

Assessing hazelnut allergens by protein- and DNA-based approaches: LC-MS/MS, ELISA and real-time PCR

Joana Costa · Parisa Ansari · Isabel Mafra ·
M. Beatriz P. P. Oliveira · Sabine Baumgartner

Received: 4 December 2013 / Revised: 28 January 2014 / Accepted: 3 February 2014 / Published online: 28 February 2014
© Springer-Verlag Berlin Heidelberg 2014

Abstract Hazelnut (*Corylus avellana* L.) is responsible for a significant part of the allergies related to nuts. Still, it is a very much appreciated nut and as consequence is widely used in all types of processed foods, such as chocolates. Correct food labelling is currently the most effective means of preventing the consumption of allergenic ingredients, namely hazelnut, by the sensitised/allergic individuals. Thus, to verify labelling compliance and to ensure allergic patient protection, the development of highly sensitive methodologies is of extreme importance. In this study, three major methodologies, namely enzyme-linked immunosorbent assays (ELISA), liquid chromatography coupled with mass spectrometry and real-time polymerase chain reaction, were evaluated for their performance regarding the detection of hazelnut allergens in model chocolates. The sandwich ELISA and respective antibodies were in-house developed and produced. With sensitivity levels of approximately 1 mg kg^{-1} and limits of quantification of $50\text{--}100 \text{ mg kg}^{-1}$, all the performed methods were considered appropriate for the identification of hazelnut in complex foods such as chocolates. To our knowledge, this was the first successful attempt to develop and compare three independent approaches for the detection of allergens in foods.

Keywords Hazelnut detection · Complex foods · Model chocolates · DNA-based methods · Protein-based techniques · Food allergy

Introduction

In the last years, food-induced allergies have been regarded with special interest. The number of individuals suffering from some kind of food allergy is not yet well established, although it is generally estimated to affect 1–10 % of the world's population [1]. Theoretically, any food can be considered as potentially allergenic; however, more than 90 % of the adverse immunological responses are caused by eight groups of foods, namely milk, egg, fish, crustaceans, tree nuts, peanut, soybean and wheat. As consequence, these foods are usually the focus of several studies, since they are also accountable for most of the severe and potentially life-threatening allergic reactions.

Included in the tree nut group, hazelnut (*Corylus avellana* L.) is responsible for a significant part of the allergies related to nuts. Often associated with birch pollinosis [2] and with an overall incidence of 7.2 % [3], allergy induced by hazelnut is one of the most extensively studied. According to the WHO-IUIS list of allergens [4] and to the Allergome database [5], 11 groups of allergenic proteins belonging to a large spectrum of protein families have been identified and characterised, namely Cor a 1, Cor a 2, Cor a 8, Cor a 9, Cor a 11, Cor a 12, Cor a 13, Cor a 14 and Cor a TLP (as food allergens) and Cor a 6 and Cor a 10 (as pollen allergens), which have been extensively reviewed by Costa et al. (Hazelnut allergens: molecular characterisation, detection and clinical relevance, in review). Clinical symptoms associated with hazelnut allergy are known to range from mild (mostly restricted to oral allergy syndrome) to potentially fatal (anaphylaxis). Until now, no effective treatments concerning hazelnut allergy are available; thus, for the sensitised/allergic individuals, the only actual means of preventing allergic reactions consists mainly of the total avoidance of the offending food (Costa et al., Hazelnut allergens: molecular characterisation, detection and clinical relevance, in review).

Accordingly, the correct labelling of processed foods is of utmost importance for maximising the quality of life of

J. Costa · P. Ansari · S. Baumgartner (✉)
Christian Doppler Laboratory for Rapid Test Systems for Allergenic Food Contaminants, Center for Analytical Chemistry, Department IFA-Tulln, University of Natural Resources and Life Sciences, Vienna, Konrad Lorenz Str. 20, 3430 Tulln, Austria
e-mail: sabine.baumgartner@boku.ac.at

J. Costa · I. Mafra · M. B. P. P. Oliveira
REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal

these individuals. In this context, to verify the compliance with labelling and to ensure the protection of such patients, adequate and reliable analytical methods are required for the control of hidden allergenic ingredients in processed foods. In the past years, several protein- and/or DNA-based methods have been advanced for the specific analysis of hazelnut in foods (Costa et al., Hazelnut allergens: molecular characterisation, detection and clinical relevance, in review). So far, the methods based on the direct detection of hazelnut allergenic and/or marker proteins have been more extensively applied. Techniques such as the enzyme-linked immunosorbent assays (ELISA) are considered easy to perform with no need for expensive equipment or specialised personnel, being therefore among the most widely used for the detection and quantification of hazelnut in foods [6–12]. However, the high propensity of the antibodies to cross-react with other plant or animal species constitutes one of the main challenges for developing these protein-based methods. More recently, to overcome these drawbacks, the mass spectrometry (MS) platforms have been regarded as excellent tools for the detection of allergenic proteins, namely hazelnut in processed foods [13–15]. In the same sense, but targeting a different biological molecule, the DNA-based techniques have also attained special emphasis for the detection of allergenic foods such as hazelnut. Although using an indirect approach for the assessment of the offending foods, the methods based on the polymerase chain reaction (PCR) have been widely employed for the detection and quantification of this nut especially in processed foods, where the proteins are known to lose their integrity upon processing [16–20]. However, none of the proposed methods have yet been made official for hazelnut or for any other allergenic food. The lack of harmonisation regarding this essential topic continues to contribute to the generalised controversy among researchers and represents key issues in the management of food allergens (Costa et al., Hazelnut allergens: molecular characterisation, detection and clinical relevance, in review).

In this work, we intended to exploit three different methodologies for the detection of hazelnut in model chocolates. The methods are based on the most representative systems used for the detection of allergens in foods: ELISA, liquid chromatography coupled with mass spectrometry (LC-MS/MS) and real-time PCR. Using the same set of matrices, all systems were developed, tested and compared for their performance regarding their capacity to detect hazelnut in chocolates. All the advantages and drawbacks of each technique were also accounted in this study. To our knowledge, this is the first successful attempt to compare these three systems based on different target analytes (protein versus DNA) for the assessment of hazelnut allergens in complex food matrices.

