



Development of a real time PCR assay for detection of allergenic trace amounts of peanut (*Arachis hypogaea*) in processed foods

Inés María López-Calleja*, Silvia de la Cruz, Nicolette Pegels, Isabel González, Teresa García, Rosario Martín

Departamento de Nutrición, Bromatología y Tecnología de los Alimentos, Facultad de Veterinaria, Avenida Puerta de Hierro s/n, Universidad Complutense de Madrid, 28040 Madrid, Spain

ARTICLE INFO

Article history:

Received 19 June 2012

Received in revised form

13 September 2012

Accepted 13 September 2012

Keywords:

TaqMan real-time PCR

Peanut (*Arachis hypogaea*)

Commercial food products

Food allergy

ABSTRACT

Peanut allergic reactions can result from the ingestion of even very small quantities of peanut and represent a severe threat to the health of sensitized individuals. In order to protect the allergic consumer, efficient and reliable methods are required for the detection of allergenic ingredients. For this purpose, we have developed a TaqMan real-time PCR method for specific detection of peanut in commercial food products. The assay uses two genetic makers in the *Arah2* (125 bp) and *ITS1* (90 bp) gene respectively, and TaqMan probes. The nuclear *18S rRNA* gene was employed as a positive amplification control. Results obtained on sensitivity of both *Arah2* and *ITS* primers with mixtures spiked with different concentrations of the target showed detection levels of 10 and 0.1 ppm, respectively. The applicability of the real-time PCR protocol to detect the presence of peanut DNA in commercial food products was determined through analysis of 123 different commercial products with the *ITS* primers which aimed higher sensitivity than *Arah2* primer pair. The performance of the real-time PCR proposed herein allows a highly sensitive detection of peanut DNA in all the different food products, which declared to contain peanut material as well as in others where the presence of peanut or its traces wasn't declared in the labeling.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

Food allergy is a serious public health problem. Around 1–2% of the population, suffer from some type of food allergy and even higher prevalence levels (up to 8%) are estimated for children (Helm & Burks, 2000; Ortolani, Ipsano, Scibilia, & Pastorello, 2001; Sicherer, Muñoz-Furlong, Murphy, Wood, & Sampson, 2003; Sicherer, Muñoz-Furlong, & Sampson, 2004). The severity of the reactions triggered by food allergens vary from mild urticaria to potentially lethal anaphylactic shocks, even after the ingestion of minimal doses of the allergenic food. It is estimated that in the United States about 30,000 anaphylactic reactions to food are treated in emergency departments and that food allergy results in 150–200 deaths each year (Sampson, 2005). Peanuts and tree nuts are responsible for the majority of deaths attributed to food allergies (Bock, Muñoz-Furlong, & Sampson, 2001; Pumphrey, 2001).

Peanut allergy is one of the most severe food allergies due to its persistency throughout the lifetime of individuals and its life

threatening character, even after the ingestion of minimal doses (Hourihane et al., 1997). The reason for this extreme response to peanut compared with other food allergens is unknown, but is of growing concern, since unwitting consumption can, in some instances, cause death. Peanuts are used widely in different preparations like snacks and biscuits, and in the confectionary industry, both in their own right and as a cheap bulking agent for other products (Hird, Lloyd, Goodier, Brown, & Reece, 2003). Thus, avoidance of peanut-containing foods can be difficult for peanut allergen sufferers and food producers.

People suffering from a food allergy usually try to avoid consuming the food that can endanger their health. Despite this, the ingestion of peanuts often happens accidentally because of mislabeling of products, rework processes which include peanut-containing foods or cross-contamination during processing (Sampson, Mendelson, & Rosen, 1992; Yunginger et al., 1988). Therefore, EU legislation has been issued aimed at informing the consumer about the content of food products by means of a mandatory labeling of allergenic food ingredients. The EU Directive 2011/1169/EC (OJEU, 2011), provides a list of 14 groups of allergenic foods that manufacturers are required to declare on the labels if any of them are used as ingredients in pre-packaged foods, regardless of their quantity. Reliable analytical methods for the

Abbreviations: PSS, peanut specific system; PAC, positive amplification control.

* Corresponding author. Tel.: +34 913943750.

E-mail addresses: ilopezcaljea@vet.ucm.es, ineslopezcaljea@gmail.com (I.M. López-Calleja).

detection of hidden allergens in foodstuff are therefore required by the food industry and food control.

Peanut contains several major protein allergens, identified by their reactivity toward IgE in sera from sensitized subjects (Hird et al., 2003). Traditionally, protein detection methods have been widely used to detect the presence of allergenic proteins. Currently, enzyme-linked immunosorbent assay (ELISA) is probably the method that is most commonly used by the food industry and official food control agencies to assess the presence of allergens like peanut in food products (Besler, 2001). Immunodetection based methods detect allergenic ingredients based on the interaction of species-specific proteins with antibodies raised in animals (Platteau, De Loose, De Meulenaer, & Taverniers, 2011; Scaravelli, Brohee, Marchelli, & Van Hengel, 2008). Several commercial ELISA test kits are available for the detection of traces of peanuts in food (Keck-Gassenmeier, Benet, Rosa, & Hischenhuber, 1999; Kiening et al., 2005; Poms et al., 2005). These immunochemical methods although highly sensitive, may be biased by cross-reactivity with other nuts. On the other hand, the detection of peanut proteins may be compromised by the fact that the food industry employs a variety of food processing methodologies like heat treatment or roasting, which can strongly affect the proteins integrity and quality (Hird et al., 2003; Immer, 2006, pp. 330–347; Taylor, Nordlee, Niemann, & Lambrecht, 2009). An alternative to the direct detection of proteins is provided by DNA-based methods. Several of such methods have recently been developed for the detection of peanut traces in food products (Hirao, Watanabe, Temmei, Hiramoto, & Kato, 2009; Hird et al., 2003; Rossi, Scaravelli, Germini, Corradini, & Marchelli, 2006; Scaravelli et al., 2008; Sforza, Scaravelli, Corradini, & Marchelli, 2005; Stephan & Vieths, 2004; Watanabe et al., 2006). The target molecules of these detection methods are not proteins but specific DNA sequences which are amplified by the polymerase chain reaction (PCR). For peanut allergen detection, several real-time PCR assays have been developed (Hird et al., 2003; Scaravelli et al., 2008) that, like ELISA, have the potential to be applied for quantitative purposes.

Recent advances in DNA technology have lead to the development of real-time PCR, at the forefront of which is Taqman technology (Applied Biosystems, Warrington, UK) (Holland, Abramson, Watson, & Gelfand, 1991). A number of published works have reported the advantages of using real-time PCR compared to conventional PCR for peanut detection (Bellagamba, Comincini, Ferretti, Valfre, & Moretti, 2006; Cawthraw et al., 2009; Fumière, Dubois, Baeten, von Holst, & Berben, 2006; Prado et al., 2009; Yancy et al., 2009). These include, for example: i) the ability to detect very small size products, since it eliminates the need for gel visualization, ii) the reduced risks for crossover contamination, as reactions are kept confined during amplification and analysis and iii) the capability for large-scale processing and high automated throughput. TaqMan probe chemistry is among the most broadly applied real-time PCR technologies to detect allergens in commercial food products. The main advantage of using the TaqMan real-time PCR platform for this purpose is the increased assay specificity, explained by the fact that detection is generated by the hydrolysis of an internal target-specific oligonucleotide (TaqMan probe) (Frezza et al., 2008; Gašparič et al., 2010; Mendoza-Romero et al., 2004; Prado et al., 2007).

In this way, the target sequence can be either designed on the gene coding for the allergenic protein or simply, on a species-specific region in the genome of the allergenic food. Despite their different targets, ELISA and real-time PCR are often designed for quantitative purposes. Comparison of a real-time PCR method and an ELISA (Engler-Blum, Raiss, & Burgmaier-Thielert, 2007), for the detection of peanut has recently been reported, whereby the relative Cp values obtained by the real-time PCR correlated well with

the amount of peanut measured by the sandwich ELISA (Stephan & Vieths, 2004). Hird et al. (2003) and Stephan and Vieths (2004), describe a real-time PCR assay with 1 and 2 ppm limit of detection respectively, which were in the range of those that can be obtained by means of ELISA (Hird et al., 2003; Stephan & Vieths, 2004). Commercial PCR based kits for the detection of peanuts are currently also available, either in the format of a real-time PCR kit (R-Biopharm, Darmstadt, Germany), a PCR combined with ELISA detection, or conventional PCR followed by gel electrophoresis analysis.

We hereby report the development and optimization of a TaqMan real-time PCR method for the specific identification of peanut, based on selective amplification of *Arah2* and *ITS* gene sequences. The applicability of the assay was evaluated through screening analysis with the *ITS* primer pair of a total of 123 different commercial food samples, consisting in a wide variety of brands and types of products. In all, this test results simple, sensitive, specific and provides a clear indication of the presence or absence of the peanut in a commercial product.

