Food Control 89 (2018) 227–234

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Evaluation of locked nucleic acid and TaqMan probes for specific detection of cashew nut in processed food by real time PCR

África Sanchiz [a], Isabel Ballesteros [b], Eric Marqués [c], M. Carmen Dieguez [d], Julia Rueda [c], Carmen Cuadrado [a, *, 1], Rosario Linacero [c, 1]

[a] Departamento de Tecnología de Alimentos, SGIT-INIA, Ctra. Coruña km 7.5, 28040 Madrid, Spain
[b] Ingeniería en Biotecnología, Facultad de Ingenierías y Ciencias Aplicadas, Universidad de las Américas, Quito, Ecuador
[c] Departamento de Genética, Fisiología y Microbiología, Facultad de Biología, Universidad Complutense de Madrid, 28040, Madrid, Spain
[d] Servicio de Alergia, Instituto de Investigación Hospital 12 de Octubre (i+12), Avenida de Córdoba s/n, 28041 Madrid, Spain

ARTICLE INFO

Article history:
Received 6 December 2017
Received in revised form
14 February 2018
Accepted 15 February 2018
Available online 16 February 2018

Keywords:
Anacardium occidentale
Allergen detection
Real time PCR
Moist thermal processing
Processed foods

ABSTRACT

Cashew (*Anacardium occidentale*) nut can trigger serious reactions in allergic patients, including anaphylaxis and death. Labelling the presence of cashew nuts in food products is mandatory and consequently, sensitive and specific analytical methods must be developed. In this study, Ana o allergen coding sequences have been sequenced in several cashew varieties. Two hydrolysis probes, locked nucleic acid (LNA) and TaqMan, have been designed and their efficiency, sensitivity, limit of detection and specificity for Ana o 1 coding-sequence detection have been compared. Reliable Real Time PCR assays to detect and quantify up to 10 ppm of cashew nuts in complex mixtures have been developed. Moreover, the influence of boiling and autoclave treatment on cashew nut detectability has been analysed by qPCR, showing both probes similar performance. This analytical method was able to detect up to 1000 ppm with good functionality in autoclave treated samples. Boiling did not affect cashew nut detectability. Both hydrolysis probes are suitable for Ana o 1 coding sequence detection. Applicability of the assay has been studied by analysing several food products, and comparing the results with those of a commercial ELISA kit.

1. Introduction

Tree nuts are valuable foods rich in proteins, minerals, vitamins, antioxidants and a considerable high content of unsaturated fatty acids (Ros, 2010), and their global production and consumption is increasing in the last years. Tree nuts allergy has also increased, although prevalence varies among individual nut and regions and it is difficult to stablish. In Europe, even though the most common allergies to tree nuts correspond to hazelnut and walnut, cashew nut allergy is getting importance over the last years according to several reports, with a variable prevalence among countries (Mendes, Costa, Vicente, Oliveira, & Mafra, 2016). Other studies indicate that allergies to walnut and cashew nuts are the most prevalent among tree nuts in the USA, achieving 20–30% and 15–30%, respectively (McWilliam et al., 2015). Originally from Brazil, cashew tree is cultivated in Africa and Asia, and mainly produced in India, and its fruits, cashews, are appreciated worldwide (Mendes et al., 2016). Cashew nuts are commonly consumed as snack or as ingredient in biscuits, sauces, sweets and several food dishes, and medical efficiency of cashew nuts has also been recently studied (Mah et al., 2017). Until the date, three major allergens have been identified, all of them being seed storage proteins (WHO-IUIS Allergen Nomenclature Sub-Comittee): Ana o 1, a 7S vicilin (Wang et al., 2002), Ana o 2, a 11S legumin (Wang, Robotham, Teuber, Sathe, & Roux, 2003), and Ana o 3, a 2S albumin (Robotham et al., 2005).