Material and methods

Reagents

All the reagents used for ELISA were of analytical grade. The water used for the preparation of buffers and solutions was either purified by reverse osmosis or drawn from a Milli-Q plus system (Millipore, Molsheim, France). Ammonium bicarbonate ($\geq 99\%$), sodium bicarbonate, sodium carbonate, 1,4-dithiothreitol (DTT, $\geq 99\%$), acetonitrile (HPLC gradient grade) and milk powder were purchased from Carl Roth GmbH (Karlsruhe, Germany). 3,3',5,5'-Tetramethylbenzidine (TMB, 89 %), tris[hydroxymethyl] aminomethane (Trizma® base, 99 %), ethylenediaminetetraacetic acid (EDTA, 99 %), bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane (Bis-Tris, $\geq 98\%$), bovine serum albumin (BSA, fraction V, $\geq 96\%$), ammonium acetate (MS grade), hydrogen peroxide (30 wt%, semiconductor grade), Tween 20, dimethyl sulfoxide (DMSO) and iodoacetamide were acquired from Sigma-Aldrich (Steinheim, Germany). Bis(2-ethylhexyl)sulfosuccinate sodium salt (DONS) was acquired from Fluka Chemie AG (Buchs, Switzerland). Ammonium acetate, ethanol (99 % denatured) and sodium azide were obtained from J.T. Baker B.V. (Deventer, Holland). Sodium chloride and citric acid were purchased from Merck (Darmstadt, Germany) and trypsin (sequencing grade modified) from Roche Applied Science (Mannheim, Germany). BCA Protein Assay kit for protein content determination was purchased from Thermo Scientific (Rockford, IL, USA).

Model chocolate preparation

Dried hazelnuts and chocolate with 41 % of cocoa used for the preparation of model chocolates were purchased at local markets in Portugal. Hazelnut kernels were grounded to a fine powder in a laboratory knife mill Grindomix GM200 (Retsch, Haan, Germany). Model chocolates spiked with 50, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005 and 0.0001 % of hazelnut were prepared. Chocolate was melted and maintained at 40 °C during the entire procedure to guarantee correct and complete homogenisation of hazelnut material. The first mixture containing 50 % of hazelnut was prepared by adding 100 g of grounded hazelnut to 100 g of melted chocolate. All the following spiked chocolates were prepared by stepwise addition of melted chocolate. The solidified model chocolates were chopped into pieces of approximately 0.3 mm of diameter (Grindomix GM200) and immediately stored at -20 °C until further analysis.

ELISA system

Protein extraction

The extraction of protein from grounded hazelnut and model hazelnut chocolates used in ELISA system was performed

according to the described by Costa et al. (Development of a sandwich ELISA-type system for the detection and quantification of hazelnut in model chocolates, submitted). One gram of grounded material was weighted out with the addition of 1 g of milk powder. To each mixture, 10 mL of 0.1 mol/L of NH_4HCO_3 was added as the extraction buffer. All mixtures were incubated at 60 °C for 30 min with occasional mixing. After incubation, they were centrifuged for 15 min ($9,400\times g$, 4 °C) and the supernatant transferred to a new 15 mL falcon tube. If supernatant presented suspended particles, it was further filtrated using a fibre glass filter MN 85/90 BFØ 45 mm (Macherey-Nagel GmbH & Co., Düren, Germany) and a cellulose acetate filter (0.2 µm/pore, Sartorius Stedim Biotech GmbH, Goettingen, Germany) to obtain a clear supernatant. The protein content of extracts was assessed with the commercial BCA protein assay, according to manufacturer's instructions.

ELISA

The anti-hazelnut antibodies (polyclonal from rabbit and monoclonal from mouse) were produced in-house as described by Costa et al. (Development of a sandwich ELISA-type system for the detection and quantification of hazelnut in model chocolates, submitted). The third antibody, anti-mouse-IgG labelled with horseradish peroxidase (anti-mouse-IgG-HRP) used for ELISA was acquired from Sigma-Aldrich (Steinheim, Germany).

ELISA was carried out using a non-competitive assay, namely sandwich ELISA developed in-house (Costa et al., Development of a sandwich ELISA-type system for the detection and quantification of hazelnut in model chocolates, submitted) using the following buffers and solutions: 0.2 mol/L of phosphate-buffered saline (PBS) (0.23 mol/L of Na_2HPO_4 , 0.022 mol/L of NaH_2PO_4 , 0.36 mol/L of NaCl, pH 7.5), coating buffer (11.5 mmol/L of Na_2CO_3 , 39 mmol/L of NaHCO_3 , 1.5 mmol/L of NaN_3 , pH 9.6), washing solution PBST (0.01 mol/L of PBS, 0.1 % Tween 20), assay buffer (0.04 mol/L of PBS, 0.08 % Tween 20, pH 7.5), substrate buffer (0.22 mol/L of citric acid, 0.67 mmol/L of k-sorbate , pH 4.0) and TMB stock solution (1.6 mmol/L of tetramethylbenzidine, 70 mmol/L of DMSO in 25 mL of methanol).

Each assay was performed in high-binding plates (Greiner bio-one, Frickenhausen, Germany) coated with 1 µg/mL of anti-hazelnut polyclonal antibody (IgG) from rabbit in coating buffer, overnight at 4 °C. Before blocking the ELISA plates, these were washed once with PBST and then blocked for 2 h at 37 °C with 2 % of milk powder in coating buffer. The plates were then incubated for 1 h at room temperature with the standards (hazelnut extracts serially diluted 1:5, ranging from 10,000 to 0.13 µg/mL) for the calibration curve and with the model chocolates diluted in blank chocolate solution to reduce the effects of the matrix. After incubation, the plates were washed twice with PBST solution and then 100 µL of anti-hazelnut monoclonal antibody from mouse (1.0 µg/mL) in assay buffer

was added to each well. Plates were again incubated for 1 h with continuous agitation. In order to obtain the colour reaction by the end of the assay, a third antibody conjugated to an enzyme was used. Thus, after washing the plate once with PBST, 100 µL of anti-mouse-IgG labelled with HRP (1.0 µg/mL) in assay buffer was added to each well and incubated for 1 h with continuous agitation. After washing again with PBST, the plates were incubated with 100 µL of substrate solution (substrate buffer pH 4.0, 0.02 % (v/v) of H_2O_2 , 30 and 0.8 % (v/v) of TMB stock solution) for approximately 2–3 min with continuous agitation and in dark conditions, until the formation of a consistent blue colour. The reaction was stopped by adding 30 µL of 1 mol/L of H_2SO_4 with the formation of yellow colour in the wells.

Calculations of the calibration curves and recovery

The calculations that allowed estimating the amount of hazelnut in the model chocolates were also performed according to Costa et al. (Development of a sandwich ELISA-type system for the detection and quantification of hazelnut in model chocolates, submitted). Briefly, the plates were read at 450 nm in a plate reader (Sunrise Remote A-5085, Tecan Group Ltd., Männedorf, Switzerland) and the absorbance results evaluated with Magellan5 software version 5.03 (Tecan Group Ltd., Männedorf, Switzerland). The absorbance values measured at 450 nm were plotted against the logarithmic concentration of the hazelnut protein standard solutions. A non-linear regression function was carried out using a sigmoid four parametric logistic function:

$$B \equiv Y = \frac{A-D}{1 + \left(\frac{X}{C}\right)^b} + D \quad (1)$$

where Y is the optical density (absorbance), A the maximum absorbance, b the slope of the calibration curve in linear range, C the 50 % inhibition concentration— IC_{50} (µg/L), D the minimum absorbance and X the analyte concentration (µg/L).

