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Development of real-time PCR assays to detect cashew (*Anacardium occidentale)* and macadamia (*Macadamia intergrifolia)* residues in market analysis of processed food products

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ABSTRACT

Real-time polymerase chain reaction (PCR)-based assays for detection of cashew (*Anacardium occidentale)* and macadamia nut (*Macadamia intergrifolia)* traces in food products are described here. The real time PCR technique proposed herein were developed based on the design of macadamia and cashew-specific primers from the *ITS* region and a TaqMan fluorescent probe. The methods were positive for cashew and macadamia respectively, and negative for all other heterologous plant and animal species tested. Using a series of model samples with defined levels of raw and heat-treated cashew and macadamia nut, respectively, within a range of concentrations of 0.1–100 000 mg kg^{-1}, practical detection limits of 0.1 mg kg^{-1} for cashew and macadamia nut were estimated. Practical applicability of the PCR methods was tested by the analysis of a total of 214 commercial foodstuffs. These PCR methods, are useful for highly selective, and sensitive detection of traces of macadamia and cashew nuts in commercial food products and is therefore proposed as a ready-to-use analytical tool to trace these target allergens in foods.

1. Introduction

Macadamia nuts of commercial importance are shelled kernels of the fruits of two species, namely, *Macadamia integrifolia* and *Macadamia tetraphylla* or their hybrids which are currently grown in various countries; Australia and USA (Hawaii) are the main producers of this crop (USDA, 2010). On the other hand, cashew (*Anacardium occidentale*) nuts are widely consumed ranking third in the international tree nut trade with over 20% of the market (Wei, Sathe, Teuber, & Roux, 2003). Nigeria is the world's largest producer of cashew nuts followed by Vietnam and India. Consumption of cashew nuts as a snack or an ingredient in diverse foods is a very popular around the world and associated allergies are reported globally (Wang et al., 2002). Macadamia allergies are less prevalent than cashew allergies, but the symptoms can be severe, including life-threatening anaphylaxis (Lerch, Egger, & Bircher, 2005). In response to this fact, European legislation (EU) 1169/2011/EC (OJEU, 2011) included these nuts in the list of components, which are required to be declared on the label of food products.

Although, due to their high price, it is not probable that undeclared macadamia and cashew nuts will be present in food products as a result of fraudulent substitution, adventitious contamination may take place when various nuts are used in the same food processing facility, particularly on shared equipment. Moreover, no cure for cashew and macadamia allergy is yet available and therefore ingestion has to be avoided by the sensitive individual. Problems may thus arise if the presence of the allergen is not discernible due to mislabeling of the products or because of unknown cross contact that may occur during industrial food processing. To protect allergic consumers, sensitive methods are needed to detect and quantify such hidden allergens in complex processed food matrixes to allow more accurate labeling of commercial food products or to facilitate quality control efforts to avoid product cross contact.

In this way, appropriate analytical methods are necessary to allow the specific and sensitive detection of macadamia and cashew nuts in food products to assure the surveillance of labeling requirements by the responsible authorities. Mainly two

Abbreviations: CSS, cashew specific system; MSS, macadamia specific system; PAC, positive amplification control; FAM, carboxyfluorescein; BHQ, black hole quencher; LOD, limit of detection.

