Detection of cashew nuts in food by real-time polymerase chain reaction

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Summary
A real-time PCR-based method for the detection of cashew (*Anacardium occidentale* L.) nuts in confectionery products is described. The method consists of DNA isolation by chaotropic solid phase extraction and the subsequent PCR with cashew-specific primers and a TaqMan fluorescent probe. The method was positive for 10 samples of cashew nuts available on the market and negative for all other tested plant materials used in food industry including almonds, brazil nuts, chestnuts, hazelnuts, peanuts, pistachio nuts and walnuts. The intrinsic detection limit of the method was 1.25 pg DNA, which corresponds to approx. 2.5 genome equivalents (1C). Using a series of model pastry samples with defined cashew nut contents, a practical detection limit of 0.01% (w/w) was determined. Practical applicability of the PCR method was tested by the analysis of 22 confectionery products. For all but one of the food samples, results obtained conformed to the labelling. The one incorrectly labelled food product was Macadamia nougat containing approx. 0.040% (w/w) of cashew nuts, which suggests that it had been contaminated rather than adulterated. The presented real-time PCR method is useful for sensitive and selective detection of cashew nuts in food samples.

Keywords
cashew; allergen; polymerase chain reaction; confectionery

Cashew (*Anacardium occidentale* L.) nuts belong to widely consumed tree nuts. Besides eaten directly, raw or roasted, they are used in a range of confectionery products like filled chocolates or wafers, nougats, in cereal müsli mixtures, and also in other food products. However, cashew nuts may be a threat to some consumers since they contain allergens and consumption of food products containing cashew nuts may cause severe allergy in a part of population [1, 2]. In response to this fact, European legislation requires labelling of food products in respect to the contents of cashew nuts [3]. Although cashew nuts are relatively expensive, they may be present undeclared in food products either as a subsitute for even more expensive nuts, or low-quality cashew nuts may be a component of nut pastes. Methods for analysis of cashew nuts in food are therefore necessary.

A method based on sandwich ELISA, which is the most sensitive technique used in the detection of tree nuts in food products, has been described [4], but it is not available commercially. An alternative method based on polymerase chain reaction (PCR) targeting the intergenic spacer region of the 5S rRNA gene has been developed [5] as well as a more sensitive real-time PCR which targets the gene encoding for the 2S albumin gene [6].

In this study, we used the latter real-time PCR primers and the 5‘-nuclease (TaqMan) probe, determined the analytical parameters and demonstrated the application of the method for analysis of confectionery products from the market in Central Europe.

MATERIALS AND METHODS

Plant and food materials

Nuts and various confectionery products were obtained from grocery and delicatessen shops in Slovakia, Austria and Czech Republic.

Preparation of model samples

Pistachio nougat (Sally Williams, Sandton, South Africa) was homogenized in a mortar with a pestle. Cashew nuts (raw; Marianna, Ivanka pri

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Tab. 1. Results of real-time PCR with various plant or food samples.

Sample	Country of origin	PCR result
Cashew nuts natural 1	India	+
Cashew nuts natural 2	India	+
Cashew nuts natural 3	Vietnam	+
Cashew nuts, roasted, salted 1	India	+
Cashew nuts, roasted, salted 2	India	+
Cashew nuts, roasted, salted 3	India	+
Cashew nuts, roasted, salted 4	India	+
Cashew nuts, roasted, salted 5	India	+
Cashew nuts, roasted, salted 6	Vietnam	+
Cashew nuts, roasted, salted 7	Nigeria	+
Almonds	Spain	–
Brazil nuts	Bolivia	–
Chestnuts	Slovakia	–
Hazelnuts	Georgia	–
Peanuts	China	–
Pistachio nuts	Iran	–
Walnuts	Slovakia	–
Sunflower seeds	Slovakia	–
Wheat flour	Slovakia	–
Maize flour	Germany	–
Soya flour	Slovakia	–
Oat flakes	Slovakia	–
Rice	Vietnam	–
Mango pulp	Brasil	–
Cocoa powder	the Netherlands	–
Milk powder	Slovakia	–
Chocolate	Slovakia	–

Tab. 2. Results of real-time PCR with food products.