The protein recovery from hazelnut was estimated using the concentration values according with the following equation:

$$\text{Recovery(\%)} = \frac{\text{measured hazelnut protein concentration (mg kg}^{-1}\text{)}}{\text{estimated hazelnut protein concentration (mg kg}^{-1}\text{)}} \times 100 \quad (2)$$

LC-MS/MS system

Protein extraction and enzymatic digestion

The protein extraction was performed as described for ELISA. Two millilitres of each protein extract was transferred to a new reaction tube and completely dried overnight at room temperature in a vacuum centrifuge. The precipitate was then resuspended in 1,000 µL of 0.1 mol/L of NH_4HCO_3 , 6 mol/L urea buffer. Protein content was evaluated with BCA kit and

set to a final concentration of 10 mg/mL of protein. At this stage, the samples were digested according to the in-solution digestion protocol (<http://www.osa.sunysb.edu/Proteomics/ProteinDigestPrep.pdf>) with minor alterations introduced by Ansari et al. [13] and then analysed by LC-MS/MS.

Liquid chromatography–mass spectrometry

The chromatographic separation of the tryptic-digested peptides was carried out as described by Ansari et al. [13] with a 1290 infinity UHPLC (Agilent, Waldbronn, Germany), using an Aquasil C18 reverse phase column (50×2.1 mm, 3 µm particle size, Thermo Electron Corporation, Marietta, GA, USA) and a C18 4×3 mm i.d. security guard cartridge (Phenomenex, Torrance, CA, USA). Separation was performed using an injection volume of 5 µL and a flow rate of 0.5 mL/min. The mobile phase consisted of eluent A (10 % of acetonitrile, 5 mmol/L of ammonium acetate) and eluent B (95 % of acetonitrile, 5 mmol/L of ammonium acetate) used to create a gradient. The LC run started with an initial hold of 0.5 min, linear gradient from 3 to 40 % of B within 11.5 min, rapidly up to 100 % of B and 4 min hold, switch back to 3 % of B within 2 min and equilibration for 5 min.

For MS analysis, a QTrap 4000 LC-MS/MS system (Applied Biosystems, Foster City, CA, USA) with a Turbo V Ion Spray source was used. Chromatographic and (tandem) mass spectrometric data was analysed using the Analyst™ software version 1.5.2. The electrospray ionisation (ESI) source parameters used were defined for positive ionisation mode; curtain gas, 20 psi; both ion source gas 1 and 2, 50 psi; source temperature, 535 °C; and +4,000 V of ion spray voltage. The collision gas (nitrogen) was defined to high. For each selected reaction monitoring (SRM) transition, a dwell time of 40 ms was set and the pause between mass ranges was defined to 5 ms.

Real-time PCR system

DNA extraction

Based on previous work, the selection of the DNA extraction method was made regarding the best results both by end-point and real-time PCR systems [17, Costa et al., Tracing tree nut allergens in chocolate: a comparison of DNA extraction protocols, submitted]. The selected method for the extraction of DNA from model chocolates was based on the use of Nucleospin Food Kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions with minor modifications. Briefly, 200 mg of grounded material was transferred to a 2-mL sterile reaction tube followed by the addition of 700 µL of lysis solution CF pre-heated at 65 °C and 10 µL of proteinase K (20 mg/mL). After 1 h incubation at 65 °C with continuous stirring, 4 µL of RNase A (10 mg/mL) was added and the mixture incubated for 10 min at 37 °C with soft stirring. Each

mixture was then centrifuged for 10 min (17,000×g at 4 °C) and the supernatant (approximately 550 µL) transferred to a new sterile reaction tube. A second centrifugation step was performed for 5 min in the same conditions and about 450 µL of supernatant was collected to a new sterile reaction tube. To each mixture, equal volumes of precipitation solution C4 and ethanol 100 % were added. After careful homogenisation by inversion, mixtures were eluted in two steps through one spin column by centrifugation at room temperature (1 min, 13,000×g). The column was then washed twice with 400 µL of CQW solution and twice with (700 and 200 µL) C5 solution, with 1 min centrifugations (13,000×g) between washings and a 2-min final centrifugation. The DNA was eluted from the column by the addition of 100 µL of CE solution at 70 °C, incubation for 5 min at room temperature and centrifugation (1 min, 13,000×g). Each hazelnut model chocolate was extracted at least twice and the DNA extracts kept at −20 °C until further analysis.

UV spectrophotometric DNA quantification was carried out on a Synergy HT multi-mode microplate reader (BioTek Instruments, Inc., Vermont, USA), using a Take3 micro-volume plate accessory. DNA content was determined using the nucleic acid quantification protocol with sample type defined for double-strand DNA in the Gen5 data analysis software version 2.01 (BioTek Instruments, Inc., Vermont, USA).

Target gene selection

To evaluate the quality of the extracted DNA, all extracts were amplified by end-point PCR using universal eukaryotic primers (18SRG-F – CTGCCCTATCAACTTTCGATGGTA and 18SRG-R – TTGGATGTGGTAGCCGTTTCTCA), targeting a conserved region of 18S ribosomal RNA gene (NCBI accession no. HQ873432.1) [21].

For the specific detection of hazelnut, primers and probe targeting the hsp1 gene, which encodes a low molecular weight heat-shock protein with the same name, were selected from the available literature. Primers HSP1F (AGCGTCGA GAGTGGCAAGTTC) and HSP1R (CCTGCTCGCCTCCG CTTTC) were retrieved from Costa et al. [16] and hydrolysis probe NOCC1P (FAM-CCTGACGATGCGATGCTCGACC AG-BHQ2) was selected from Píknová et al. [22]. The oligonucleotides were synthesised by Eurofins MWG Operon (Ebersberg, Germany).

Real-time PCR

Real-time PCR assays were performed in 20 µL of total reaction volume. Each reaction tube contained 2 µL of DNA (20 ng), 1× of SsoFast Probes Supermix (BioRad, Hercules, CA), 300 nmol/L of each primer (Hsp1F/Hsp1R) and 150 nmol/L of hydrolysis probe Nocc1P. The real-time PCR assays were carried on a fluorometric thermal cycler CFX96 Real-time PCR Detection System (BioRad, Hercules, CA)

according to the following temperature protocol: 95 °C for 5 min, 50 cycles at 95 °C for 15 s and 66 °C for 45 s, with the collection of fluorescence signal at the end of each cycle. Data were collected and analysed using the software BioRad CFX Manager 3.0 (BioRad, Hercules, CA). Cycle threshold (Ct) values were calculated using the software at automatic threshold settings. Real-time PCR trials were repeated in three independent runs using four replicates each, respectively.