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techniques, protein-based immunoassays and DNA-based polymerase chain reaction, are currently used for the analysis of allergenic food. Currently, immunoassays have been developed for the detection of cashew nuts in food products (Gaskin & Taylor, 2011; Rejeb, Abbott, Davies, Cléroux, & Delahaut, 2005; Wei, Sathe, Teuber, & Roux, 2003). In the case of macadamia nuts, the development of immunoassays is not yet reported except for the recent existence of commercial allergy-testing kits (*Biofront*; *R-Biopharm*; *Romer, Genon Laboratories* etc …), which are available for macadmia and for cashew detection. These kits can either be based in Lateral Flow Device (LFD) or immunological (ELISA) based kits. Such as *Ribda®Quick from R-Biopharm*, Inc. (Washington) which has Lateral Flow Device Test Kits available for the detection of macadamia and cashew nut detection with a 1 mg kg^{-1} LOD of depending on matrix. Also, *Romer labs* offer agrastrip LFD for the detection of macadamia and cashew nut with a LOD of 2 and 5 mg kg^{-1} respectively depending on the matrices in which the allergen is present. While *Bioavid diagnostics* based in LFD reach a LOD of up to 1 mg kg^{-1} in macadamia depending in the food matrix. On the other hand, Biadiagnostics developed commercially available ELSIA kits for the detection of cashew and macadamia nuts with a LOD of 0.6 and 7.4 mg kg^{-1} respectively. Also, BioFront technologies based in the use of ELISA kits allowed the detection of cashew nuts with a LOD of 0.2 mg kg^{-1}". While *Biopharm labs*, detects macadamia with a LOD of 1 mg kg^{-1} using a sandwich enzyme immunoassay. These immunoassays although highly sensitive, present numerous problems mainly due to the cross-reactivity with non target proteins and the low resistance of proteins to food processing during thermal processing, because it can cause conformational changes in the tridimensional structure of the epitopes (e.g., heat induced denaturation) and/or protein cleavage, affecting linear epitopes (e.g., fermentation).

On the other hand, DNA-based methods have been increasingly used as highly sensitive and specific alternatives for the detection of residues of allergenic foods taking. In this way, real-time PCR technology has proved to be very effective for the detection of allergenic foods (López-Calleja et al., 2013; Pegels et al., 2011). Several real-time PCR approaches have been proposed to detect cashew nuts (Brzezinski, 2006; Ehlert, Hupfer, Demmel, Engel, & Busch, 2008; Koppel, Velsen-Zimmerli, & Bucher, 2012; Piknova & Kuchta, 2007). However, currently only one publication has been reported so far for macadamia detection (Brezna, Piknova, & Kuchta, 2009).

Here we present specific, sensitive and quantitative methods for the detection of traces of cashew nut and macadamia DNA, by real-time PCR in different food matrixes.

Table 1
Specificity of cashew and macadamia real-time PCR system.