2. Materials and methods

2.1. Sample selection

Reference samples were supplied by the Institute for Reference Materials and Measurements provided by the European Commission (Belgium), and consisted on five peanut varieties from different geographical origins exposed to different heat treatments (Table 1).

Peanut, nuts and various commercial brands of food were purchased from different local stores and several delicatessen markets and stored at room temperature in the dark. A wide range of plant and animal species was also included in the assays for specificity control purposes (Table 2).

Peanuts were finely grounded and binary mixtures of raw and heat treated peanut (160 °C for 13 min) in wheat flour and cocoa containing 0.1, 1, 10, 100, 1000 and 10,000 ppm of peanuts were prepared to a final weight of 500 g using a kitchen robot (Thermomix, Vorwerk).

2.2. DNA extraction

Two hundred milligrams of each sample were homogenized with 860 µL of extraction buffer, pH 8.0 (10 mM Tris, 150 mM NaCl, 2 mM EDTA and 1% SDS), 100 µL of 5 M guanidine hydrochloride and 40 µL of 20 mg/mL proteinase K (Merck, Darmstadt Germany), and incubated overnight at 55 °C with shaking at 60 rpm. Then, the samples were left to cool at room temperature. Five hundred mL of chloroform (Sigma Aldrich) were added to the lysate before centrifugation at 16,438 g for 10 min.

Table 1
Sensitivity of the peanut real-time PCR system in IRMM-481 test material.^a

Peanut treatment	Peanut variety origin	<i>Arah2</i> P.S.S. (C _p)	<i>ITS</i> P.S.S. (C _p)
Blanched only	Jumbo Runners, USA	20.96 ± 0.02	14.53 ± 0.01
Blanched, oil-roasted at 140 °C for 9 min	Virginia, China	21.53 ± 0.03	14.73 ± 0.01
Blanched, oil-roasted at 140 °C for 20 min	Runners, Argentina	22.85 ± 0.02	15.71 ± 0.02
Blanched, oil-roasted at 145 °C for 25 min	Virgin, USA	23.12 ± 0.03	16.02 ± 0.02
Raw, air roasted at 160 °C for 13 min	Common Natal, South-Africa	23.51 ± 0.01	16.42 ± 0.02

^a IRMM (Institute for Reference Materials and Measurements)-481 provided by the European Commission (Belgium).

Table 2
Specificity of the peanut real-time PCR system

Common Name	Scientific name	Arah2 P.S.S	ITS P.S.S	18S rRNA P.A.C
Peanut	<i>Arachis hypogaea</i>	19.84 ± 0.01 ^a	13.87 ± 0.01 ^a	16.61 ± 0.01
Nut	<i>Juglans regia</i>	–	–	15.02 ± 0.04
Pistachio	<i>Pistacia vera</i>	–	–	14.15 ± 0.06
Macadamia	<i>Macadamia intergrifolia</i>	–	–	16.17 ± 0.01
Hazelnut	<i>Corylus avellana</i>	–	–	14.53 ± 0.02
Cashew nut	<i>Anacardium occidentale</i>	–	–	15.13 ± 0.04
Almond	<i>Prunus dulcis</i>	–	–	14.79 ± 0.03
Brazil nut	<i>Bertholletia excelsa</i>	–	–	16.13 ± 0.01
Pecan	<i>Carya illinoensis</i>	–	–	16.20 ± 0.01
Soybean	<i>Glycine max</i>	–	–	16.52 ± 0.06
Tiger Nut	<i>Cyperus esculentum</i>	–	–	14.76 ± 0.04
Lupine	<i>Lupinus albus</i>	–	–	15.48 ± 0.03
Acorn	<i>Quercus ilex</i>	–	–	14.72 ± 0.02
Chestnut	<i>Aesculus hippocastanum</i>	–	–	16.38 ± 0.03
Sesame	<i>Sesamum indicum</i>	–	–	15.78 ± 0.01
Pine nut	<i>Pinus pinea</i>	–	–	16.34 ± 0.01
Barley	<i>Hordeum vulgare</i>	–	–	14.32 ± 0.06
Oat	<i>Avena sativa</i>	–	–	14.01 ± 0.03
Rye	<i>Secale cereale</i>	–	–	14.23 ± 0.02
Rice	<i>Oryza sativa</i>	–	–	14.56 ± 0.04
Sunflower	<i>Helianthus annuus</i>	–	–	14.11 ± 0.02
Maize	<i>Zea mays</i>	–	–	15.11 ± 0.06
Wheat	<i>Triticum aestivum</i>	–	–	13.55 ± 0.01
Cocoa	<i>Theobroma cacao</i>	–	–	15.05 ± 0.05
Orange	<i>Citrus Sinesis</i>	–	–	13.87 ± 0.01
Potato	<i>Solanum tuberosum</i>	–	–	14.02 ± 0.02
Olive	<i>Olea europaea</i>	–	–	16.26 ± 0.07
Cattle	<i>Bos Taurus</i>	–	–	13.98 ± 0.00
Sheep	<i>Ovis aries</i>	–	–	14.12 ± 0.02
Goat	<i>Capra hircus</i>	–	–	15.44 ± 0.01
Swine	<i>Sus scrofa domestica</i>	–	–	14.34 ± 0.07

Arah2 P.S.S: Peanut-specific system on the Arah2 gene (*PeanutArahdir* and *PeanutArahinv*).

ITS P.S.S: Peanut-specific system on the Internal Transcribed Spacer (*PeanutITSdir* and *PeanutITSinv*). 18S rRNA P.A.C: Positive amplification control (*18Sdir/18Sinv* and *18SP*) for the peanut system on the 18S rRNA gene.

^a Average Cp value ± SD shown from triplicate PCR reactions from each DNA extraction. Minus sign indicates no positive signal after 50 PCR cycles.

Genomic DNA from the clear aqueous supernatant obtained after the centrifugation (500 µL) was purified using the Wizard DNA Clean-up System kit (Promega, Madison, WI, USA) as described by López-Calleja et al. (2007). The DNA was eluted in 50 µL of sterile deionized water. DNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Montchanin, DE). Unless otherwise stated, three DNA replicates were extracted from each sample. A negative control sample was included in every DNA extraction. The DNA extracted from raw and heat-treated peanut was electrophoresed in a 1% D1 (Hispanlab S.A., Torrejón, Spain) agarose gel containing 1 µg/mL ethidium bromide in Tris–acetate buffer (0.04 M Tris–acetate and 0.001 M EDTA, pH 8.0) for 30 min at 110 V. Total DNA was visualized by UV transillumination using a Geldoc 1000 UV Fluorescent Gel Documentation System-PC (Bio-Rad Laboratories, Hercules, CA).

2.3. Oligonucleotide primers and probes

The oligonucleotides used in the real-time PCR assay were designed upon the 2S Albumin (*Arah2*) gene, the Internal Transcribed Spacer (*ITS*) gene and the nuclear 18S rRNA gene sequences from various plant and animal species available in the NCBI (National Center for Biotechnology Information) database.

Alignment and computer analysis of *Arah2* and *ITS1* sequences allowed the design of peanut-specific primers: *PeanutArahdir/PeanutArahinv* and *PeanutITSdir/PeanutITSinv* for the amplification of a DNA fragment of 125 bp and 90 bp, respectively. Besides, a dual-labeled TaqMan probe *PeanutArahP* and *PeanutITSP* with a reporter fluorophore at the 5' end (6-carboxyfluorescein, FAM) and a quencher fluorophore at the 3' end (Blackberry, BBQ), were designed to anneal within the *Arah2* and the *ITS1* gene fragment generated by amplification of the corresponding target.

As a positive amplification control of real-time PCR experiments, a pair of universal primers (*18Sdir/18Sinv*) and a TaqMan probe (*18SP*) were designed on a conserved 18S rRNA gene fragment in all eukaryotic cells. This PCR system was expected to yield amplicons of the same length (approximately 77 bp) in all species analyzed in this work.

The EMMA program included in the EMBOS software package version 2.0 and the Primer Express 2.0 software (Perkin–Elmer/Applied Biosystems Division, Foster City, CA) were used for sequence alignment and primer design. TaqMan probes were designed and synthesized by TibMolBiol (Berlin, Germany). The sequences and description of every primer and probe used in this study are listed in Table 3.