Results and discussion

Application of ELISA to detect hazelnut in chocolate

The ELISA system used for the evaluation of model chocolates spiked with known amounts of hazelnut was previously developed and validated (Costa et al., Development of a sandwich ELISA-type system for the detection and quantification of hazelnut in model chocolates, submitted). The estimated results for the relative contents of hazelnut in chocolates are presented in Table 1.

In general, the estimated values for hazelnut were in good agreement with the reference values for each model mixture. With the calculation of bias parameter, it was possible to determine that the samples until the spiked level of 0.005 % (50 mg/kg) of hazelnut in chocolate were within the acceptance criteria (≤ 25 %) [23]. In accordance to this, the limit of quantification (LOQ) set for this system corresponded to the lowest concentration of hazelnut in chocolate (50 mg/kg) with bias below 25 %. The model chocolates with the concentration of hazelnut below the LOQ could also be detected with the proposed ELISA, although they can only be interpreted as qualitative information. In comparison with the other ELISA systems, namely the ones developed by Rejeb et al. [10] and Holzhauser et al. [24], the proposed ELISA also enable to detect down to

1 mg/kg of hazelnut in chocolate. The accuracy and the repeatability of the method were ensured by the performance of four independent ELISA, using four replicates of each protein extract. Mean recovery and the relative repeatability standard deviation (RSD_r) were calculated for the four assays and are represented on Table 1. The results of recovery were also in good accordance with the parameters defined for the acceptance criteria of a quantitative protein-based method. Repeatability values ranged from 79.3 to 106.8 %, which are within the interval (70–120 %) recommended for this type of assays [23].

Application of LC-MS/MS to detect hazelnut in chocolate

Based on previously reported work [13], eight peptides from three hazelnut allergens (Cor a 8, Cor a 9 and Cor a 11) were detected in all model chocolates. The sequences of the eight peptides (Cor a 801, Cor a 901, Cor a 902, Cor a 903, Cor a 904, Cor a 1101, Cor a 1102 and Cor a 1103) and the performance parameters of LC-MS/MS runs are presented in Table 2. Results from hazelnut detection in model chocolates by LC-MS/MS, namely the concentrations of selected hazelnut peptides detected in the digested samples expressed in microgram of peptide per kilogram of chocolate and respective recoveries, are presented in Table 3. Figure 1 represents examples of extracted ion chromatograms (XIC) for Cor a 902 peptide of blank chocolate (A), digested dilution of hazelnut (50 %) (B) and model chocolate spiked with 50 % of hazelnut (C) with a recovery of 92.5 %. All the MS runs presented very good performance, which is confirmed by the mean correlation coefficients (R^2) above 0.98 as evidenced in Table 2. Recoveries were calculated as a ratio in percentage (%) of model chocolates with hazelnut and added digested nut at the same concentration. In general, the model chocolates evaluated by LC-MS/MS evidenced recoveries within the acceptance criteria (70–120 %), with only few exceptions revealing values below this

Table 1 Application of ELISA to model chocolates spiked with known amounts of hazelnut. Estimation of the quantity of hazelnut (%), bias, recovery and respective relative standard deviation for each model chocolate tested

Model chocolates spiked with hazelnut (%)	Estimated amount of hazelnut (%) $\pm$ SD ^a	Bias (%)	Recovery (%) ^b	RSD_r (%) ^c
50	52 $\pm$ 4.5	4.7	104.7	8.7
10	9.4 $\pm$ 0.5	6.1	93.9	4.8
5.0	5.3 $\pm$ 0.6	6.8	106.8	11.7
1.0	0.79 $\pm$ 0.1	20.7	79.3	12.1
0.50	0.51 $\pm$ 0.2	2.4	98.1	33.8
0.10	0.10 $\pm$ 0.01	1.2	101.2	6.2
0.050	0.052 $\pm$ 0.003	3.9	103.9	5.2
0.010	0.0086 $\pm$ 0.0002	13.9	86.1	2.6
0.0050	0.0048 $\pm$ 0.0010	4.0	96.0	20.8
0.0010	0.0032 $\pm$ 0.0003	222	–	–
0.00050	0.0024 $\pm$ 0.0003	381	–	–
0.00010	0.0019 $\pm$ 0.0003	1,815	–	–

^a Mean and standard deviation (SD)

^b Mean recovery (%)

^c Relative repeatability standard deviation (RSD_r) of the estimated value of hazelnut in chocolates for $n=16$ replicates performed in four independent assays

Table 2 MS performance parameters for the quantitative analysis of hazelnut peptides

Allergen	Peptide	MW (Da)	LOD (ng/mL)	LOQ (ng/mL)	Calibration curve $Y=b(\pm Sb) X$	Correlation coefficient (R^2)	Linear dynamic range (ng/mL)
Cor a 801	GIAGLNPNLAAGLPGK	1,461.8	1	10	257 ± 56	0.9987 ± 0.0005	6,250–1.25
Cor a 901	ALPDDVLANAFQISR	1,628.9	1	3	201 ± 43	0.9971 ± 0.0006	6,250–1.25
Cor a 902	QGQVLTIPQNFAVAK	1,612.9	1	5	212 ± 48	0.9975 ± 0.0022	6,250–1.25
Cor a 903	INTVNSNTLPVLR	1,439.8	1	3	211 ± 42	0.9988 ± 0.0010	6,250–1.25
Cor a 904	WLQLSAER	1,001.5	1	5	435 ± 64	0.9965 ± 0.0027	6,250–1.25
Cor a 1101	AFSWEVLEAALK	1,362.7	1	10	139 ± 28	0.9973 ± 0.0016	6,250–1.25
Cor a 1102	LLSGIENFR	1,047.6	1	10	784 ± 80	0.9977 ± 0.0013	6,250–1.25
Cor a 1103	ELAFNLPSR	1,045.6	1	10	399 ± 39	0.9965 ± 0.0016	6,250–1.25

interval (Table 3). The worst recoveries were retrieved from the analysis of peptides Cor a 1102 and Cor a 1103, probably due to the low concentration of these peptides in digested hazelnut, which is in good accordance with the results reported by Ansari et al. [13] during the development of the method.

According to MS analysis, the eight peptides were successfully detected in all model chocolates down to the lowest hazelnut spiking level of 0.0001 % (1 mg/kg), although the established LOQ values differed with the peptide. Regarding their quantification from different hazelnut allergens, the highest estimated amounts concerned the peptides from protein Cor a 9. This allergen is considered a major storage protein in hazelnut, which was confirmed by the results obtained in the MS analysis (Table 3). Considering MS performance parameters for the four peptides from Cor a 9 allergen, the LOQ was lower in the case of peptides Cor a 901 and Cor a 903, while for Cor a 902 and Cor a 904 the LOQ was approximately double. Although Cor a 8 and Cor a 11 peptides could also be detected down to the spiked level of 1 mg/kg of hazelnut in model chocolates (Table 3), the respective peptide LOQ values increased to 10 ng/mL, due to different ionisation characteristics (Table 2).