In order to protect the safety of the allergic patients, European regulation obliges to advise the presence of tree nuts in food labels (Official Journal of the European Union (22/11/2011), 2011 Regulation (EU) No 1169/2011). The presence of allergenic ingredients in foods can be either a consequence of fraudulent substitution or adventitious contamination in the food facility. Last option is more likely for cashew nuts because of its high price. There is no treatment for food allergies, and sensitized individuals have to totally

* Corresponding author.
E-mail address: cuadrado@inia.es (C. Cuadrado).
[1] Equal contribution.

https://doi.org/10.1016/j.foodcont.2018.02.021
0956-7135/

avoid the consumption of offending ingredients. Therefore, a reliable and specific tool for detecting traces of specific food allergens is indeed essential to improve the quality of life of sensitized individuals. Enzyme-linked immunosorbent assay (ELISA) is the common method for detecting small amounts of proteins from specific foods, as cashew nuts, and it is possible to find several ELISA tests on the market. One of the disadvantages of this technique is the possible effect of food processing on protein solubility, because the subsequent detection with protein-based techniques might be altered (Mattison et al., 2016). In the last years, DNA-based methodologies, such as Real Time PCR, microarrays and also DNA biosensors, have been proposed as specific, sensitive and reliable alternatives to ELISA (Sun, Guan, Shan, Zhang, & Li, 2012), since DNA molecules can preserve its integrity better than proteins. Several studies have been carried out to develop Real Time PCR methods for detecting allergens in foods (Costa, Fernandes, Villa, Oliveira, & Mafra, 2017; Prado et al., 2016), cashew nuts included (López-Calleja, Cruz, González, García, & Martín, 2015a; Piknová & Kuchta, 2007), with different approaches and chemistries, and obtaining promising results.

The selection of the specific target and chemistry are essential parts of a new Real Time PCR experiment, because specificity, sensitivity and potential of quantification of the method directly depend on them. Some researchers have used multi copy genes such as mitochondrial, chloroplast or repetitive sequences as Internal Transcriber Sequences (ITS), to detect an allergenic ingredient in food (Demmel, Hupfer, Hampe, Busch, & Engel, 2008; Lopez-Calleja et al., 2013), while others have published detection methods using allergen coding sequences as targets, e.g. Ara h 2 in peanut (Hird, Lloyd, Goodier, Brown, & Reece, 2003), Cor a 9, 11 and 13 in hazelnut (Iniesto et al., 2013), Jug r 1 and 3 in walnut (Linacero et al., 2016), Pis v 1 (Sanchiz et al., 2017) or Ana o 3 in cashew nuts (Brzezinski, 2006; Piknová & Kuchta, 2007), among others.

On the other hand, Real Time PCR assay can be performed using several chemistries commercially available: intercalating dyes, primer-based or probe-based chemistries. However, very few studies have been published addressing a practical comparison among different chemistries for application to the detection of GMO or allergen traces (Buh Gašparič et al., 2010).

Locked Nucleic Acid (LNA) and classical Taqman probes are both hydrolysis probes, also called 5′-nuclease probes, commonly located between the PCR primers as fluorescence oligonucleotides. Thus, high specificity can be achieved compared to other alternatives such as intercalating dyes. LNA are modified DNA analogues primers with high melting temperature that allows them to be shorter and highly specific when compared to classical TaqMan probes. Both probes carry a fluorophore attached to one extreme and a quencher attached to the other; when they are physically close, the quencher prevents fluorescence. Once the probe is cleaved by the DNA polymerase, the reporter emits fluorescence (Valasek & Repa, 2005).

Tree nuts, as other foods, usually undergo thermal treatment to improve organoleptic properties, ensure food safety and sometimes modify allergenic properties. Recently, the influence of enzymatic hydrolysis and thermal treatment on cashew nut allergens have been studied (Cuadrado et al., 2018; Sanchiz et al., 2018). Fragmentation and/or degradation of DNA molecules have been reported by several authors after severe treatments (Costa, Oliveira, & Mafra, 2013; Gryson, 2010; López-Andreo, Aldeguer, Guillén, Gabaldón, & Puyet, 2012) and some studies have been performed in order to analyse the effect of processing, such as thermal treatment with and without applying pressure (boiling, High Hydrostatic Pressure HHP, autoclave, frying, roasting), on the detection of different DNA targets, in peanut, hazelnut, walnut, almond and pistachio (Iniesto et al., 2013; Linacero et al., 2016; Prieto et al., 2014; Sanchiz et al., 2017; Scaravelli, Brohée, Marchelli, & Van Hengel, 2009).