2.4. Real-time PCR

Real-time PCR was run under generic cycling conditions. The optimum PCR concentrations of primers yielding the highest endpoint fluorescence and the lowest Cp were experimentally determined for each set of primers: 300 nM for forward primers and 900 nM for reverse primers. The PCR reactions were carried out using the LightCycler® TaqMan® Master (Roche Diagnostics GmbH, Mannheim, Germany). 2 pmol of each TaqMan probe (TibMolBiol), and 2 µL of extracted DNA. Amplification reactions were done in a total reaction volume of 10 µL in a glass capillary tube and were run on the LightCycler 2.0 Instrument (Roche Applied Science, Ponsberg, Germany) with the following program: an amplification program of 55 cycles at 95 °C for 5 s and 60 °C for 30 s. Samples were then cooled to 40 °C for 30 s. This program was used to amplify the species-specific PCR system, along with the positive amplification control. Unless otherwise indicated, all real-time PCR reactions were carried out in triplicate for each DNA extract.

The crossing point value (C_p), which refers to the cycle number where the sample's fluorescence significantly increases above the background level, was calculated automatically by the LightCycler software as the first maximum of the second derivative of the curve. The continual measurement of fluorescence is related to the amount of amplicon in the real time PCR, yielding a qualitative result on the presence of the target species.

2.5. Construction of standard curves and data analysis

To assess the efficiency, linear range and analytical sensitivity of the peanut-specific system (*Arah2* & *ITS1*), standard curves were constructed using three arrays of binary mixtures of known peanuts' content (Figs. 2 and 3: raw peanut/wheat flour, heat treated peanut/wheat flour and raw peanut/cocoa) rendered under homogeneous conditions and containing increasing amounts of target material. The amount of target DNA in an unknown sample can be then measured by extrapolation of the C_p value obtained in the unknown sample in the corresponding standard curve of C_p values generated from known DNA concentrations of the target species. In addition, the correlation between the variables, crossing point (C_p) and concentration ($[]$) is semilogarithmic:

Table 3

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)
<i>PeanutArahdir</i>	20	GAACCAGAGCGATAGGTTGC	Peanut-specific forward primer	<i>Arah2</i>	125
<i>PeanutArahinv</i>	18	CGCCATTTCGACTTCCAA	Peanut-specific reverse primer		
<i>PeanutITSdir</i>	20	AAGCCAAACGTTTCTGCTCT	Peanut-specific forward primer	<i>ITS</i>	90
<i>PeanutITSinv</i>	17	CGTTGCCGAGAGTCGTT	Peanut-specific reverse primer		
<i>18Sdir</i>	16	TGGTGCCAGCAGCCGC	Positive control forward primer	<i>18S rRNA</i>	77
<i>18Sinv</i>	25	TCCAACTACGAGCTTTTAACTGCA	Positive control reverse primer		
Probes					
<i>PeanutArahP</i>	24	6FAM-AGCTCCCTCTTGAAGTGTGCTCC-BBQ	Peanut probe	<i>Arah2</i>	
<i>PeanutITSP</i>	24	6FAM-TAACTCTTGCTGCTACTCGTCGCC-BBQ	Peanut probe	<i>ITS</i>	

$$C_p = b \log[\] + a$$

Where b is the slope and a is the intercept.

Linearity test, sensitivity, accuracy and precision parameters of the species-specific real-time PCR systems were evaluated according to previously described methods (Camacho, Torres, Gil-Alegre, Obregón, & Ruz, 1993; International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use [ICH], 2005). To carry out the validation of the real-time PCR technique developed in this work, three separate DNA extractions of each peanut concentration were assayed in different days, using three replicates of each sample. Precision of the assay was evaluated by comparing the coefficient of variation (CV) values obtained in three different days versus those obtained in the same day, to assess the influence of random events (day of analysis, analyst, equipment, etc.) in the data obtained.

3. Results and discussion

3.1. Peanut-specific system on the *Arah2* gene

3.1.1. TaqMan real-time PCR

The real-time PCR assay developed in this work was initially based on the design of a species-specific primer pair and a TaqMan

probe constructed on the 2S *Albumin* (*Arah2*) gene. This seed storage protein is a major peanut allergen recognized by serum IgE on peanut allergic patients. This glycoprotein is known for its high heat stability (Koppelman et al., 2001; Scaravelli, Brohee, Marchelli, & Van Hengel, 2009). Since a critical point in PCR-based food commercial products analysis is heat degradation of DNA, the selection of a gene that presents high heat stability together with the selection of a short length DNA target, results crucial for successful system development. In this way, a short *Arah2* amplicon (125 bp) was chosen for real-time PCR analysis aimed to detection of peanut in commercial food products. In addition, the use of a conserved region in the nuclear *18S rRNA* as positive control is important to avoid false-negative results due to factors affecting amplification such as DNA degradation, expired reagents, etc.

3.1.2. Specificity and sensitivity

Specificity and sensitivity are two important indices of performance of qualitative assays. The close phylogenetic relationships among peanut and tree nut species, together with the varied number of plants and animals components that can be present in different commercial food products indicates the need to check the cross reactivity of the PCR against a wide range of species. Specificity of the peanut real-time PCR system (primers and probe) on the *Arah2* gene was therefore assessed by analysis of the DNA extracted from 31 plant species and 4 animal species. As expected, the peanut specific PCR system on the *Arah2* gene successfully detected a DNA fragment of 125 bp from peanut (19.84 ± 0.01), while no positive amplification signal was obtained on the animal and plant species tested. The *18S rRNA* eukaryotic system amplified a 77 bp fragment from all samples tested (Table 2).

A part from the demand for specificity, real-time PCR methods intended for species detection in commercial food products should be aimed at reaching a good sensitivity level when highly degraded DNA is present as consequence of high temperature treatments (Van Raamsdonk et al., 2007). Prior to consumption, peanuts usually undergo heat treatments like blanching or roasting. The IRMM-481 peanut test material, which was used in this study, includes five peanut varieties from different geographical origins exposed to five different industrial heat treatments (Table 1). Therefore, it represents a material that might be comparable to the peanuts the consumer is exposed to. As can be seen in Fig. 1, gel electrophoresis of total genomic DNA extracted from raw peanut and from the five peanut varieties submitted to different heat treatments, shows increasing degradation as the heat treatment becomes more intense. Whereas DNA of raw and those peanut varieties subjected to a less severe heat treatment appear more intact and of a higher molecular weight. However, real time PCR C_p values obtained were very similar in raw and in the heat treated peanut varieties (Table 1). In this way, real time PCR results indicate effective amplification of the desired DNA segments in all raw and heat treated samples, confirming the ability of the PCR to amplify relatively short segments in highly damaged DNA.

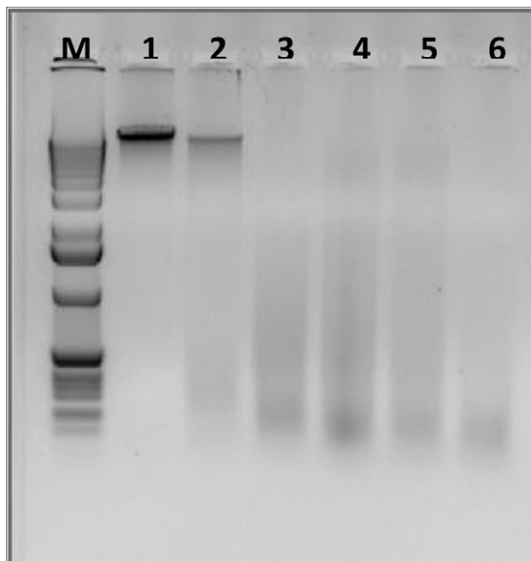


Fig. 1. Gel electrophoresis of total genomic DNA extracted from peanut. Sample in lane 1 was obtained from raw peanut, and samples in lanes 2–6 were obtained from peanut heat-treated at: Blanching only (2), Blanching, oil-roasted at 140 °C for 9 min (3), Blanching, oil-roasted at 140 °C for 20 min (4), Blanching, oil-roasted at 145 °C for 25 min (5) and raw, air roasted 160 °C/13 min (6). M, molecular weight marker 1 kb plus DNA ladder (Invitrogen).