According to these results, the limit of detection (LOD) described in this study (1 mg/kg) is comparable to the LOD of 5 and 1.3 mg/kg reported by Heick et al. [15] and Bignardi et al. [14], respectively, although the LOQ presented in this study were slightly higher. In addition, eight different marker peptides were used in this study, while in the previous reports only that four [15] and two peptides [14] were targeted, which also enable to undoubtedly identify hazelnut in difficult and highly complex matrices such as chocolates.

Application of real-time PCR to detect hazelnut in chocolate

For the successful application of PCR-based methods, a determinant factor regards the quality and purity of DNA extracts. In the case of complex food matrices such as chocolates, the extraction of amplifiable DNA with adequate quality is highly dependent on the chosen DNA extraction method. According

to previous data (Costa et al., Tracing tree nut allergens in chocolate: a comparison of DNA extraction protocols, submitted), DNA from chocolates was extracted with the commercial Nucleospin Food Kit, presenting adequate concentration (50.7 ± 7.0 ng/ μ L) and high purity (1.7 ± 0.2). In spite of these results and in order to guarantee the absence of any possible false-negative results, all DNA extracts were previously evaluated for their amplifiability with primers 18SRG-F/18SRG-R to target a universal eukaryotic fragment [21]. All tested model chocolates amplified positively for the expected product of 113 bp, confirming that the DNA extracts presented the proper quality and purity for PCR amplification.

For the specific detection of hazelnut, the nucleotide sequence encoding the Hsp1 protein was targeted. Based on previous study [16], the designed primers were tested using 18 different cultivars of *C. avellana*, presenting positive amplification for all cultivars. Although in silico analysis also exhibit cross-reactivity with another close related hazel, namely *Corylus heterophylla*, this particular species was not tested. Besides this species from *Corylus* genus, the chosen primers did not present any cross-reactivity with other 28 species of plant foods, namely with several tree nuts.

For the evaluation of the developed real-time PCR system, several parameters were considered. The prerequisites used to test the real-time PCR were based on the available documents of the definition of minimum performance requirements for analytical methods of genetically modified organism testing [25] and the MIQE guidelines for the minimum information for publication of quantitative real-time PCR experiments [26].

The real-time PCR assays were performed using the already described reference mixtures ranging from 50 % down to 0.0001 % of hazelnut in chocolate. Figure 2 represents one of the calibration curves for real-time PCR system using model chocolates spiked with 50, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005 and 0.0001 % of hazelnut. The method allowed amplifying positively down to the relative level of 1 mg/kg, with an established LOD of 50 mg/kg (0.005 %) of hazelnut in chocolate (Table 4), which is in good accordance with other research works [16, 17, Costa et al.,

Table 3 Concentrations (μg of peptide per kg of chocolate) and recoveries of detected spiked hazelnut in model chocolates by LC-MS/MS

Model chocolates spiked with hazelnut (%)	Cor a 801		Cor a 901		Cor a 902		Cor a 903		Cor a 904		Cor a 1101		Cor a 1102		Cor a 1103	
	(μg peptide/ k g chocolate)	Recovery $\pm$ SD (%)	(μg peptide/ k g chocolate)	Recovery $\pm$ SD (%)	(μg peptide/ k g chocolate)	Recovery $\pm$ SD (%)	(μg peptide/ k g chocolate)	Recovery $\pm$ SD (%)	(μg peptide/ k g chocolate)	Recovery $\pm$ SD (%)	(μg peptide/ k g chocolate)	Recovery $\pm$ SD (%)	(μg peptide/ k g chocolate)	Recovery $\pm$ SD (%)	(μg peptide/ k g chocolate)	Recovery $\pm$ SD (%)
50	33,811.0	102.1 $\pm$ 5.6	329,886.4	97.0 $\pm$ 8.9	278,171.9	100.7 $\pm$ 8.9	420,620.8	92.8 $\pm$ 11.1	101,928.1	96.3 $\pm$ 7.3	20,919.3	91.9 $\pm$ 12.2	2,541.2	81.6 $\pm$ 14.0	3,345.2	91.1 $\pm$ 7.6
10	2,745.1	85.9 $\pm$ 16.0	57,138.4	99.2 $\pm$ 15.7	48,094.2	91.5 $\pm$ 15.9	76,316.2	98.9 $\pm$ 17.1	15,259.6	85.2 $\pm$ 16.4	4,833.4	101.5 $\pm$ 7.4	709.3	80.7 $\pm$ 9.9	937.6	76.1 $\pm$ 21.8
5.0	1212.3	74.9 $\pm$ 14.6	34,250.3	95.3 $\pm$ 6.2	26,277.3	93.0 $\pm$ 8.5	41,496.1	91.3 $\pm$ 8.6	7,344.2	96.7 $\pm$ 7.1	1,855.0	94.5 $\pm$ 8.0	393.3	65.7 $\pm$ 8.6	608.7	78.3 $\pm$ 14.5
1.0	331.7	56.1 $\pm$ 6.0	5,617.7	100.0 $\pm$ 22.9	4,782.3	96.9 $\pm$ 10.9	7,889.7	93.2 $\pm$ 15.4	1,331.8	94.3 $\pm$ 5.0	393.3	92.5 $\pm$ 11.5	257.5	60.6 $\pm$ 8.6	383.2	69.6 $\pm$ 19.2
0.50	314.3	58.9 $\pm$ 12.9	3,849.4	90.5 $\pm$ 16.4	2,998.7	93.1 $\pm$ 10.4	4,632.5	85.1 $\pm$ 13.0	806.5	89.5 $\pm$ 11.8	423.8	96.1 $\pm$ 7.5	232.4	68.1 $\pm$ 20.8	400.6	55.0 $\pm$ 3.2
0.10	275.7	96.2 $\pm$ 1.6	779.1	95.5 $\pm$ 22.8	743.2	68.6 $\pm$ 16.4	977.6	102.0 $\pm$ 16.1	195.7	89.114.3	226.7	74.3 $\pm$ 19.9	191.9	65.8 $\pm$ 24.4	320.0	51.9 $\pm$ 2.3
0.050	259.8	87.3 $\pm$ 9.8	478.0	83.5 $\pm$ 18.8	565.4	71.5 $\pm$ 18.2	658.1	96.1 $\pm$ 16.9	83.0	83.0 $\pm$ 16.1	222.6	76.4 $\pm$ 17.8	178.5	77.1 $\pm$ 23.3	284.3	64.2 $\pm$ 20.3
0.010	200.5	91.0 $\pm$ 13.3	237.2	82.7 $\pm$ 19.4	305.7	86.3 $\pm$ 2.1	301.6	76.1 $\pm$ 19.1	47.2	79.4 $\pm$ 14.4	185.5	74.1 $\pm$ 18.0	125.2	88.2 $\pm$ 19.4	234.8	50.7 $\pm$ 0.7
0.0050	240.7	92.5 $\pm$ 16.0	254.7	96.4 $\pm$ 16.7	338.6	60.8 $\pm$ 2.1	344.4	73.1 $\pm$ 11.6	38.4	57.5 $\pm$ 16.8	233.3	85.3 $\pm$ 11.3	174.1	56.1 $\pm$ 13.1	286.5	60.5 $\pm$ 2.5
0.0010	251.7	91.9 $\pm$ 11.1	307.0	103.6 $\pm$ 16.6	883.7	84.7 $\pm$ 15.0	382.0	73.6 $\pm$ 12.4	42.0	81.3 $\pm$ 18.1	238.5	90.8 $\pm$ 11.9	164.3	78.3 $\pm$ 20.9	293.3	50.8 $\pm$ 0.5
0.00050	217.1	95.2 $\pm$ 5.7	257.4	84.6 $\pm$ 16.3	895.3	93.1 $\pm$ 13.7	304.8	77.3 $\pm$ 18.9	43.1	72.6 $\pm$ 17.3	271.2	98.2 $\pm$ 11.9	145.8	65.8 $\pm$ 20.3	263.9	53.5 $\pm$ 2.0
0.00010	321.5	97.6 $\pm$ 4.3	314.2	85.3 $\pm$ 19.9	1,372.2	91.5 $\pm$ 11.3	461.8	81.4 $\pm$ 20.9	48.3	68.3 $\pm$ 17.6	238.7	97.4 $\pm$ 4.2	218.9	79.9 $\pm$ 19.9	337.0	63.3 $\pm$ 22.9