Food product	Country of origin	Cashew nut declaration	PCR result
Cashew snack	Greece	+	+
Cashew nougat	South Africa	+	+
Cashews in chocolate	Germany	+	+
Corn flakes 1	Austria	+	+
Corn flakes 2	Austria	–	–
Fruits and nuts snack 1	Germany	+	+
Fruits and nuts snack 2	Germany	–	–
Fruits and nuts snack 3	Germany	+	+
Fruits and nuts snack 4	Germany	–	–
Müsli 1	Austria	+	+
Müsli 2	Austria	–	–
Pistachio nougat	France	–	–
Macadamia nougat	South Africa	–	+[a]
Macadamia nuts in chocolate	Germany	–	–
Biscuits with nut filling 1	Slovakia	–	–
Biscuits with nut filling 2	Slovakia	–	–
Chocolate with nut filling 1	Czech Republic	–	–
Chocolate with nut filling 2	Czech Republic	–	–
Chocolate with nut filling 3	Czech Republic	–	–
Chocolate with nut filling 4	Slovakia	–	–
Chocolate with nougat filling	Belgium	–	–
Almond snack	Greece	–	–

a - content of cashew nuts was 0.040% (w/w).

Dunaji, Slovakia) were milled in a coffee mill and mixtures containing 0; 0.01; 0.02; 0.05; 0.1; 0.2; 0.5; 1; 2; 5 and 10% (w/w) cashew nuts were prepared. The size of each model sample was 5 g.

DNA extraction, quantification and amplifiability determination

Food samples were homogenized using mortar and pestle, and DNA was isolated by chaotropic solid-phase extraction using the NucleoSpin kit (Macherey-Nagel, Düren, Germany). DNA was quantified by fluorimetry using Quant-iT PicoGreen kit (Invitrogen Molecular Probes, Eugene, Oregon, USA) using λ-phage DNA for the construction of the calibration line. Amplifiability of the isolated DNA was determined using universal eukaryotic real-time PCR [7].

Polymerase chain reaction

PCR was performed in a volume of 25 µl. Each reaction contained 5 µl of template DNA, 1.5 U DNA polymerase HotStarTaq Plus (Qiagen, Hilden, Germany), reaction buffer for the DNA polymerase, 2.5 mmol.l^{-1} $MgCl_2$, 200 µmol.l^{-1} dNTP mixture (Applied Biosystems, Foster City, California, USA), 150 nmol.l^{-1} of the primer cashF (tgccaggagttgcaggaagt), 150 nmol.l^{-1} of the primer cashR (gctgcctcaccatttgctcta) and 200 nmol.l^{-1} of the probe cashP (acagaaggtgccgctgccagaa) labelled with FAM (6-carboxyfluorescein) and quenched with TAMRA (tetratmethylrhodamine) [6]. Oligonucleotides were synthesized by Qiagen Operon, Köln, Germany. PCR was performed in a PTC-200 PCR cycler with an optical unit Chromo 4 (MJ Research, Waltham, Massachusetts, USA). The PCR programme consisted of the initial denaturation at 95 °C for 5 min followed by 60 cycles of denaturation at 95 °C for 15 s and annealing with polymerization at 60 °C for 60 s. Fluorescence was measured in FAM channel. The threshold cycle c_T was calculated for individual samples by the internal software of the cycler using the manual threshold setting at a fluorescence value of 0.025. To construct a calibration line, averaged threshold cycle values were plotted against the decadic logarithm of concentrations and parameters were calculated using Prism 4 software (GraphPad Software, San Diego, California, USA).

RESULTS AND DISCUSSION

Real-time PCR was used to analyse DNA isolated from 10 samples of cashew nuts and 17 other plant or food samples. Positive results were obtained for all samples of cashew nuts and negative results were obtained for all other samples (Tab. 1). This means that the inclusivity of the method was 100% and the exclusivity was 100%.