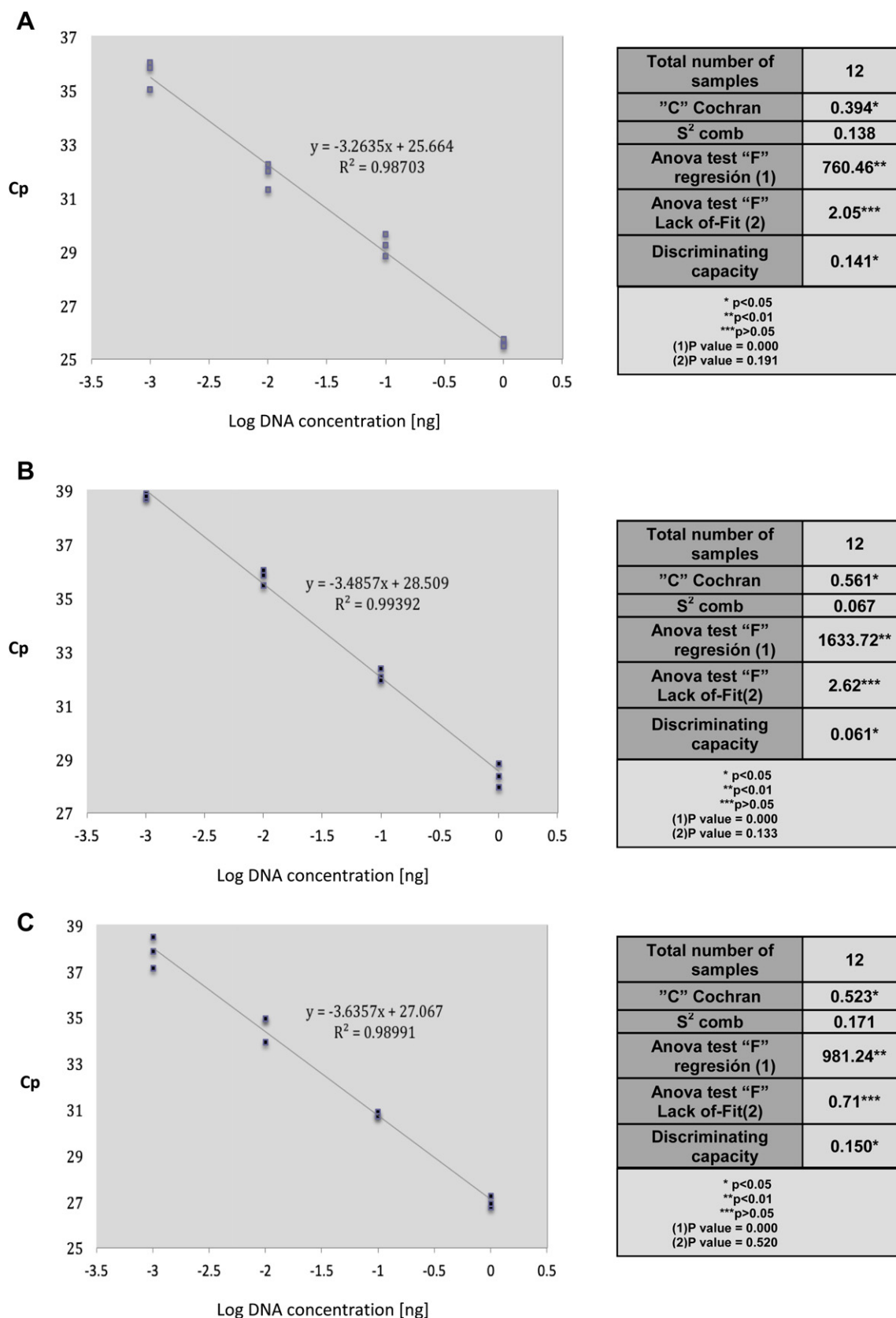


Fig. 2. Linearity test, regression line and sensitivity parameters of the peanut-specific TaqMan system on the *Arah2* gene, using three different DNA extractions of four different peanut concentrations (10,000, 1000, 100, 10 ppm) of each binary mixture: (A) Raw peanut/wheat flour mixture. (B) Raw peanut/cocoa mixture. (C) Heat treated peanut/wheat flour.

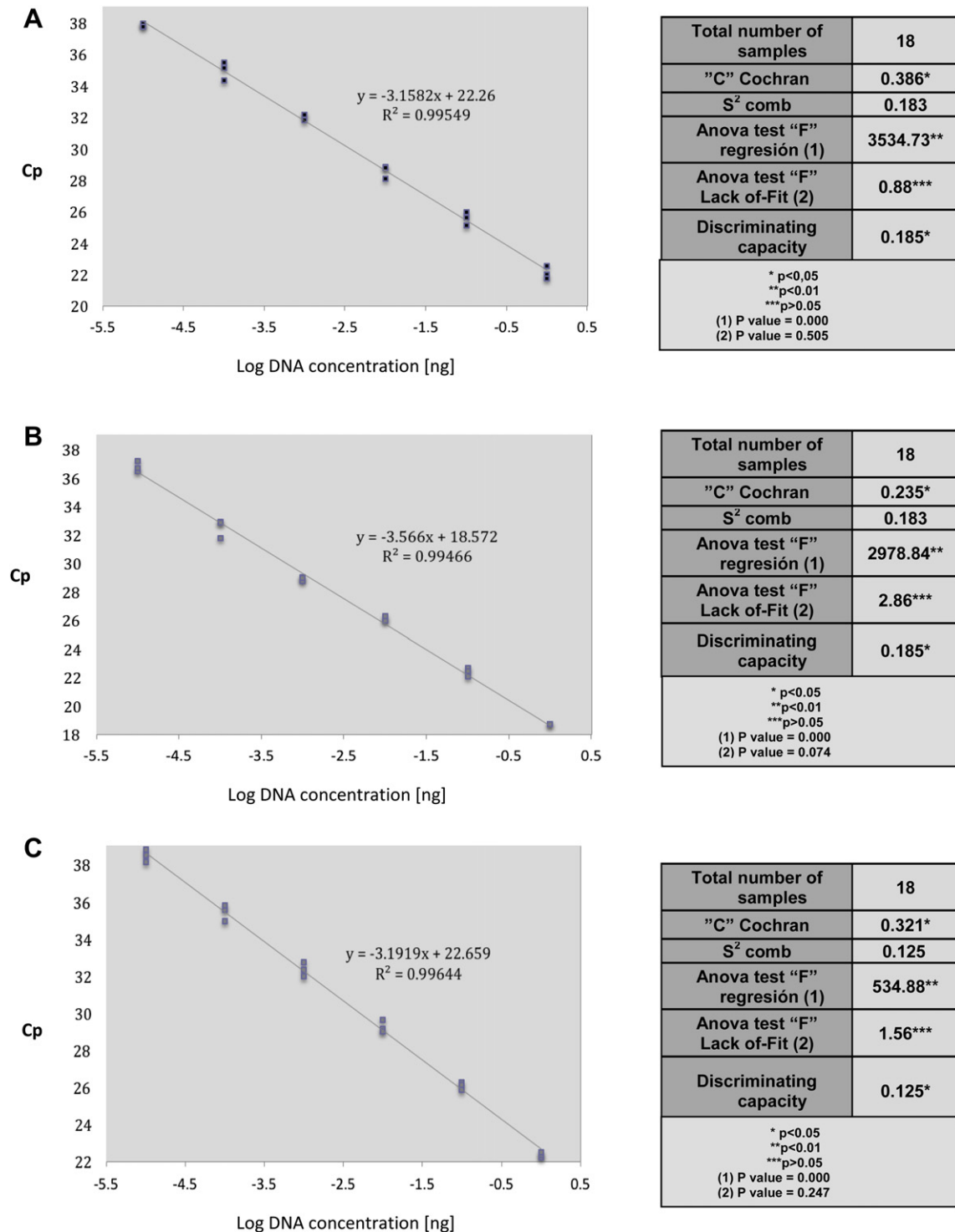


Fig. 3. Linearity test, regression line and sensitivity parameters of the peanut-specific TaqMan system on the *ITS* gene, using three different DNA extractions of six different peanut concentrations (10,000, 1000, 100, 10, 1, 0.1 ppm) of each binary mixture: (A) Raw peanut/wheat flour mixture. (B) Raw peanut/cocoa mixture. (C) Heat treated peanut/wheat flour.

Three arrays of binary mixtures of raw and heat treated peanut (160 °C for 13 min) in wheat flour and cocoa containing increasing amounts of the target peanut were used to construct the calibration curves using a simple regression model with the log input DNA concentration versus the C_p (Fig. 2).

As shown in Fig. 2, the resulting slopes of the linear equations are -3.26 for raw peanut/wheat *Arah2* peanut-specific system (Fig. 2A), -3.48 for raw peanut/cocoa *Arah2* peanut-specific system

(Fig. 2B) and -3.63 for heat treated peanut/wheat *Arah2* peanut-specific system (Fig. 2C).

C_p values obtained from peanut calibration curves, plotted against the logarithm of the DNA concentrations, were used to test the sensitivity of the real-time PCR method. Fig. 2 also shows the discriminating capacity, which is the least difference in logarithm of target DNA concentration in the sample that the analytical method can discriminate with a significant level. Linearity of the

peanut real-time PCR response was also analyzed. In each PCR system, the following parameters were evaluated: (a) Cochran's test, which determined whether the variances of the responses obtained for each concentration of peanut DNA in the reference feeds were homogeneous, (b) regression analysis and (c) variance analysis with lack of fit. All parameters were evaluated following previously described validation protocols (Camacho et al., 1993; Fajardo et al., 2009; ICH, 2005).

