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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 thread 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.

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, Cléroux, 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-Biofarm®, 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 (Brezná, Dudásová, & Kuchta, 2010; Hubalkova & Rencova, 2011; Röder, Filbert, & Holzhauser, 2010), but only that of Röder 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	*Ber e 1* BNS[a]	18S rRNA PAC[b]
Brazil nut	*Bertholletia excelsa*	20.50 ± 0.08[c]	13.90 ± 0.02
Almond	*Prunus dulcis*	–[d]	13.11 ± 0.02
Cashew nut	*Anacardium occidentale*	–	13.86 ± 0.07
Chestnut	*Aesculus hippocastanum*	–	15.88 ± 0.02
Hazelnut	*Corylus avellana*	–	11.37 ± 0.01
Macadamia	*Macadamia integrifolia*	–	12.97 ± 0.04
Peanut	*Arachis hypogaea*	–	14.56 ± 0.04
Pecan nut	*Carya illinoinensis*	–	13.43 ± 0.02
Pine nut	*Pinus pinea*	–	15.11 ± 0.06
Pistachio	*Pistacia vera*	–	13.38 ± 0.01
Walnut	*Juglans regia*	–	11.30 ± 0.01
Barley	*Hordeum vulgare*	–	15.02 ± 0.04
Chocolate	*Theobroma cacao*	–	16.41 ± 0.04
Chufa sedge	*Cyperus esculentus*	–	14.79 ± 0.03
Lupine	*Lupinus albus*	–	16.52 ± 0.06
Maize	*Zea mays*	–	16.38 ± 0.03
Mung bean	*Vigna radiata*	–	15.78 ± 0.01
Oats	*Avena sativa*	–	14.32 ± 0.06
Olive	*Olea europaea*	–	14.01 ± 0.03
Orange	*Citrus sinesis*	–	14.23 ± 0.02
Potato	*Solanum tuberosum*	–	15.05 ± 0.05
Rice	*Oryza sativa*	–	13.87 ± 0.01
Rye	*Secale cereale*	–	14.02 ± 0.02
Sesame	*Sesamum indicum*	–	16.26 ± 0.07
Soybean	*Glycine max*	–	14.12 ± 0.02
Sunflower	*Helianthus annuus*	–	15.44 ± 0.01
Wheat	*Triticum aestivum*	–	15.72 ± 0.06
Cattle	*Bos taurus*	–	16.17 ± 0.01
Goat	*Capra hircus*	–	15.02 ± 0.04
Sheep	*Ovis aries*	–	13.98 ± 0.00
Swine	*Sus scrofa domestica*	–	14.34 ± 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 ± SD obtained from triplicate PCR reactions from each DNA extraction.
[d] Minus sign indicates no positive signal after 50 PCR cycles.