When dilutions of DNA isolated from cashew nuts (raw, Marianna) were analysed by real-time PCR in six parallels, a linear calibration curve was constructed and a detection limit of 1.25 pg was determined (data not shown). This corresponds to approx. 2.5 genome equivalents, given the genome size (2C) of *Anacardium occidentale* is 1.02 pg [8].

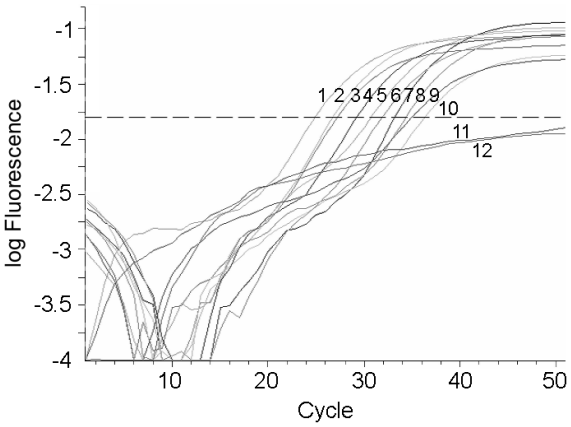

Fig. 1. A record of the cashew-specific real-time PCR analysis.
Model samples contained 10% (w/w, curve 1), 5% (w/w, curve 2), 2% (w/w, curve 3), 1% (w/w, curve 4), 0.5% (w/w, curve 5), 0.2% (w/w, curve 6), 0.1% (w/w, curve 7), 0.05% (w/w, curve 8), 0.02% (w/w, curve 9), 0.01% (w/w, curve 10) and 0% (w/w, curve 11) of the cashew nut component. Curve 12 is the negative control with no template DNA. The template DNA amount was adjusted to 100 ng per reaction.

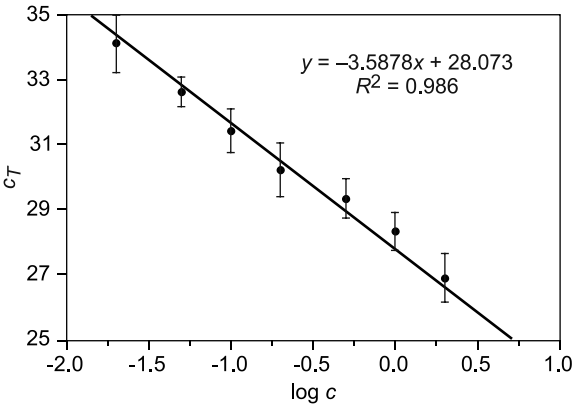

Fig. 2. Relationship between the cashew nut contents in the model samples and the threshold cycle c_T in real-time PCR.
Average values of six parallels ± standard deviation are shown. The template DNA amount was adjusted to 100 ng per reaction.

At the analysis of model samples with defined cashew nut contents, a linear relationship between the logarithm of the cashew nut content and the threshold cycle, c_T was observed and a practical detection limit of 0.01% (w/w) was determined (Fig. 1, Fig. 2). These parameters also suggest that the method has a potential to be quantitative, at least for discrimination between the presence of traces of the analyte (in case of adventitious contamination) from the presence of several percent of the analyte (in case of adulteration).

Practical applicability of the real-time PCR-based method was tested by the analysis of 22 confectionery products. For all but one of the food samples, results obtained conformed to the labelling (Tab. 2). The one incorrectly labelled food product was Macadamia nougat containing approx. 0.040% (w/w) of cashew nuts. Such cashew nut content suggests that the product had been contaminated rather than adulterated.

CONCLUSIONS

The presented real-time PCR-based method is useful for sensitive and selective detection of cashew nuts in food. The method is relatively straightforward and fast, the analysis can be performed in one working day.

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