In order to determine the accuracy of the *Arah2* peanut-specific system six concentrations on the *Arah2* (0.1, 1, 10, 100, 1000 and 10,000 ppm) of reference samples were elaborated and analyzed. Accuracy is reported as percentage recovery by the assay of known added amounts of analyte. *Arah2* primers were only able to detect up to 10 ppm. Using the Snedecor *F*-test, it was verified that the concentration of DNA present in a sample did not affect the variation of the results. The values for Student's *t*-test obtained demonstrated that the method was accurate, since no significant differences between 100% and the mean recovery values were detected. The influence of day of analysis on the precision of the assay was also tested by comparing the coefficients of variation (CV) of three separate DNA extractions performed on the 10 ppm *Arah2*, analyzed in duplicate three times on the same day, versus the values obtained for the same samples on three different days. Results showed significant differences with higher CV values when the assay was done on different days with respect to the same day. Thus, a standard curve should be determined on each day of analysis.

Nevertheless, in order to achieve higher sensitivity with the real time PCR assay and therefore lower LOD for further detection of peanut in commercial food samples another primer pair was designed based in the multi copy *ITS* gene. The level of 10 ppm is within the range of detection limits that are relevant for the detection of potentially hazardous residues of undeclared allergens in foods (Hefle & Koppelman, 2006; pp. 313–329; Scaravelli et al., 2008). However, lower detection limits result very convenient, since minimal amounts of the target allergen can be critical.

3.2. Peanut-specific system on the *ITS1* gene

3.2.1. TaqMan real-time PCR

In order to improve the detection limit obtained with the *Arah2* peanut specific system, we designed a new primer pair on the *Internal Transcribed Spacer 1* region. The *ITS* region results an ideal candidate for primer designing, due to the high copy number of rRNA genes easy to amplify even from small quantities of DNA which contribute to increase the sensitivity of the present PCR. Also, the presence of a multi-copy gene makes easier the recovery of at least a few copies when subjected to intense thermal processing conditions. Commercial production processes of peanuts and tree nuts involve heat treatment or roasting which degrades DNA, thereby the selection of a short *ITS1* amplicon of 90 bp is relevant for real-time PCR analysis aimed to detection of peanut in commercial food products. Moreover, the *ITS* gene also presents a high degree of sequence variation required for identification of closely related species. In addition, the use of a conserved region in the nuclear *18S rRNA* gene would provide a positive control as described before.

3.2.2. Specificity and sensitivity

Specificity of the peanut real-time PCR system (primers and probe) on the *ITS* gene was therefore assessed by analysis of the DNA extracted from 31 plant species and 4 animal species. As expected, the peanut specific PCR systems on the *ITS* gene successfully detected, a DNA fragment of 90 bp from peanut (13.87 ± 0.01), while no positive amplification signal was obtained on the rest of the species tested. The *18S rRNA* eukaryotic system amplified a 77

bp fragment from all samples tested (Table 2). Sensitivity level was also checked on IRMM-481 peanut test material. C_p values obtained with *ITS* peanut specific system oscillated between 14 and 18. As shown in Table 1, C_p values decrease as sterilization conditions applied to the samples become less severe.

Moreover, the results showed that no matter the intensity of the heat treatment applied, all of them were amplifiable by the two peanut-specific systems proposed. However, C_p s reached by *ITS* peanut specific system were lower than the ones obtained with *Arah2*. This is probably due to the fact that it is a multi copy gene and to a lower size amplicon. It should be noted that although heat treatments have an indisputable effect on DNA damage (Arslan, Irfan Ilhak, & Calicioglu, 2006; Hird et al., 2006), positive amplification signals were observed with the P.S.S. in peanut samples subjected to processing temperatures as high as 160 °C. The short length of the selected amplicons minimized possible PCR failures due to DNA degradation.

3.2.3. Efficiency, quantitation limit and statistical analysis

Three arrays of binary mixtures of raw and heat treated peanut (160 °C for 13 min) in wheat flour and cocoa containing increasing amounts of the target peanut were used to construct the calibration curves using a simple regression model with the log input DNA concentration versus the C_p (Fig. 3).

As shown in Fig. 3, the resulting slopes of the linear equations are -3.15 for raw peanut/wheat *ITS* peanut-specific system (Fig. 3A), -3.56 for raw peanut/cocoa *ITS* peanut-specific system

Table 4

Results of the peanut real time PCR analysis of 123 commercial products.

Commercial food products that declared peanuts in the labeling	Number of samples analyzed	<i>ITS</i> P.S.S. (C_p)	<i>18S</i> rRNA (C_p)
Chocolate	6	+	+
Bonbon	5	+	+
Biscuits	1	+	+
Nut bar	5	+	+
Cereal	2	+	+
Ice cream	1	+	+
Commercial food products that declared traces of peanuts	Number of samples analyzed	<i>ITS</i> P.S.S. (C_p)	<i>18S</i> rRNA (C_p)
Chocolate	2	+	+
Bonbon	16	+	+
Biscuits	2	+	+
Nut bar	1	+	+
Cereal	5	+(3)/(2)	+
Ice cream	2	+	+
Cashew cream	1	+	+
Precooked meal	1	+	+
Commercial food products that not declared peanut nor traces on the label	Number of samples analyzed	<i>ITS</i> P.S.S. (C_p)	<i>18S</i> rRNA (C_p)
Chocolate	18	+(8)/(10)	+
Bonbon	7	+(1)/(6)	+
Biscuits	14	+(7)/(7)	+
Nut bar	12	+(4)/(8)	+
Cereal	4	+(1)/(3)	+
Ice cream	2	–	+
Bread	3	–	+
Chocolate cream	3	+(2)/(1)	+
Beverage	4	+(2)/(2)	+
Sauce	1	–	+
Precooked meal	1	–	+
Rice cake	2	+	+
Yogurt	1	–	+
Nougat	1	–	+

(+) Plus sign indicates positive signal before 50 PCR cycles.

(–) Minus sign indicates no positive signal after 50 PCR cycles.

Table 5

Real-time PCR results obtained with the *ITS* peanut-specific system and *18S* rRNA positive amplification control in the analysis of commercial food products that not declared peanut nor traces on the label.