Recoveries were calculated using $n=3$ to $n=9$ replicates in three independent runs

Tracing tree nut allergens in chocolate: a comparison of DNA extraction protocols, submitted]. According to Mazzara et al. [25] and Bustin et al. [26], the LOD should be defined as the lowest concentration presenting 95 % of positive replicates for the target analyte, which in this case was determined accounting with the total number of positive replicates ($n=12$) in all the performed real-time PCR assays. Below the LOD, model chocolates containing 10 and 5 mg/kg of hazelnut produced positive amplification in 8 out of 12 replicates. The lowest model chocolate spiked with hazelnut (0.0001 %) was only positive in 25 % of the total replicates.

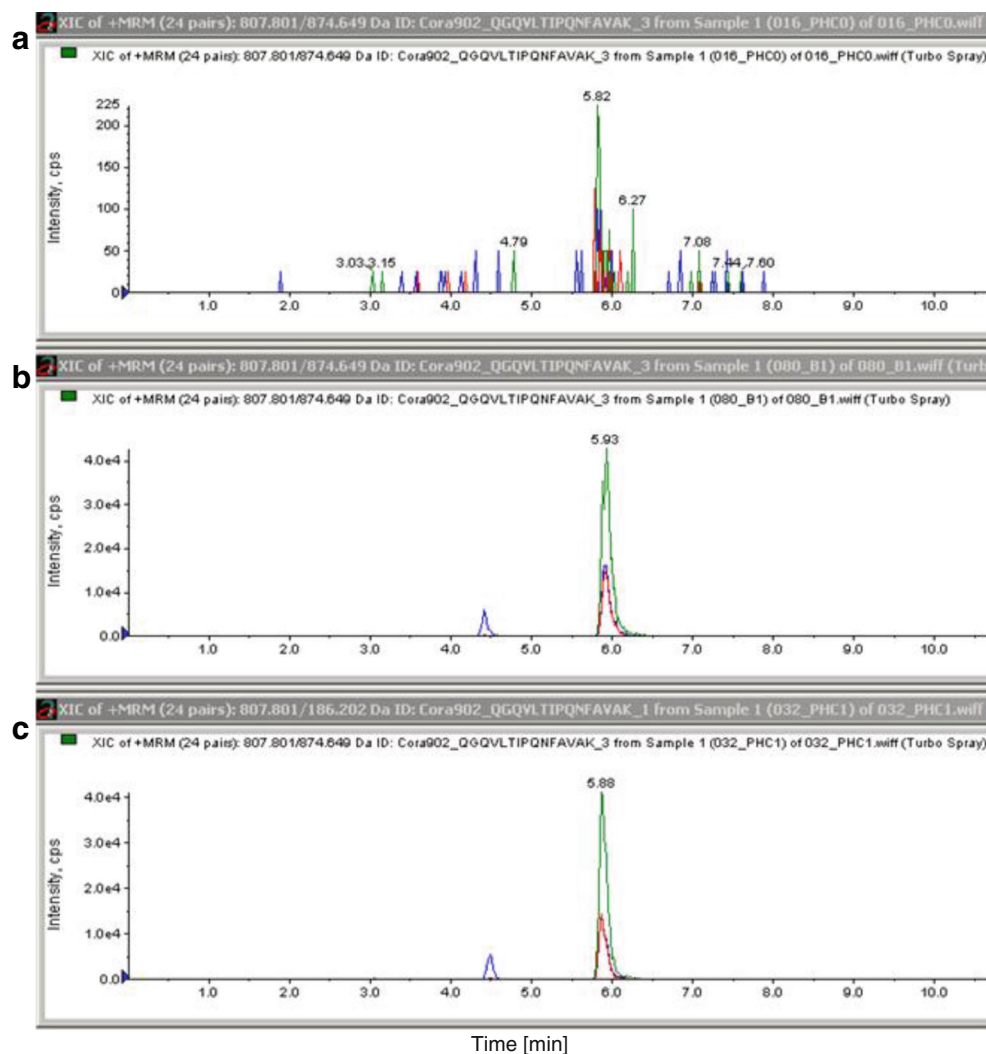
The LOQ was found to be equal to the LOD (50 mg/kg of hazelnut chocolate) since the lowest amount of hazelnut amplified in the system was within the linear range of the calibration curve. Under the definition of minimum performance requirements, the correlation coefficient (R^2) of standard curves should be above 0.98 and the PCR efficiency between 90 and 110 %, implicating slopes ranging from -3.6 and -3.1 , respectively [25]. The parameters of the reactions exhibited average values for R^2 of 0.9913, slope of -3.1376 and PCR efficiency of 108.3 % (Table 4), being also in good accordance with the acceptance criteria for method performance [25, 26].

The number of DNA copies was calculated according to the genome size of hazelnut (0.48 pg) and assuming that the targeted sequences are single copy genes [27]. Considering the total quantity of DNA (20 ng) used in real-time PCR for each extract of spiked chocolate, it was possible to estimate the number of hazelnut DNA copies (Table 4). In all the assays, the estimated LOD of 50 mg/kg of hazelnut in chocolate corresponded to a total of approximately two amplified DNA copies. Model chocolates spiked with hazelnut below this concentration have less than one DNA copy, which could explain the lack of reproducibility in the number of positive replicates, since there is a strong possibility that the target DNA might not be present in all wells. In contrast with the results from ELISA, the 0 % model hazelnut chocolate (100 % milk chocolate) failed to amplify in all the replicates and in all the real-time PCR assays. This fact was already expected and further confirms the previous data, suggesting that the chocolate used for model chocolates was free from hazelnut residues. When analysing complex samples such as chocolates, the sensitivity values attained in this study are in good accordance with other published works [16, 17, Costa et al., Tracing tree nut allergens in chocolate: a comparison of DNA extraction protocols, submitted].