The present study is aimed to set up a reliable and suitable real time PCR-based detection assay for Ana o 1 allergen coding sequence from cashew nuts using two different hydrolysis probes, Locked Nucleic Acid and classical TaqMan, which have been compared regarding efficiency, sensitivity, specificity and applicability in several commercial food products. Comparison with a commercial ELISA kit has also been performed. We particularly focused the analysis on the influence of six different treatments, based on boiling and autoclave, on the detectability of cashew nut target Ana o 1 by Real Time PCR.

2. Materials and methods

2.1. Samples

Five varieties of raw *Anacardium occidentale* L. (CCP006, 1001, BRS189, BRS274, Embrapa 50) nuts were analysed in this work, provided by Germplasm Bank of Embrapa, Brazil. A commercial ready-to-eat variety of cashew nut from India (type 320) was provided by Productos Manzanares S.L. (Cuenca, Spain). Other plant species commonly found as food ingredients were also used as well as food products which were purchased in local stores, ground with a kitchen robot (Thermomix 31-1, Vorwerk Elektrowerke, GmbH & Co. KG, Wüppertal, Germany) and stored at −20 °C.

Several points of binary mixtures (0.5, 1, 5, 10, 100, 1000, 10000, 100000 mg/kg) were performed by mixing known amounts of defatted cashew nut flour in spelt wheat (*Triticum spelta* L.) flour, in a final weight of 100 g. The mixture containing 10% cashew nuts (100.000 mg/kg) was prepared by adding 10 g of cashew nut flour to 90 g of spelt wheat flour, and followed by 10 or 5-fold dilutions to 0.5 mg/kg, all of them being homogenized with the kitchen robot.

2.2. Thermal and pressure treatments

One hundred grams of cashew nuts type 320 were boiled in distilled water (1:5 w/v) for 30 or 60 min, or autoclaved using a Compact 40 Benchtop autoclave (Priorclave, London, UK) at 121 °C (120 kPa) or at 138 °C (256 kPa) for 15 or 30 min. Untreated and treated cashew nuts were freeze-dried (Telstar Cryodos frezze-dryer), ground using a Cyclotec 1093 Sample Mill with a sieve of 1 mm, defatted with n-hexane for 4 h (34 ml/g of flour) and air-dried. Binary mixtures in spelt wheat flour were performed as described before with defatted treated cashew nut flours, containing 10, 100, 1000, 10000 and to 100000 mg/kg (0.01%–10% cashew nuts). All samples were stored at 4 °C.

2.3. DNA extraction

One hundred milligrams of cashew nuts from the six varieties were extracted using i-Genomic Plant DNA Extraction Mini Kit (iNtRON biotechnology), following the manufacturer instructions. At the same time, 100 mg of each binary mixture were homogenized in 1000 μl of Cetyl trimethylammonium bromide (CTAB) with 1% of polyvinylpyrrolidone (PVP) and 3 μl of 25 mg/ml RNAse, and were incubated at 65 °C for 30 min, shaking every 10 min. Then, 800 μl of chloroform were added to the sample before centrifugation at 13000 rpm for 10 min. Genomic DNA was obtained from 800 μl of aqueous supernatant, which were processed with the Power Plant DNA isolation kit (MoBio, CA USA) and finally eluted in 100 μL of Tris HCl pH 8.3.

Two hundred milligrams of each food sample were homogenized in liquid nitrogen and genomic DNA was obtained using the NucleoSpin kit (Macherey-Nagel, Düren, Germany) following the