Commercial food product	Ingredients declared on the label	P.S.S. (Cp)	P.A.C. (Cp)	PPMS
Chocolate-1	Sweetener, hazelnuts 25%, maltitol, cocoa butter, cocoa mass, milk powder, skimmed milk powder, emulsifier, soy lecithin, aroma. This product may contain traces of almonds, hazelnuts and milk.	34.38 ± 0.02	14.67 ± 0.01	1.5 ppm
Chocolate-2	Sweetener, hazelnuts, maltitol, cocoa butter, cocoa mass, emulsifier, soy lecithin, aroma. This product may contain traces of almonds and hazelnuts.	32.96 ± 0.02	16.01 ± 0.01	4.1 ppm
Chocolate-3	Sweetener, maltitol, cocoa butter, cocoa mass, vegetable fat, skimmed milk powder, whey powder, fat reduced cocoa powder, emulsifier, soy lecithin, aroma. This product may contain traces of almonds and hazelnuts.	35.41 ± 0.01	15.23 ± 0.01	0.7 ppm
Chocolate-4	Sweetener, maltitol, cocoa butter, cocoa mass, vegetable fat, skimmed milk powder, whey powder, fat reduced cocoa powder, emulsifier, soy lecithin, aroma. This product may contain traces of almonds and hazelnuts.	35.51 ± 0.03	16.23 ± 0.03	0.7 ppm
Chocolate-5	Sweetener, almonds, maltitol, cocoa butter, cocoa mass, vegetable fat, skimmed milk powder, whey powder, fat reduced cocoa powder, emulsifier, soy lecithin, aroma. This product may contain traces of hazelnuts.	34.82 ± 0.01	13.78 ± 0.01	1.1 ppm
Chocolate-6	Sugar, cocoa butter, chocolate, milk powder, lactose, cocoa paste, skim powder milk, milk whey powder, sunflower lecithin, vanilla flavor. May contain traces of nuts and gluten.	32.30 ± 0.01	16.67 ± 0.02	6.6 ppm
Chocolate-7	Sugar, cocoa butter, cocoa paste, sunflower lecithin and flavor. May contain traces of nuts and milk.	35.59 ± 0.02	15.56 ± 0.02	0.6 ppm
Chocolate-8	Cocoa mass, sugar, cocoa butter, milk, milk whey powder, sunflower lecithin. May contain traces of nuts and milk.	34.66 ± 0.03	14.21 ± 0.02	1.2 ppm
Bonbon-1	Milk chocolate (sugar, cocoa butter, cocoa liquor, skimmed milk powder, whey powder, milk fat, hazelnut paste, emulsifier: soy lecithin, vanillin, in milk chocolate cocoa solid minimum 30%) hazelnuts 48%, glazing agent (acacia gum, beeswax), vegetable oil. Contains wheat, milk, gluten, soy and hazelnut, and may contain other nuts.	34.91 ± 0.01	14.92 ± 0.03	0.9 ppm
Biscuits-1	Sugar, enriched flour, high oleic canola oil, soybean oil, high fructose corn syrup, cornstarch, salt, soy lecithin, vanillin-an artificial flavor, chocolate, contains: wheat, soy and milk.	29.14 ± 0.02	14.01 ± 0.01	>50 ppm
Biscuits-2	Unbleached enriched wheat flour, milk chocolate, sugar, dark chocolate, vegetable oils, pecan nuts 11%, butter, brown sugar, eggs, coconut, contains 2 percent or less of: leavening, salt, cornstarch, natural flavors ground nuts (pecan, macadamia, walnut, cashew, almond).	33.17 ± 0.01	16.23 ± 0.01	3.5 ppm
Biscuits-3	Unbleached enriched wheat flour (flour, niacin, reduced iron, thiamin mononitrate (vitamin B1), Riboflavin (vitamin B2), folic acid), sugar, semi-sweet chocolate (sugar, chocolate liquor, cocoa butter, dextrose, soy lecithin added as an emulsifier, vanilla extract), fructose, vegetable oils (palm oil), invert sugar, butter, cocoa processed with alkali (dutch), nonfat milk, whole eggs, contains 2% or less of: corn syrup solids, leavening (baking soda, cream of tartar, ammonium bicarbonate), rice starch (caramel color added), salt, natural flavors, pectin, canola oil, wheat flour, wheat gluten, sodium stearoyl lactylate, calcium stearoyl lactylate and date (dough conditioner).	33.77 ± 0.01	16.78 ± 0.08	2.3 ppm
Biscuits-4	Unbleached enriched wheat flour, milk chocolate, sugar, vegetable oils, macadamia nuts, butter, brown sugar, eggs, coconut, contains 2 percent or less of: leavening, salt, cornstarch, natural flavors. Tree nut meal.	33.39 ± 0.01	14.78 ± 0.01	3 ppm
Biscuits-5	Semi-sweet chocolate (sugar, chocolate liquor, cocoa butter, dextrose, soy lecithin added as an emulsifier, vanilla extract), unbleached enriched wheat flour (flour, niacin, reduced iron, thiamin mononitrate (vitamin B1), riboflavin (vitamin B2), folic acid), sugar, (palm oil), butter (milk), whole eggs, brown sugar, contains 2 percent or less of: leavening (baking soda, ammonium bicarbonate, cream of tartar), butter oil, salt, natural flavor and caramel color.	34.52 ± 0.03	14.23 ± 0.01	1.3 ppm
Biscuit-6	Wheat flour, chocolate chunks 15% (sugar, cocoa mass, cocoa butter, emulsifier: soy lecithin; natural vanilla), butter, sugar, hazelnuts 9%, vegetable oils, milk chocolate chunks 4% (sugar, cocoa butter, milk powder, cocoa mass, emulsifier: soy lecithin; natural vanilla), invert sugar syrup, skimmed milk powder, raising agents (sodium bicarbonate, acid sodium pyrophosphate), salt, natural flavoring. Contains nuts, milk, wheat, gluten, soy.	25.78 ± 0.02	15.01 ± 0.02	>50 ppm
Biscuit-7	Cereal flours (oats, rice, corn), sugar, plant oil, hazelnuts 4.5%, chocolate 4.5% (sugar, cocoa paste, thin powder cocoa, flavor), thin cocoa powders, skimmed dried milk, lactose, salt, almonds, dyes (carotenoid, rocou), emulsifier (lecithin of sunflower), antioxidant (palmitate of ascorbyl, alpha-tocopherol), vitamins (PP, B6, B2, B1, B9, B12), iron. Allergens: hazelnut, almond and other nuts, milk, oats.	35.95 ± 0.01	13.78 ± 0.01	0.5 ppm

(continued on next page)

Table 5 (continued)

Commercial food product	Ingredients declared on the label	P.S.S. (Cp)	P.A.C. (Cp)	PPMS
Nut bar-1	Almonds, raisins, sesame seeds, agave nectar, sunflower seeds, brown rice syrup, pumpkin seeds, whole grain brown rice crisps. Certified organic. May contain sesame, tree nuts and soy.	30.42 ± 0.01	15.12 ± 0.08	26 ppm
Nut bar-2	Raisins 23.3%: vegetable oil, glucose syrup, toasted wheat flakes, oats, crisped rice 8.7%: rice flour 7.8%, malted barley flour, sugar 0.4%, sunflower oil, emulsifier: sunflower lecithin, apricots 5.8%: apricots 5.7%, vegetable oil, preservative: sulfur dioxide], honey 5.7%, sugar 5.0%, vegetable fat, humectants: vegetable glycerine, flavoring, acidity regulator: citric acid. Contains wheat, barley, oats, gluten and sulfur dioxide. May contain traces of nuts and/or seeds.	34.12 ± 0.01	14.12 ± 0.01	1.8 ppm
Nut bar-3	Raisins 20%: vegetable oil, crisped rice 20%: rice flour 17.9%, malted barley flour, sugar 1%, sunflower oil, emulsifier: sunflower lecithin, glucose syrup, plain chocolate chips 9%: sugar 4.5%, cocoa mass 4.1%, cocoa butter 0.3%, emulsifier: soya lecithin, invert sugar syrup 6.6%: sugar 5.4%, water, oats, toasted wheat flakes, vegetable fat, honey 2.5%, milk chocolate 2.4%: sugar 1.1%, cocoa butter 0.5%, whole milk powder, cocoa mass 0.3%, whey powder, emulsifier: soya lecithin, invert sugar syrup 2%: sugar 1.6%, water, humectants: glycerine. Contains wheat, barley, oats, gluten, milk and soy. May contain traces of nuts and/or seeds.	35.31 ± 0.01	14.12 ± 0.01	0.7 ppm
Nut bar-4	Raisins 22.7%: vegetable oil, glucose syrup, crisped rice 12.3%: rice flour 11%, malted barley flour, sugar 0.6%, sunflower oil, emulsifier: sunflower lecithin, toasted wheat flakes, sweetened dried cranberries 6.3%: sugar 3.8%, cranberries 2.4%, sunflower oil, honey 5.9%, barley flakes, sugar 5.7%, oats, vegetable fat, humectants vegetable glycerine, blueberries 1.6%: blueberries 1.0%, honey 0.5%, sunflower oil, acidity regulator: citric acid, flavoring. Contains wheat, barley, oats and gluten. May contain traces of nuts and/or seeds.	35.12 ± 0.01	14.31 ± 0.01	0.8 ppm
Cereal-1	Rolled oats (33%), wheat (19%), sugar, sunflower oil (10%), glucose syrup, Brazil nuts (3.3%), hazelnuts (3.3%), toasted almonds (2.8%), pecan nuts (2.0%), rice flour (1.4%), wheat flour, honey (0.5%), color (plain caramel), hazelnut flavoring, antioxidant (tocopherol). May contain nuts.	18.14 ± 0.02	15.12 ± 0.08	>50 ppm
Chocolate Cream-1	Skim milk 46%, chocolate with hazelnuts 28% (cocoa paste, sugar, cocoa butter, hazelnuts 21%, emulsifier, soy lecithin, natural vanilla), sugar, fiber, cocoa powder, cocoa paste, modified starch, maltodextrin.	33.41 ± 0.01	14.26 ± 0.01	2.9 ppm
Chocolate Cream-2	Skim milk 48%, chocolate 21% (cocoa paste, sugar, cocoa butter, emulsifier, soy lecithin, natural vanilla), sugar, fiber, cocoa powder, cocoa paste, modified starch, maltodextrin.	33.19 ± 0.02	15.45 ± 0.02	3.5 ppm
Beverage-1	Water, hazelnuts 7%, raw cane sugar, corn maltodextrin.	33.02 ± 0.01	14.45 ± 0.02	3.9 ppm
Beverage-2	Water, almond 7%, raw cane sugar, corn maltodextrin.	35.25 ± 0.01	16.11 ± 0.08	0.8 ppm
Rice cake-1	Dark chocolate 54%, sugar, cocoa mass, cocoa butter, cocoa powder, soy lecithin. This product is manufactured in a facility that uses soy, gluten, milk and nuts.	34.88 ± 0.01	14.34 ± 0.08	1 ppm
Rice cake-2	Corn 66%, dark chocolate 34%, sugar, cocoa mass, cocoa butter, cocoa powder, soy lecithin, salt, sunflower oil. This product is manufactured in a facility that uses soy, gluten, milk and nuts.	35.30 ± 0.02	15.02 ± 0.08	0.7 ppm

P.S.S.: Peanut-specific system on the *Internal Transcribed Spacer*.