Common name	Scientific name	*ITS* C.S.S./M.S.S[a]	*18S r*RNA P.A.C.[a]
Cashew nut	*Anacardium occidentale*	13.97 ± 0.01	15.23 ± 0.04
Macadamia	*Macadamia intergrifolia*	12.34 ± 0.01	16.17 ± 0.01
Almond	*Prunus dulcis*	–	14.79 ± 0.03
Hazelnut	*Corylus avellana*	–	14.53 ± 0.02
Peanut	*Arachis hypogaea*	–	16.61 ± 0.01
Walnut	*Juglans regia*	–	15.02 ± 0.04
Pistachio	*Pistacia vera*	–	14.15 ± 0.06
Brazil nut	*Bertholletia excelsa*	–	16.13 ± 0.01
Pecan	*Carya illinoinensis*	–	16.20 ± 0.01
Soybean	*Glycine max*	–	16.52 ± 0.06
Green bean	*Phaseolus vulgaris*	–	16.01 ± 0.02
Green pea	*Pisum sativum*	–	15.01 ± 0.01
Chickpea	*Cicer arietinum*	–	14.87 ± 0.01
Lentil	*Lens culinaris*	–	15.21 ± 0.02
Tiger Nut	*Cyperus esculentum*	–	15.46 ± 0.04
Lupine	*Lupinus albus*	–	15.48 ± 0.03
Acorn	*Quercus ilex*	–	14.72 ± 0.02
Chestnut	*Aesculus hippocastanum*	–	16.38 ± 0.03
Sesame	*Sesamum indicum*	–	15.78 ± 0.01
Pine nut	*Pinus pinea*	–	16.34 ± 0.01
Barley	*Hordeum vulgare*	–	14.32 ± 0.06
Oat	*Avena sativa*	–	14.01 ± 0.03
Rye	*Secale cereale*	–	14.23 ± 0.02
Rice	*Oryza sativa*	–	14.56 ± 0.04
Sunflower	*Helianthus annuus*	–	14.11 ± 0.02
Maize	*Zea mays*	–	15.11 ± 0.06
Wheat	*Tritucum aestivum*	–	13.55 ± 0.01
Cocoa	*Theobroma cacao*	–	15.05 ± 0.05
Grape	*vitis vinifera*	–	15.04 ± 0.01
Pear	*Pryus rosaceae*	–	14.81 ± 0.01
Apple	*Malus domestica*	–	14.43 ± 0.01
Peach	*prunus persica*	–	15.03 ± 0.04
Plum	*prunus domestica*	–	16.02 ± 0.02
Banana	*Musa cavendishii*	–	15.87 ± 0.01
Kiwifruit	*Actinidia deliciosa*	–	13.98 ± 0.01
Watermelon	*Citrullus lanatus*	–	14.67 ± 0.02
Orange	*Citrus Sinesis*	–	13.87 ± 0.01
Potato	*Solanum tuberosum*	–	14.02 ± 0.02
Garlic	*Allium sativum*	–	15.09 ± 0.01
Onion	*Allium cepa*	–	14.73 ± 0.02
Eggplant	*solanum melongena*	–	15.21 ± 0.01
Zucchini	*Cucurbita pepo*	–	16.34 ± 0.03
Tomatoe	*Solanum lycopersicum*	–	13.92 ± 0.01
Asparagus	*Asparagus officinalis*	–	14.87 ± 0.02
Carrot	*Daucus sativus*	–	15.45 ± 0.02
Olive	*Olea europaea*	–	16.26 ± 0.07
Cattle	*Bos taurus*	–	13.98 ± 0.00
Sheep	*Ovis aries*	–	14.12 ± 0.02
Goat	*Capra hircus*	–	15.44 ± 0.01
Swine	*Sus scrofa domestica*	–	14.34 ± 0.07

***ITS* C.S.S./M.S.S.**: Cashew and macadamia-specific system on the *Internal Transcribed Spacer* (*CashewITSdir & CashewITSinv/MacITSdir & MacITSinv*).
***18S* rRNA P.A.C**: Positive amplification control (*18Sdir*/*18Sinv* and *18SP*) for eukaryotics on the *18S* rRNA gene.
[a] Average Crossing Point Value (Cp) value ± SD shown from triplicate PCR reactions from each DNA extraction. Minus sign indicates no positive signal after 50 PCR cycles.

2. Materials and methods

2.1. Sample selection

Cashews, macadamias, other tree nuts, peanuts and various commercial brands of food were purchased from different local stores and several delicatessen markets and stored at room temperature in the dark. Commercial cashews from Vietnam, Nigeria and India and macadamias from Australia, New Zealand and Guatemala were considered for analysis. Moreover, a wide range of plant (flesh and kernels) and animal (flesh) species was also included in the assays to assess specificity control purposes (Table 1).

Cashews (India) were finely ground and two separate series of binary mixtures: raw in wheat flour and heat treated cashew (160 °C for 13 min) in wheat flour containing 0.1, 1, 10, 100, 1000, 10 000 and 100 000 mg kg^{-1} of cashews were prepared for each series, to a final weight of 500 g using a kitchen robot (Thermomix, Vorwerk, Wuppertal, Germany). Wheat flour samples containing 100 000 mg kg^{-1} of cashew were prepared by diluting 50 g of ground cashew either raw or heat treated with 450 g of wheat flour by using the Thermomix at maximum speed during 5 min ten-fold dilutions were repeated until 0.1 mg kg^{-1} samples were obtained. Macadamia binary mixtures: raw macadamia (Australia) in wheat flour and heat treated in wheat flour, were elaborated under identical conditions (160 °C for 13 min) as described before.

2.2. DNA extraction

Two hundred milligrams of each sample were homogenized with 860 μL of extraction buffer, pH 8.0 (0.01 mol/L Tris, 0.15 mol/L,