree nuts			
Biscuit	4	–	+
Bread	2	–	+
Chocolate	5	–	+
Cereal	1	+	+
Rice cake	1	–	+
Food bar	3	+(2)/(–1)	+
Granola	1	–	+
Ice cream	1	–	+
Candy bar	1	–	+
Commercial food products that declared to contain traces of nuts different from Brazil nut			
Chocolate	3	–	+
Cashew cream	1	–	+
Cereal	1	–	+
Beverage	3	–	+
Biscuit	3	–	+
Tomato sauce	1	–	+
Precooked meal	1	–	+
Ice cream	1	–	+

(+) Indicates positive signal before 50 PCR cycles.

(–) Indicates no positive signal after 50 PCR cycles.

comparison of the coefficients of variation (CV) of the average percentage of recovery values obtained in triplicate assays from 3 independent real-time PCR experiments, either performed on the same day or on three different days. CV for the same day were 11.95 and 10.29 for raw and roasted Brazil nut, respectively. When the assay was performed on different days the CV values were slightly higher (12.29 and 13.06). Nevertheless, the ANOVA test demonstrated that the factor “day of analysis” did not have a

statistically significant effect on the average percentage of recovery at a 95.0% confidence level.

Currently, PCR techniques described in bibliography for Brazil nut detection are scarce. Hubalkova and Rencova (2011) developed a multiplex PCR assay for detection of pecan and Brazil nut based on the *Ber e 1* gene with a LOD of 1000 mg/kg. Similarly, Brezná et al. (2010) developed a real-time PCR method for Brazil nut on the gene encoding the 11S globulin, which also achieved a detection limit of 1000 mg/kg. Nevertheless, these methods lacked of sufficient sensitivity to achieve detection in the range of 1–100 mg/kg. The LOD of the assay described in the present work is similar to the 5 mg/kg reported by Röder et al. (2010), being in the range of sensitivity recommended for detection of nut allergens in food, but they only analyzed a total of 15 retail samples, of which just 2 contained Brazil nut. Therefore, it would have been desirable to analyze a higher number of commercial samples in order to assess the applicability of that method.

3.3. Analysis of retail samples

Availability of techniques for detection of Brazil nut in foodstuffs is crucial not only because an accidental contamination with these tree nuts could cause a serious health problem in sensitized individuals, but also because Brazil nuts are susceptible targets for fraudulent substitution, due to their higher price when compared to other tree nuts or peanut (Woolfe & Primrose, 2004).

The suitability of the developed method for detection of Brazil nut DNA in foodstuffs was assessed through analysis of 66 commercial food products that could contain Brazil nut in their composition. Among the samples analyzed, 18 were labeled as including Brazil nut as an ingredient, 15 labeled as containing traces of Brazil nut, 19 labeled as ‘may contain traces of tree nuts’, and 14 declaring to contain tree nuts, but not Brazil nut (Table 3).

Real-time PCR analysis supported labeling in most of the samples. However, Brazil nut DNA was not amplified from 3 chocolates which declared to contain Brazil nut, but did not state the percentage of this ingredient. Result was also negative for 14 chocolates which declared to contain traces of Brazil nuts. Due to its high economic value, the absence of DNA amplification in the 3 samples

Table 4

Real-time PCR results obtained in the analysis of commercial food products that contained Brazil nut according to the Brazil nut specific system.