P.A.C.: Positive amplification control for the peanut system on the *18S rRNA* gene.

(Fig. 3B) and −3.19 for heat treated peanut/wheat *ITS* peanut-specific system (Fig. 3C), being near to the slope's value of −3.32 corresponding to a 100% PCR efficiency.

C_p values obtained from peanut calibration curves, plotted against the logarithm of the DNA concentrations, were used to test the sensitivity of the real-time PCR method. Fig. 3 also shows the discriminating capacity, which is the least difference in logarithm of target DNA concentration in the sample that the analytical method can discriminate with a significant level. Linearity of the peanut real-time PCR response was also analyzed. In each PCR system, the following parameters were evaluated: (a) Cochran's test, which determined whether the variances of the responses obtained for each concentration of peanut DNA in the reference feeds were homogeneous, (b) regression analysis and (c) variance analysis with lack of-fit. All parameters were evaluated following previously described validation protocols.

The accuracy of the peanut assays was determined from the six P.S.S. percentages on the *ITS* (0.1, 1, 10, 100, 1000 and 10,000 ppm) of reference samples. Accuracy is reported as percentage recovery by the assay of known added amounts of analyte. Using the Snedecor *F*-test, it was verified that the concentration of DNA present in a sample did not affect the variation of the results. The values for

Student's *t*-test obtained demonstrated that the method was accurate, since no significant differences between 100% and the mean recovery values were detected.

3.2.4. Commercial food products analysis

The method was also tested with regard to its suitability for the detection of peanut DNA in commercial food products. In this way, the *ITS* peanut-specific system was applied to the analysis of a collection of 123 commercial food products of different brands with different label declaration: 20 containing peanut as an ingredient, 30 with traces of peanut, 66 that had traces of tree nuts and 7 that had no traces of peanuts nor tree nuts. PCR results were found to conform to the labeling in all samples except for 27 food samples with undeclared peanut in the labeling (Table 4).

To assess the quantification ability of the assay, extrapolation analysis of the C_p data obtained for different commercial products (chocolates, cereal, nut bars, ice cream etc.) was carried out using the linear regression equations of the reference curves in order to determine whether the detected peanut content (%) of each sample fitted with the declared (real) one. Table 5 shows the average C_p values obtained for all the food commercial samples and the content estimated for these food products by substitution of the C_p s in the

corresponding P.S.S. equations. From the extrapolation of the different C_p s obtained for this food samples, we estimated that: 23 of the 27 samples with an undeclared peanut content contained less than 10 ppm of peanut, whereas from the other four: 1 contained 26 ppm and the other 3 contained more than 50 ppm of peanut. For those commercial food products that gave positive the presence of peanut, it was probably due to contamination during the production, as they were produced by companies, which also process peanuts.

The degree of contamination of commercial food samples with peanuts, whether fraudulent or accidental, can cause a serious public health problem in sensitive individuals and, therefore, detection methods should provide a selective and sensitive detection level. One of the achievements of the assay described in this work is the ability to specifically detect down to 0.1 ppm of the target in food samples. This represents a major advantage compared to the very limited working range of detection of several ELISA kits (Keck-Gassenmeier et al., 1999; Poms et al., 2005) and a lower detection level achieved in comparison to many other immunoassay techniques described up to date (Pomés et al., 2003).

On the other hand among PCR techniques, real-time PCR approaches (Hird et al., 2003) report higher or similar detection levels when attempting the identification of peanut: Scaravelli et al. (2008) reported a real time PCR based on the *Arah3* gene with a detection limit of 10 ppm. Whereas Stephan and Vieths (2004), described a real time PCR on the *Arah2* gene combined with a sandwich ELISA with a detection limit of 2 ppm Koppel et al. (2010), developed two tetraplex real time PCR assays for the detection of several allergens, with a detection limit of 100 ppm on peanut based on *Arah2* gene. Finally, Watanabe et al. (2006) reported a PCR assay based on *Arah3* combined with gel electrophoresis achieving a limit of detection of 10 ppm. Recently Pollet et al. (2011) has described for the first time an optical fiber SPR biosensor for peanut detection based on *Arah1*, with a limit of detection between 0.1 and 1 ppm.

This observation suggests that the real-time PCR method proposed herein is beneficial because due to its high sensitivity it facilitates the detection of peanut traces in commercial food products. Moreover, this real-time PCR can also be useful for monitoring the effectiveness of cleaning processes from production units of the food industry, and as consequence, they can help to prevent the food allergic consumer from unintentional ingestion of hidden allergens.

4. Conclusions

The ITS peanut specific system was applied to the analysis of a collection of 123 commercial food products, and PCR results were found to conform to the labeling in all samples but 27 which gave positive results by real time PCR, and didn't declare the presence of peanut nor it's traces in the labeling. This demonstrates the potential of the real-time PCR-based method to produce accurate results at the analysis of commercial products. As demonstrated, the presented PCR method is highly sensitive and selective, which makes it suitable for the detection of small amounts of peanuts in food samples. The method is relatively straightforward, it can be easily implemented in any analytical laboratory routinely performing real-time PCR. Consequently, this methodology could be used in inspection programs to enforce accurate labeling of commercial food products, thereby protecting both producers and consumers against peanut adulteration and misrepresentation.

Acknowledgments

This work was supported by the Programa de Vigilancia Sanitaria 2009/AGR/1489 of the Comunidad de Madrid (Spain) and by

a project (AGL2010/15279) from the Ministerio de Ciencia e Innovación (Spain). Inés María López-Calleja Díaz is recipient of a Juan de la Cierva grant from the Ministerio de Ciencia e Innovación (Spain).

References

- Arslan, A., Irfan Ilhak, O., & Calicioglu, M. (2006). Effect of method of cooking on identification of heat processed beef using polymerase chain reaction (PCR) technique. *Meat Science*, 72, 326–330.
- Bellagamba, F., Comincini, S., Ferretti, L., Valfre, F., & Moretti, V. M. (2006). Application of quantitative real-time PCR in the detection of prion-protein gene species-specific DNA sequences in animal meals and feedstuffs. *Journal of Food Protection*, 69, 891–896.
- Besler, M. (2001). Determination of allergens in food. *Trends in Analytical Chemistry*, 20, 662–672.
- Bock, S. A., Muñoz-Furlong, A., & Sampson, H. A. (2001). Fatalities due to anaphylactic reactions to foods. *Journal of Allergy and Clinical Immunology*, 107, 191–193.
- Camacho, M. A., Torres, A. I., Gil-Alegre, M. E., Obregón, M. M., & Ruz, V. (1993). Validation protocol of analytical methods for finished pharmaceutical products. *STP Pharma Pratiques*, 3, 197–202.
- Cawthraw, S., Saunders, G. C., Martin, T. C., Sawyer, J., Windl, O., & Readney, S. D. (2009). Real-time PCR detection and identification of prohibited mammalian and avian material in animal feeds. *Journal of Food Protection*, 72, 1055–1062.
- Engler-Blum, G., Raiss, Ch., & Burgmaier-Thielert, E. (2007). Detection of allergens in j food using real-time polymerase chain reaction and immunoassays: sensitive methods for detection of peanut and hazelnut – a comparative approach. *Deutsche Lebensmittel- Rundschau*, 103, 101–108.
- Fajardo, V., González, I., Martín, I., Rojas, M., Hernández, P. E., García, T., et al. (2009). A LightCycler TaqMan PCR assay for quantitative detection of chamois (*Rupicapra rupicapra*) and Pyrenean ibex (*Capra pyrenaica*) in experimental meat mixtures. *International Journal of Food Science and Technology*, 44, 1997–2004.
- Frezza, D., Giambra, V., Chegiani, F., Fontana, C., Maccabiani, G., Losio, N., et al. (2008). Standard and Light-Cycler PCR methods for animal DNA species detection in animal feedstuffs. *Innovative Food Science and Emerging Technology*, 9, 18–23.
- Fumière, O., Dubois, M., Baeten, V., von Holst, C., & Berben, G. (2006). Effective PCR detection of animal species in highly processed animal byproducts and compound feeds. *Analytical and Bioanalytical Chemistry*, 385, 1045–1054.
- Gašparić, M. B., Tengs, T., La Paz, J. L., Holst-Jensen, A., Pla, M., Esteve, M., et al. (2010). Comparison of nine different real-time PCR chemistries for qualitative and quantitative applications in GMO detection. *Analytical and Bioanalytical Chemistry*, 396, 2023–2029.
- Hefle, S., & Koppelman, S. (2006). *Detecting allergens in food*. Cambridge, UK: Woodhead Publishing Limited.
- Helm, R. M., & Burks, A. W. (2000). Mechanisms of food allergy. *Current Opinion in Immunology*, 12, 647–665.
- Hirao, T., Watanabe, S., Temmei, Y., Hiramoto, M., & Kato, H. (2009). Qualitative polymerase chain reaction methods for detecting major food allergens (peanut, soybean, and wheat) by using Internal Transcribed Spacer region. *Journal of AOAC International*, 92, 1464–1471.
- Hird, H., Lloyd, J., Goodier, R., Brown, J., & Reece, P. (2003). Detection of peanut using real-time polymerase chain reaction. *European Food Research and Technology*, 217, 265–268.
- Hird, H., Chisholm, J., Sánchez, A., Hernández, M., Goodier, R., Schneede, K., et al. (2006). Effect of heat and pressure processing on DNA fragmentation and implications for the detection of meat using a real-time polymerase chain reaction. *Food Additives and Contaminants*, 23, 645–650.
- Holland, P. M., Abramson, R. D., Watson, R., & Gelfand, D. H. (1991). Detection of specific polymerase chain reaction product by utilizing the 5' to 3' exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proceedings of the National Academy of Sciences USA*, 88, 7276–7280.
- Hourihane, J. O' B., Kilburn, S. A., Nordlee, J. A., Hefle, S. L., Taylor, S. L., et al. (1997). An evaluation of the sensitivity of subjects with peanut allergy to very low doses of peanut protein: a randomized, double-blind, placebo-controlled food challenge study. *Journal of Allergy and Clinical Immunology*, 100, 596–600.
- Immer, U. (2006). *Detecting allergens in food*. Cambridge: Woodhead Publishing Limited.
- International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). (2005). *Harmonised tripartite guideline. Validation of analytical procedures: Text and methodology Q2 (R1)*. Available at <http://drugtiger.com/d/gdoc/media417.pdf> <http://drugtiger.com/d/gdoc/media417.pdf>.
- Keck-Gassenmeier, B., Benet, S., Rosa, C., & Hischenhuber, C. (1999). Determination of peanut traces in food by a commercially-available ELISA test. *Food and Agricultural Immunology*, 11, 243–250.
- Kiening, M., Niessner, R., Drs, E., Baumgartner, S., Skrsa, R., Bremer, M., et al. (2005). Sandwich immunoassays for the determination of peanut and hazelnut traces in foods. *Journal of Agricultural and Food Chemistry*, 53, 3321–3327.
- Koppelman, S. J., Vlooswijk, R. A., Knippels, L. M., Hessing, M., Knol, E. F., Van Reijssen, F. C., et al. (2001). Quantification of major peanut allergens *Arah1* and *Arah2* in the peanut varieties Runner, Spanish, Virginia, and Valencia, bred in different parts of the world. *Allergy*, 56, 132–137.