Conclusion

ELISA and more recently MS are among the most commonly used techniques for the direct detection of allergens in foods. In addition to these two protein-based approaches, real-time PCR is also considered one of the most relevant molecular methodologies applied to the indirect assessment of allergens

Fig. 1 Extracted ion chromatograms (XIC) of peptide Cor a 902 in model chocolate with 50 % hazelnut. **A** Chromatogram of blank chocolate (0 % hazelnut); **B** chromatogram of 50 % of hazelnut diluted in 0.1 M of NH_4HCO_3 used for the calculation of recovery and **C** chromatogram of model chocolate with 50 % of hazelnut



in foods. Still, the application of the proposed techniques for allergen detection faces different challenges, which might

explain the difficulty of officialising one or more methods that fulfil all the crucial criteria.

Fig. 2 Example of one of the calibration curves for real-time PCR system applied to model chocolates spiked with known amounts of hazelnut. ($n=4$) replicates per run

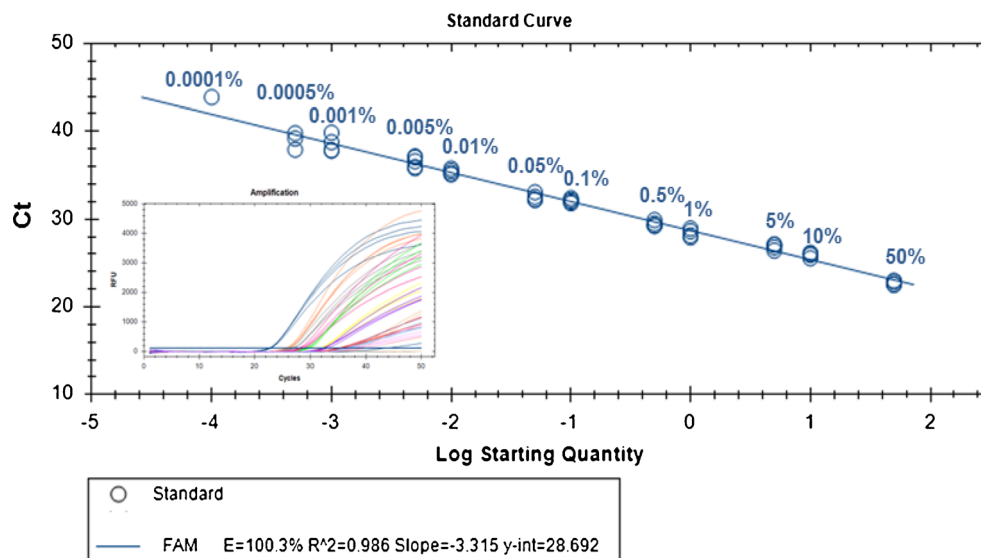


Table 4 Results of detection of spiked hazelnut in model chocolates by real-time PCR

Model chocolates spiked with hazelnut (%)	Ct±SD ^a	CV (%)	DNA copies ^b
50	22.98±0.07 (12/12)	0.31	20,833
10	25.47±0.15 (12/12)	0.60	4,167
5.0	26.41±0.21 (12/12)	0.81	2,083
1.0	28.21±0.40 (12/12)	1.41	417
0.50	29.17±0.27 (12/12)	0.93	208
0.10	31.57±0.27 (12/12)	0.87	41.7
0.050	31.78±0.68 (12/12)	2.13	20.8
0.010	35.44±1.54 (12/12)	4.33	4.17
0.0050	36.69±1.00 (12/12)	2.73	2.08
0.0010	37.26±1.55 (8/12)	4.16	0.42
0.00050	38.13±1.93 (8/12)	5.07	0.21
0.00010	41.23±0.87 (3/12)	2.10	0.042
0	nd (0/12)	na	na
Correlation coefficient (R^2)	0.9913		
Slope	-3.1376		
PCR efficiency (%)	108.3		

CV coefficient of variance, nd not detected, na not applicable

^a Mean cycle threshold (Ct) values±standard deviation (SD) ($n=12$ replicates in three independent real-time PCR runs)

^b Number of hazelnut haploid genome copies (0.48 pg)

In this study, the developed ELISA was considered a very reliable method allowing detecting traces of hazelnut proteins down to the spiked level of 1 mg/kg, but the interference of the matrix cannot be neglected. In the case of the analysis of model chocolates containing hazelnut, the application of ELISA enabled quantifying hazelnut protein until the spiking level of 50 mg/kg with good bias and adequate recoveries. The same results were also attained when applying a real-time PCR system for the detection of hazelnut in model chocolates. Positive amplifications were obtained for the lowest concentration (1 mg/kg) of hazelnut in chocolate. For quantification purposes, the level of 50 mg/kg of hazelnut in chocolate was defined as the LOQ for the proposed real-time PCR system, which according to the hazelnut genome size represent approximately two single copy genes. MS analysis enables detecting hazelnut peptides down to 1 mg/kg, but with different LOQ depending on the target.

Taking into consideration the application of the three methodologies to detect trace amounts of hazelnut, the presented findings exhibit similar and adequate performance, achieving limits of detection and quantification in the same order of magnitude. These facts evidenced that each of the techniques could be widely applied for the identification of hazelnut in complex matrices such as milk chocolates with similar levels of sensitivity. However, when choosing the technique for allergen detection in foods, some factors have to be considered, such as

the availability of expensive equipment and specialised personnel, time consuming per analysis, cost of analysis, among others. For instance, the time needed for sample preparation was higher when applying MS platform (until 3 days), followed by real-time PCR approach (approximately 2 days). In the case of ELISA, the time for sample preparation was short, but in order to setup the assay at least 1 to 2 days were needed. In terms of cost per analysis, MS platform was the most expensive technology employed in this study.

In summary, all the methods presented major advantages and also some drawbacks; still all can be employed with high sensitivity to evaluate the presence of hazelnut in chocolates. There is no recommendation for just one of the methods as the best usable because depending on the problem all of the three methods do have their qualification for allergen analysis, e.g. for immediate process monitoring, immunoanalytical techniques like lateral flow device (LFD) or ELISA can be used. PCR offers an alternative detection where protein assays are limited. And the MS methods come into place where confirmatory methods are needed as there is at the moment no comparison possibility for immunoanalytical protein methods.