Commercial food product	Percentage of Brazil nut declared on the label	BNS (Cp) ^a	PAC (Cp) ^b	Estimated Brazil nut percentage
Chocolate-1	Brazil nuts (15%)	24.27 ± 0.04	13.24 ± 0.09	9.052
Chocolate-2	Brazil nuts	26.67 ± 0.06	13.36 ± 0.07	1.889
Food-bar-1	Brazil nuts	26.99 ± 0.09	15.19 ± 0.06	1.533
Food bar-2	Brazil nuts	30.12 ± 0.08	13.80 ± 0.03	0.199
Food bar-3	Brazil nuts	25.20 ± 0.30	14.12 ± 0.08	4.932
Food bar-4	Brazil nuts	29.33 ± 0.12	15.46 ± 0.04	0.333
Food bar-5	Brazil nuts (8%)	26.06 ± 0.01	15.07 ± 0.05	2.813
Food bar-6	Brazil nuts (6.2%)	23.78 ± 0.02	16.19 ± 0.02	12.465
Muesli-1	Brazil nuts (2.8%)	30.22 ± 0.03	14.10 ± 0.03	0.186
Breakfast cereals-1	Brazil nuts (3.3%)	26.94 ± 0.03	14.75 ± 0.07	1.584
Breakfast cereals-2	Nuts (6.5%) (Brazil nuts, pecan nuts)	24.93 ± 0.04	13.30 ± 0.05	5.883
Breakfast cereals-3	Brazil nuts (3.5%)	29.74 ± 0.04	16.72 ± 0.04	0.254
Breakfast cereals-4	Nuts (4%) (Brazil nuts, pecan nuts)	29.98 ± 0.01	13.46 ± 0.01	0.218
Milled Flaxseed-1	Brazil nuts (10%)	25.02 ± 0.01	14.02 ± 0.02	5.547
Biscuits-1	Brazil nuts (18%)	26.57 ± 0.09	14.40 ± 0.01	2.016
Commercial food product	Traces of nuts declared on the label	BNS (Cp)	PAC (Cp)	Estimated Brazil nut percentage
Chocolate-8	Traces of Brazil nuts	34.88 ± 0.06	15.34 ± 0.07	0.009
Breakfast cereals-10	May contain traces of nuts	35.34 ± 0.04	13.75 ± 0.06	0.007
Food bar-7	May contain traces of nuts	36.47 ± 0.03	15.33 ± 0.01	0.003
Food-bar-8	May contain traces of nuts	35.84 ± 0.09	16.37 ± 0.07	0.005

^a BNS: Brazil nut specific system on the *Ber e 1* gene. Average Cp value ± standard deviation of triplicate analysis.^b PAC: Positive amplification control on the 18S rRNA gene.

of chocolates that declared to contain Brazil nut corresponds to a fraudulent substitution by cheaper nuts. Nevertheless, the lack of positive results in products labeled as 'may contain traces of Brazil nut' could be explained because its content was below LOD, or because Brazil nut-containing products are manufactured at the same factory and the company prefers to alert about a possible contamination in order to safeguard allergic consumers.

In an attempt to estimate Brazil nut content, Cp values obtained from commercial food products which yielded positive results in the BNS were interpolated in the linear regression equation obtained from binary mixtures of wheat flour and roasted Brazil nut. Table 4 shows the estimated Brazil nut percentages of the commercial products after substitution of the Cp values in the regression equation. According to the results obtained, the estimated Brazil nut content was lower than that stated in the labels for most products, except for Food bar-6 and Breakfast cereals-2. It is important to note that food processing encompass different time–temperature combinations, and also that a wide number of ingredients are employed. Even though some approaches have been recently purposed to compensate for the variability in DNA extraction and amplification in food materials (Demmel, Hupfer, Busch, & Engel, 2012; Hirao, Hiramoto, Imai, & Kato, 2006), quantification of Brazil nut in commercial products is affected by the heat treatments and food matrices analyzed. Thus, accurate quantification would require different standard curves for different products, according to their composition and processing conditions.

4. Conclusions

The real-time PCR system developed is highly sensitive and specific, which makes it particularly suitable for detection of small amounts of Brazil nut DNA in food products, with a LOD of 2.5 mg/kg. False negative results are avoided by the use of a positive amplification control based on the 18S rRNA gene. In addition to experimental mixtures, the method has proven to be appropriate for analysis of a wide range of processed commercial food products, including breakfast cereals, cakes, chocolates, and energetic bars, that may contain Brazil nut as an ingredient or contaminant. The results obtained were found to confirm the product labels in most cases. However, some samples which declared Brazil nut as an ingredient yielded a negative result. Due to the high economic value of Brazil nut, fraudulent substitution with a cheaper ingredient is the most probable reason for the negative results in these products. The method is rapid and could be performed as a routine analysis to enforce compliance with food labeling legislation, in order to protect allergic consumers against Brazil nut's allergens, and also to avoid fraudulent replacement of Brazil nut by more economic ingredients.

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