- Koppel, R., Dvorak, V., Zimmerli, F., Breitenmoser, A., Eugster, A., & Waiblinger, H. U. (2010). Two tetraplex real-time PCR for the detection and quantification of DNA from eight allergens in food. *European Food Research and Technology*, 230, 367–374.
- López-Calleja, I., González, I., Fajardo, V., Martín, I., Hernández, P. E., García, T., et al. (2007). Quantitative detection of goats' milk in sheep's milk by real-time PCR. *Food Control*, 18, 1466–1473.
- Mendoza-Romero, L., Verkaar, E. L. C., Savelkoul, P. H., Catsburg, A., Aarts, H. J. M., Buntjer, J. B., et al. (2004). Real-time PCR detection of ruminant DNA. *Journal of Food Protection*, 67, 550–554.
- Official Journal of the European Union. (2011). Regulation (EU) 1169/2011/EC of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004, L304, 18–43.
- Ortolani, C., Ipsano, M., Scibilia, J., & Pastorello, E. A. (2001). Introducing chemists to food allergy. *Allergy*, 56, 5.
- Platteau, C., De Loose, M., De Meulenaer, B., & Taverniers, I. (2011). Detection of allergenic ingredients using real-time PCR: a case study on hazelnut (*Corylus avellana*) and soy (*Glycine max*). *Journal of Agricultural and Food Chemistry*, 59, 10803–10814.
- Pollet, J., Delpont, F., Janssen, K. P., Tran, D. T., Wouters, J., Verbiest, T., et al. (2011). Fast and accurate peanut allergen detection with nanobead enhanced optical fiber SPR biosensor. *Talanta*, 83, 1436–1441.
- Pomés, A., Helm, R. M., Bannon, G. A., Burks, A. W., Tsay, A., & Chapman, M. D. (2003). Monitoring peanut allergen in food products by measuring *AraH1*. *Journal of Allergy and Clinical Immunology*, 111, 640–645.
- Poms, R. E., Agazzi, M. E., Bau, A., Brohée, M., Capelletti, C., Nørgaard, J. V., et al. (2005). Interlaboratory validation study of five commercial ELISA test kits for the determination of peanut proteins in biscuits and dark chocolate. *Food Additives and Contaminants*, 22, 104–112.
- Prado, M., Berben, G., Fumière, O., van Duijn, G., Mensinga-Kruize, J., Reaney, S., et al. (2007). Detection of ruminant meat and bone meals in animal feed by real-time polymerase chain reaction: result of an interlaboratory study. *Journal of Agricultural and Food Chemistry*, 55, 7495–7501.
- Prado, M., Fumière, O., Boix, A., Marien, A., Berben, G., & von Holst, C. (2009). Novel approach for interlaboratory transfer of real-time PCR methods: detecting bovine meat and bone meal in feed. *Analytical and Bioanalytical Chemistry*, 394, 1423–1431.
- Pumphrey, R. (2001). Fatal allergic reactions to food. *Food Allergy Intolerance*, 2, 91.
- Rossi, S., Scaravelli, E., Germini, A., Corradini, R., & Marchelli, R. (2006). A PNA-array platform for the detection of hidden allergens in foodstuffs. *European Food Research and Technology*, 223, 1–6.
- Sampson, H., Mendelson, L., & Rosen, J. (1992). Fatal and near-fatal anaphylactic reactions to food in children and adolescents. *New England Journal of Medicine*, 327, 380–384.
- Sampson, H. A. (2005). Food allergy: accurately identifying clinical reactivity. *Allergy*, 60, 19–24.
- Scaravelli, E., Brohee, M., Marchelli, R., & Van Hengel, A. (2008). Development of three real-time PCR assays to detect peanut allergen residue in processed food products. *European Food Research and Technology*, 227, 857–869.
- Scaravelli, E., Brohee, M., Marchelli, R., & Van Hengel, A. (2009). The effect of heat treatment on the detection of peanut allergens as determined by ELISA and real-time PCR. *Analytical and Bioanalytical Chemistry*, 395, 127–137.
- Sforza, S., Scaravelli, E., Corradini, R., & Marchelli, R. (2005). Unconventional method based on circular dichroism to detect peanut DNA in food by means of a PNA probe and a cyanine dye. *Chirality*, 17, 515–521.
- Sicherer, S. H., Muñoz-Furlong, A., Murphy, R., Wood, R. A., & Sampson, H. A. (2003). Symposium: pediatric food allergy. *Pediatrics*, 111, 1591.
- Sicherer, S. H., Muñoz-Furlong, A., & Sampson, H. A. (2004). Prevalence of seafood allergy in the United States determined by a random telephone survey. *Journal of Allergy and Clinical Immunology*, 114, 159–165.
- Stephan, O., & Vieths, S. (2004). Development of a real-time PCR and a sandwich ELISA for detection of potentially allergenic trace amounts of peanut (*Arachis hypogaea*) in processed foods. *Journal of Agricultural and Food Chemistry*, 52, 3754–3760.
- Taylor, S. L., Nordlee, J. A., Niemann, L. M., & Lambrecht, D. M. (2009). Allergen immunoassays—considerations for use of naturally incurred standards. *Analytical and Bioanalytical Chemistry*, 395, 83–92.
- Van Raamsdonk, L. W. D., von Holst, C., Baeten, V., Berben, G., Boix, A., & de Jong, J. (2007). New developments in the detection and identification of processed animal proteins in feeds. *Animal Feed Science and Technology*, 133, 63–83.
- Watanabe, T., Akiyama, H., Maleki, S., Yamakawa, H., Iijima, K., Yamazaki, F., et al. (2006). A specific qualitative detection method for peanut (*Arachis hypogaea*) in foods using polymerase chain reaction. *Journal of Food Biochemistry*, 30, 215–233.
- Yancy, H. F., Washington, J. D., Callahan, L., Mason, J. A., Deaver, C. M., Farrell, D. E., et al. (2009). Development, evaluation, and peer verification of a rapid real-time PCR method for the detection of animal material. *Journal of Food Protection*, 72, 2368–2374.
- Yunginger, J., Sweeney, K., Sturmer, W., Giannandrea, L., Teigland, J., Bray, M., et al. (1988). Fatal food-induced anaphylaxis. *Journal of the American Medical Association*, 260, 1450–1452.