Acknowledgments The ELISA and MS systems were developed and performed in the Christian Doppler Laboratory “Rapid Test Systems for Allergenic Food Contaminants”, which is funded by the Christian Doppler Research Association and RomerLabs. The authors also thank Marcela Hermann, Institute of Medical Biochemistry, Department of Molecular Genetics, University Vienna, for the immunisation of rabbits as well as Thomas Kolbe, University of Veterinary Medicine Vienna, for the immunisation of mice.

This work has been supported by Fundação para a Ciência e a Tecnologia (FCT) through grant no. PEst-C/EQB/LA0006/2013, NORTE-07-0124-FEDER-000069-Food Science and by University of Porto “Projectos Pluridisciplinares” IJUP. Joana Costa is grateful to FCT PhD grant (SFRH/BD/64523/2009) financed by POPH-QREN (subsidised by FSE and MCTES).

References

1. Chafen JJS, Newberry SJ, Riedl MA, Bravata DM, Maglione M, Suttrop MJ, Sundaram V, Paige NM, Towfigh A, Hulley BJ, Shekelle PG (2010) Diagnosing and managing common food allergies: a systematic review. *JAMA* 303:1848–1856
2. Roux KH, Teuber SS, Sathe SK (2003) Tree nut allergens. *Int Arch Allergy Immunol* 131:234–244
3. Burney P, Summers C, Chinn S, Hooper R, Van Ree R, Lidholm J (2010) Prevalence and distribution of sensitization to foods in the European Community Respiratory Health Survey: a EuroPrevall analysis. *Allergy* 65:1182–1188
4. ALLERGEN, (2013). Official site for the systematic allergen nomenclature. <http://www.allergen.org/> Accessed 14 March 2013
5. ALLERGOME, (2013). Allergome database, the platform for allergen knowledge, Latina, Italy. <http://www.allergome.org/> Accessed 14 March 2013
6. Akkerdaas JH, Wensing M, Knulst AC, Stephan O, Hefle SL, Aalberse RC, van Ree R (2004) A novel approach for the detection of potentially hazardous pepsin stable hazelnut proteins as contaminants in chocolate-based food. *J Agric Food Chem* 52:7726–7731

7. Drs E, Baumgartner S, Bremer M, Kemmers-Voncken A, Smits N, Haasnoot W, Banks J, Reece P, Danks C, Tomkies V, Immer U, Schmitt K, Krska R (2004) Detection of hidden hazelnut protein in food by IgY-based indirect competitive enzyme-immunoassay. *Anal Chim Acta* 520:223–228
8. Kiening M, Niessner R, Drs E, Baumgartner S, Krska R, Bremer M, Tomkies V, Reece P, Danks C, Immer U, Weller MG (2005) Sandwich immunoassays for the determination of peanut and hazelnut traces in foods. *J Agric Food Chem* 53:3321–3327
9. Pele M, Brohee M, Anklaam E, van Hengel AJ (2007) Peanut and hazelnut traces in cookies and chocolates: relationship between analytical results and declaration of food allergens on product labels. *Food Addit Contam* 24:1334–1344
10. Rejeb SB, Abbott M, Davies D, Cl  roux C, Delahaut P (2005) Multi-allergen screening immunoassay for the detection of protein markers of peanut and four tree nuts in chocolate. *Food Addit Contam* 22: 709–715
11. R  der M, Vieths S, Holzhauser T (2009) Commercial lateral flow devices for rapid detection of peanut (*Arachis hypogaea*) and hazelnut (*Corylus avellana*) cross-contamination in the industrial production of cookies. *Anal Bioanal Chem* 395:103–109
12. Trashin SA, Cucu T, Devreese B, Adriaens A, De Meulenaer B (2011) Development of a highly sensitive and robust Cor a 9 specific enzyme-linked immunosorbent assay for the detection of hazelnut traces. *Anal Chim Acta* 708:116–122
13. Ansari P, Stoppacher N, Baumgartner S (2012) Marker peptide selection for the determination of hazelnut by LC-MS/MS and occurrence in other nuts. *Anal Bioanal Chem* 402:2607–2615
14. Bignardi C, Mattarozzi M, Penna A, Sidoli S, Elviri L, Careri M, Mangia A (2013) A rapid size-exclusion solid-phase extraction step for enhanced sensitivity in multi-allergen determination in dark chocolate and biscuits by liquid chromatography–tandem mass spectrometry. *Food Anal Methods* 6:1144–1152
15. Heick J, Fischer M, P  pping B (2011) First screening method for the simultaneous detection of seven allergens by liquid chromatography mass spectrometry. *J Chromatogr A* 1218:938–943
16. Costa J, Mafra I, Kuchta T, Oliveira MBPP (2012) Single-tube nested real-time PCR as a new highly sensitive approach to trace hazelnut. *J Agric Food Chem* 60:8103–8110
17. Costa J, Oliveira M, Mafra I (2013) Applicability of a real-time PCR system to verify labelling compliance of nut allergens in chocolates. *Clin Trans Allergy* 3(Suppl 3):P129
18. D'Andrea M, Coisson JD, Locatelli M, Garino C, Cereti E, Arlorio M (2011) Validating allergen coding genes (Cor a 1, Cor a 8, Cor a 14) as target sequences for hazelnut detection via real-time PCR. *Food Chem* 124:1164–1171
19. K  ppel R, Velsen-Zimmerli F, Bucher T (2012) Two quantitative hexaplex real-time PCR systems for the detection and quantification of DNA from twelve allergens in food. *Eur Food Res Technol* 235: 843–852
20. 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*). *J Agric Food Chem* 59:10803–10814
21. Costa J, Oliveira MBPP, Mafra I (2013) Effect of thermal processing on the performance of the novel single-tube nested real-time PCR for the detection of walnut allergens in sponge cakes. *Food Res Int* 54: 1722–1729
22. P  knov   L, Pangallo D, Kuchta T (2008) A novel real-time polymerase chain reaction (PCR) method for the detection of hazelnuts in food. *Eur Food Res Technol* 226:1155–1158
23. CAC/GL 74–2010 (2010) Guidelines on performance criteria and validation of methods for detection, identification and quantification of specific DNA sequences and specific proteins in foods, Codex Alimentarius International Food Standards, Rome, Italy http://www.codexalimentarius.org/standards/list-of-standards/en/?no_cache=1. Accessed 2 December 2012
24. Holzhauser T, Vieths S (1999) Quantitative sandwich ELISA for determination of traces of hazelnut (*Corylus avellana*) protein in complex food matrixes. *J Agric Food Chem* 47:4209–4218
25. Mazzara M, Savini C, Charles de Delobel C, Broll H, Damant A, Paoletti C, van den Eede G (2008) European Network of GMO Laboratories (ENGL). Definition of minimum performance requirements for analytical methods of GMO testing, European Commission, Brussels. <http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm> Accessed 5 July 2013
26. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 55:611–622
27. Plant DNA C-values database, Royal Botanic Gardens, Kew, Richmond, Surrey, USA. <http://data.kew.org/cvalues/> Accessed 22 July 2013